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Formation and Release of aging-relevant aldehydes in lager beer – Prediction of flavor instability via bound state aldehydes

Florian Lehnhardt

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Vorsitz:

Prof. Dr. Corinna Dawid

Prüfer der Dissertation:

1. Prof. Dr.-Ing. Thomas Becker

2. Prof. Dr. Michael Rychlik

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Preface and Peer-Reviewed Publications

The results and publications of this thesis were produced from 2016 to 2021 at the Technical University of Munich, Chair of Brewing and Beverage Technology, Research Group Raw Material Based Brewing and Beverage Technology under the supervision of Prof. Dr.-Ing. Thomas Becker.

This cumulative thesis is based on the following peer-reviewed publications:

I. Lehnhardt, F., Gastl, M., and Becker, T.: Prediction Power and Accuracy of Forced Ageing - Matching Sensory and Analytical Results for Lager Beer. Brewing Science. 71(May/June). (2018): 39-48 (DOI: https://doi.org/10.23763/BrSc18-05lehnhardt).

II. Lehnhardt, F., Becker, T., Gastl, M.: Flavor stability assessment of lager beer: what we can learn by comparing established methods. European Food Research and Technology 246 (2020): 1105-1118 (DOI: https://doi.org/10.1007/s00217-020-03477-0).

III. Lehnhardt, F., Gastl, M., and Becker, T.: Forced into aging: Analytical prediction of the flavor-stability of lager beer. A review. Critical Reviews in Food Science and Nutrition. 59/16 (2018): 2642-2653 (DOI: doi:10.1080/10408398.2018.1462761).

IV. Nobis, A.*, Lehnhardt F.*, M. Gebauer, T. Becker and M. Gastl.: The Influence of Proteolytic Malt Modification on the Aging Potential of Final Wort. Foods. 10/2320 (2021): 1-18 (DOI: 10.3390/foods10102320).

V. Lehnhardt F.*, Nobis, A.*, A. Skornia, T. Becker and M. Gastl.: A comprehensive evaluation of flavor instability of beer (part 1): Influence of release of bound state aldehydes. Foods. 10/2432 (2021): 1-15 (DOI: 10.3390/foods10102432).

Contents

Acknowledgements ..... **II**

Preface and Peer-Reviewed Publications ..... **III**

Contents ..... **IV**

Abbreviations ..... **VI**

Summary ..... **VII**

Zusammenfassung ..... **IX**

1 Introduction **1**

1.1. Flow of beer around the globe 2

1.2. Beer volatilome – Aroma of fresh lager beer 2

1.3. Sensory and analytical changes during aging 3

1.4. Mechanisms of aldehyde formation during beer aging 5

1.4.1. Direct oxidation – the formation of ROS 7

1.4.2. Maillard reaction 8

1.4.3. Strecker degradation 9

1.4.4. Bound state aldehydes 10

1.5. Assessment of flavor instability 13

1.5.1. Singular approaches 13

1.5.2. Antioxidative capacity 13

1.5.3. Prediction by forced aging 14

1.6. Determination of aroma active volatiles 14

1.7. Motivation and thesis outline 15

2 Methods 18

3 Results 20

3.1 Summary of main results 20

3.2 Thesis Publications 26

3.2.1 Prediction Power and Accuracy of Forced Ageing - Matching Sensory and Analytical Results for Lager Beer 26

3.2.2 Flavor stability assessment of lager beer: what we can learn by comparing established methods 36

3.2.3 Forced into aging: Analytical prediction of the flavor-stability of lager beer. A review 50

3.2.4 The Influence of Proteolytic Malt Modification on the Aging Potential of Final Wort 62

3.2.5 A comprehensive evaluation of flavor instability of beer (part 1): Influence of release of bound state aldehydes 80

4 Discussion 95

4.1 Deficiencies of established methods for the assessment of flavor instability 95

4.2 Structure-dependent interactions of aldehydes in complex matrices ... 99

4.3 Development and degradation of amino group-related adducts 104

4.4 The use of bound state aldehydes for the prediction of flavor instability 106

5 Conclusions and Outlook 109

6 References 111

7 Appendix 121

7.1 Non-peer reviewed publications 121

7.2 Conference Contributions 121

7.2.1 Oral 121

7.2.2 Poster 121

Abbreviations

Abbreviation	Full meaning
2MB	2-Methylbutanal
2MP	2-Methylpropanal
3-DG	3-desoxyglucosone
3-DGal	3-desoxygalactosone
3MB	3-Methylbutanal
4VP	4-Vinylpyridine
ACA	Acetaldehyde
BENZ	Benzaldehyde
CATA	Check all that apply
DLG	Deutsche Landwirtschaftsgesellschaft
ESR	Electron spin resonance spectroscopy
GC-MS	Gas chromatography-mass spectrometry
GC-O	Gas chromatography-olfactometry
HEX	Hexanal
HMF	Hydroxymethyl furfural
HPLC-UV	High pressure liquid chromatography ultraviolet detection
HSO_3^-	Bisulfite
HS-SPME	Headspace solid-phase microextraction
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
METH	Methional
O_2	Oxygen
PA	Phenyl acetaldehyde
QDA	Quantitative descriptive analysis
ROS	Reactive oxygen species
SAFE	Solvent-assisted flavor evaporation
SD	Steam distillation
SO_2	Sulfur dioxide
t	Time
T2N	(E)-2-Nonenal
ΔT	Temperature

Summary

Quality loss – especially of desirable sensory qualities – during aging within shelf life is typical and well known for various beverages and foods. The sensory quality of beer – in particular pale lager beer – decreases constantly during aging and consequently consumer acceptance decreases. Aldehydes from Maillard reaction, Strecker degradation and lipid degradation influence the quality substantially due to their high aroma activity. The molecular mechanisms responsible for the observed increases in free aldehydes during aging are various but can be classified into *de novo* formation and release from bound states.

To assess and predict the so-called flavor instability of beer, the brewing industry needs reliable sensory and analytical tools. Yet, the established methods – an endpoint estimation via thermal stress (forced aging) in combination with extraction of volatile compounds via steam distillation – show major drawbacks. Therefore, this thesis aimed to reveal these drawbacks, uncover the molecular differences and open new possibilities to improve the prediction of flavor instability.

In part 1 of this thesis, the differences between natural and forced aging were uncovered. It was found, that the sensory profiles differ substantially between both methods. In addition, free aldehydes mostly showed a linear behavior in forced aged samples in contrast to natural aged samples indicating a diverging formation. Part 2 of this study revealed deficiencies during the extraction of volatiles during the sample preparation for analytical measurements. Especially steam distillation was found to be detrimental due to the high temperature intake resulting in considerable changes during sample workup. Fundamental differences in the profile of aging indicators (qualitative and quantitative) was observed in comparison to other, more gentle extraction techniques.

To uncover the underlying mechanisms explaining the observed differences, a comprehensive literature review was performed as a third part of this thesis. It highlighted the potential impact of bound state aldehydes in the context of beer flavor instability. Their chemistry – presence, structures and stabilities – in the beer matrix remain an intensely discussed topic in literature. These compounds provide a great potential as indicators for the prediction of flavor instability. Thus, possible strategies for their determination were gathered.

The indirect measurement of bound state aldehydes after chemical release was performed in the last parts of this thesis. In part 4, it was discovered that during wort boiling, an amino group-related pool of bound state aldehydes is built up. Furthermore, the previously discussed substance group of cysteinylated aldehydes was found to be inconsequential for flavor instability. In part 5, it was revealed that the equilibrium of free and bound state aldehydes is shifted to the free form during aging. Therefore, the assessment of bound state aldehydes proved promising for the prediction of flavor instability – especially in the early and medium stage of aging. The advantages lie in the ease of implementation (in combination with HS-SPME) as well as speed of analysis and results. The findings of this work provide the brewing industry with important mechanistic insights into beer aging as well as strong analytical tools for the early-stage assessment of flavor instability of lager beer.

Zusammenfassung

Qualitätsverluste während der Alterung innerhalb des Mindesthaltbarkeitsdatums sind typisch für verschiedene Getränke und Lebensmittel. Besonders die sensorische Qualität von Hellen Vollbieren nimmt während der Alterung kontinuierlich ab, wodurch die Verbraucherakzeptanz abnimmt. Vor allem die Zunahme an Aldehyden aus der Maillard-Reaktion, dem Strecker-Abbau and der Lipidoxidation beeinflusst die Qualität aufgrund ihrer hohen Aromaaktivität. Die molekularen Mechanismen, die diese Aldehydzunahme verursachen, können in die *de novo*-Bildung und die Freisetzung aus gebundenen Vorstufen unterteilt werden.

Um die sogenannte Aromainstabilität von Bier beurteilen und vorhersagen zu können, benötigt die Brau- und Getränkeindustrie verlässliche sensorische und analytische Methoden. Bislang beruhen die etablierten Methoden auf einer reinen Endpunktabschätzung, welcher thermisch in der sogenannten forcierten Alterung hervorgerufen wird. Die Beurteilung dieses Punktes erfolgt meist durch die Extraktion von flüchtigen Stoffen durch Wasserdampfdestillation. Beide Methoden weisen deutliche Nachteile auf. Deswegen war das Ziel dieser Arbeit diese Nachteile die zugrundeliegenden molekularen Unterschiede aufzuzeigen und neue Möglichkeiten zur Vorhersage der Alterungsinstabilität zu eröffnen.

Im ersten Teil dieser Arbeit wurden die Unterschiede zwischen natürlicher und forcierter Alterung dargestellt. Es wurde gezeigt, dass sich die jeweiligen sensorischen Profile grundsätzlich zwischen den beiden Alterungsarten unterscheiden. Zusätzlich wurde festgestellt, dass freie Aldehyde meist linear während der forcierten Alterung ansteigen, sich jedoch nichtlinear in der natürlichen Alterung verhalten, was auf unterschiedliche Alterungsmechanismen hindeutet. Im zweiten Teil dieser Arbeit wurden die Nachteile der Extraktion von flüchtigen Stoffen mittels Wasserdampfdestillation. Die erhöhte Temperatur während der Aufarbeitung führte zu deutlichen Unterschieden im Profil an Alterungsindikatoren (qualitativ und quantitativ) im Vergleich zu nicht-invasiven Aufarbeitungs- und Analysemethoden.

Um die zugrundeliegenden Mechanismen aufzudecken, wurde im dritten Teil dieser Arbeit eine umfassende Literaturrecherche durchgeführt. Diese zeigte vor allem den potentiellen Einfluss von gebundenen Aldehyden auf die Aromainstabilität und deren Möglichkeit zum Einsatz als Alterungsindikatoren auf. Die Chemie – Vorkommen,

Struktur und Stabilität – dieser Stoffe in der Biermatrix wird in der Literatur diskutiert. Deshalb wurden Strategien zur analytischen Bestimmung gebundener Aldehyde erstellt.

Die Anwendung der indirekten Bestimmung gebundener Aldehyde nach chemischer Freisetzung wurde zuletzt untersucht. Im vierten Teil wurde der Aufbau eines Aminogruppen-abhängigen Pools an gebundenen Aldehyden entdeckt. Zudem wurde gezeigt, dass die zuvor diskutierten cysteinylierten Aldehyde als irrelevant für die Bieralterung zu erachten sind. Im letzten Teil dieser Arbeit wurde gezeigt, dass sich das Gleichgewicht zwischen freien und gebundenen Aldehyden im Laufe der Alterung verschiebt. Aus diesem Grund stellte sich die Bestimmung von gebundenen Aldehyden als vielversprechend zur Vorhersage der Aromainstabilität besonders während der frühen bis mittleren Phase heraus. Die Vorteile liegen in der einfachen Implementierung mit bestehenden Methoden (HS-SPME), sowie in der Analysengeschwindigkeit. Die Erkenntnisse dieser Arbeit eröffnen der Brau- und Getränkeindustrie wichtige mechanistische Einblicke in die Bieralterung und stellen robuste analytische Methoden zur frühzeitigen Bestimmung der Aromainstabilität von Hellen Vollbieren zur Verfügung.

1 Introduction

Flavor instability of beer has been a focus of brewers and brewing chemists, who strive for the highest consistency and stability, for decades. In times of growing consumer awareness, increasing competition between breweries and advancing globalization, very high demands in regard to beer quality are made [1]. Especially pale lager beers – the most consumed beer style around the world – are prone to quality losses during aging within the shelf life due to their flavor profile and matrix [2–4].

The responsible chemical mechanisms for beer aging are inevitable and inherited in the brewing process and additionally, the phenomenon is not fully understood nor can be controlled entirely. Still, ways of describing and predicting flavor instability during beer aging exist [5–8]. These usually include the assessment of volatile aldehydes – the most established group of aging indicators due to their increase during aging and their high aroma impact – in combination with forced aging. The latter aims to accelerate aging solely due to increased temperature for a specified time [2, 6, 9–11].

Up to now, aldehydes are only used as aging indicators in their aroma active free form. Yet, they can also be present in a bound state from which they are hypothesized to be released. It is assumed that these compounds have a substantial impact on flavor instability [12].

This thesis aims to demonstrate drawbacks of established ways of assessing and predicting flavor instability such as forced aging and further to reveal the impact of bound state aldehydes on beer aging. In the regard, the thesis focusses on adducts of aldehydes and amino groups (imines and cysteinylated aldehydes) and their variation in various malting and brewing trials. Ultimately, bound state aldehydes are discussed as potential indicators of flavor instability already in fresh pale lager beer.

Therefore, the following lines describe the global transport of beer, the aroma of pale lager beer including the responsible compounds and the sensory as well as analytical changes during aging. Thereafter, the different mechanisms of beer aging and the importance of methods to assess the compounds involved are discussed. Finally, the underlying hypotheses of this thesis as well as the outline are presented.

1.1. Flow of beer around the globe

Exportation and importation of beer, especially pale lager beer, has been increasing continuously over the last years. The five leading countries in exportation of beer are Mexico (export value: $ 4.7 B), Netherlands ($ 2.1 B), Belgium ($ 2.0 B), Germany ($ 1.3 B) and USA ($ 0.6 B). The top 5 importing countries are USA ($ 6.0 B), France ($ 1.2 B), UK ($ 0.7 B), Italy ($ 0.5 B), and Netherlands ($ 0.5 B). The total volume of exportation around the globe in 2020 was $ 14.37 B. Thereby, the highest flow happens from Mexico to USA with a total volume of $ 4.25 B [13].

While some of these distances can be covered by trucks, overseas shipping takes place via cargo with increased shipping times, as well as physical and thermal stress. For example, a shipment from Germany to Taiwan takes more than 40 days by cargo. Temperature logging revealed that the shipped products can be exposed to temperatures above 40 °C [14]. This is detrimental for beer quality, especially the sensory aspects, as it will be discussed in the following chapters.

A consumer study focusing on the relationship between preference, drinkability and age of beers revealed that fresh lager beer shows the highest acceptance [15]. Therefore, aging of lager beer is considered as one of the key problems, brewers are facing today in a globalized world [1, 16].

1.2. Beer volatilome – Aroma of fresh lager beer

The chemistry of beer is highly sophisticated resulting in complex aroma profiles. Through a multi-stage process from the raw materials water, barley, hops and yeast, a product arises with an immense set of volatiles from different chemical families. Recently, 329 volatiles from different chemical families (acids, alcohols, esters, monoterpenic compounds, norisoprenoids, sesquiterpenic compounds, sulfur compounds, and volatile phenols) were analyzed by multidimensional gas chromatography, from which 96 were reported for the first time in lager beer [17]. In fact, the total volatilome of lager beers is even richer, since the authors did not report any aldehydes or ketones. Other authors reported that the total metabolome of beer – analysed in a set of 120 diverse beer samples assessed by non-targeted profiling – consists in average of 2800 compounds [18].

The aroma active compounds range in concentrations from ng/L to g/L and have different origins, odor thresholds, and aroma impressions. In 2014, a meta-analysis

revealed that only 17–20 key aroma compounds create the aroma perception of fresh bottom-fermented beers [19]. These include several esters, higher alcohols, sulfides, short-chain fatty acids, representing aromas such as *banana, gummy bears, roses, citrus, caramel, cooked corn,* and *cheese* [20, 21].

The aroma situation in beer is further influenced by interactions of volatiles with the beer matrix (matrix effects) and among other volatiles (interactive effects). Carbohydrates are known to push aroma compounds into the headspace of a sample (salting out effect), while proteins promote the retention in the liquid phase. Of course, this behavior is greatly dependent on the chemical structures of the volatiles [22].

Interactive effects of aroma compounds – either in the headspace or directly at the human olfactory epithelium – can also enhance or the decrease the resulting aroma impression. On the one hand, thresholds of mixtures can be (substantially) lower than of the individual compounds (additive and synergistic effects). An example is the mixture of (E)-2-nonenal (T2N) and (E,E)-2,4-decadienal that shows a threshold of only 24 % when present in the same matrix. On the other hand, thresholds can also increase in mixtures (masking effect). In this regard, isoamyl acetate is able to mask 2-methylbutanal (2MB) but not phenyl acetaldehyde (PA) in beer [23].

Due to this aroma situation, pale lager beers are especially vulnerable during aging. Their matrix rather tends to release volatiles instead of retaining them. At the same time, the aroma profile is made up from low concentrations of volatiles compared to other beer styles [4].

1.3. Sensory and analytical changes during aging

The sensory profile of beer, especially pale lager beer, is constantly changing within its regular shelf life. This dynamic phenomenon is inherent to beer and is thus regarded as “flavor instability”. Therefore, it should be used instead of “flavor stability” [24].

The changes during beer aging affect all the sensory properties of beer – aroma, taste, bitterness, and mouthfeel – and have been described in literature [25, 26]. Frequently mentioned descriptors for aging impressions are *fruity-sweetish, ribes, cardboard, berry-like* and *bready* in the earlier stages of aging of up to 6 months. The descriptors *caramel, honey* and *sherry* occur in the later stages (more than 6 months) [4, 25, 27].

During beer aging, quantitative changes in aroma compounds are observed. On the one hand, desirable compounds such as 2-methylbutyl isobutyrate, a primary ester of

hops can decrease dramatically and with them their potential masking capacity [28]. On the other hand, undesirable compounds can increase and result in an aged aroma. Among other substance classes, **aldehydes** represent the most potent odor-active compounds that increase during aging.

Previously, volatile aging indicators have been classified into different groups according to their main influences during aging. Those compounds were selected due to their increases in various aging setups, not due to their impact on the aroma. The amounts of the respective compounds are summed up to indices on exposure to oxygen, temperature and (general) aging [11]. Most of the mentioned compounds are aldehydes. Table 1 gives an overview on these established indicators and their classification according to Lustig [11].

Table 1: Oxygen, heat and aging indicators as proposed by Lustig [11]; X indicates the affiliation to the resp. group of indicators

Compound	**Oxygen indicator**	**Heat indicator**	**Aging indicator**
2-methylbutanal	X		
3-methylbutanal	X		X
Benzaldehyde	X		X
Phenylacetaldehyde	X		X
Furfural		X	X
γ-nonalactone		X	X
5-methyl furfural			X
Succinic acid diethyl ester			X
Phenylacetic acid ethyl ester			X
2-acetyl furan			X
2-propionyl furan			X

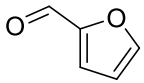
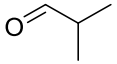
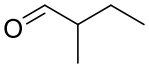
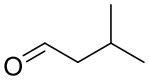
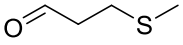
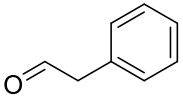
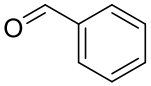
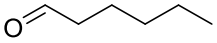
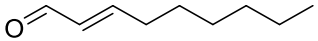
The aldehydes that are discussed in this thesis can reach concentrations above or close to their respective odor thresholds. Especially, 2-methylpropanal (2MP), 3-methylbutanal (3MB), methional (METH) and T2N are known to exhibit high odor activity values (OAV). Only furfural (FUR) and benzaldehyde (BENZ) are usually present in lower concentrations as a recent overview reported [29]. Nevertheless, these compounds are known to be important aging indicators.

1.4. Mechanisms of aldehyde formation during beer aging

In general, in foods and beverages, aldehydes can arise from a variety of oxidative and non-oxidative reactions. The most important reactions comprise degradations of carbohydrates, amino compounds, and lipids. Principally, these reactions are possible throughout the process – from raw materials to final product – but are of course favored in different stages. The individual reaction parameters are discussed in the resp. chapters.

Furthermore, these compounds are not only formed newly (**de novo**) in the reactions that are described below but they can also interact covalently with other compounds in the malt, mash, wort and beer matrix. Since these reactions are reversible and greatly dependent on the matrix, a labile equilibrium (**bound state aldehydes**) is formed [12]. In the following chapter, mechanisms of the formation of free and bound aldehydes will be discussed in detail. These comprise direct oxidation, Maillard reaction, Strecker degradation and the release of bound state aldehydes. Table 2 gives an overview of the relevant aldehydes.

Table 2: Most relevant aldehydes in beer aging, their origin, structure, aroma impressions, thresholds in beer according to Saison et al. [23], and concentrations ranges according to Dennenlöhr et al. [29]

Aging-relevant aldehyde	**Origin**	**Structure**	**Aroma impression**	**Threshold [µg/L][23]**	**Concentration ranges [µg/L][29]**
Furfural	Maillard reaction		Caramel, bready	15157	29.5–356.9
2-methylpropanal	Strecker degradation		Grainy, fruity	86	8.84–95.2
2-methybutanal	Strecker degradation		Almond, apple	45	1.68–26.2
3-methylbutanal	Strecker degradation		Malty, chocolate	56	3.91–63.7
Methional	Strecker degradation		Cooked potatoes	4.2	1.02–39.0
Phenyl acetaldehyde	Strecker degradation		Floral, roses	105	6.91–95.9
Benzaldehyde	Strecker degradation + oxidation		Almond, cherry stone	515	<0.5–2.78
Hexanal	Lipid degradation		Winey	88	0.38–1.86
(E)-2-nonenal	Lipid degradation		Cardboard	0.03	0.07–0.34

1.4.1. Direct oxidation – the formation of ROS

Oxygen has been considered as the most important factor in beer aging for a long time. Brewers strive to achieve less than 0.1 mg/L total oxygen in bottled beer. Yet, in this way of packaging, ingress of oxygen is possible via the crown corks [30]. Certain antioxidants, the most abundant in beer being SO_2, thiols, and products of Maillard reaction can counteract flavor instability through oxidation and thus delay sensory deterioration. Furthermore, isohumulones and phenolic substances show antioxidative properties [31].

Triplet oxygen ($^3O_2$), the ground state of oxygen in beer, is relatively inert. Yet, in activated forms it can impair the beer aroma through various oxidation reactions. Reactive oxygen species (ROS) such as the singlet oxygen ($^1O_2$), peroxide anion (HOO^-), superoxide ($O_2^{\bullet-}$), hydroperoxyl radical ($^\bullet OOH$), hydroxyl radical ($^\bullet OH$), and hydroxyethyl radical ($EtO^\bullet$) are formed via catalysis of Fe^{2+} or Cu^+ in the Fenton Reaction. The hydroxyethyl radical is regarded as the predominant radical since ethanol is the most abundant compound in beer [32].

For direct radical oxidation, a variety of potential reaction partners is available in beer. Andersen et al. showed that the 88 % of hydroxyethyl radical in a commercial pilsner beer reacted with isohumulones, 9 % with thiols, 2 % with other compounds, and only 1 % towards acetaldehyde and cause further oxidative damage. Oxidized isohumulones result in a changed bitterness, while oxidized thiols can undergo dimerization, irreversible thiol oxidation, or oxidize other beer components [33].

The direct oxidation of amino acids to corresponding aldehydes in model systems and beers is described in the literature. In the same experiments it was observed that addition of Fe^{2+} did not elevate the aldehyde levels after aging. This suggests that minimal levels of Fe^{2+} are needed for the catalytic formation of ROS and the resulting Fe^{3+} is rapidly reduced again in a beer environment [34, 35]. Recently, it was demonstrated in wine that the oxidation of higher alcohols to their respective aldehydes had only very little influence on the level of corresponding aldehydes despite the drastically elevated O_2 contents compared to beer. The authors found that the oxidation of amino acids is more relevant [36]. An indicator for the oxidation of (higher) alcohols could be the increase in acetaldehyde from the oxidation of ethanol. It is suggested that other alcohols would react to their corresponding aldehydes at similar extents.

Another relevant target for oxidation reactions are lipids and fatty acids, especially with unsaturated C-C binding systems. In the latter case, ROS will abstract an electron from e. g. linoleic acid. After addition of O_2, either 13- or 9-hydroperoxy fatty acids result, giving rise to the aroma active compounds hexanal (HEX) and T2N, respectively [30]. While this might hold true in model systems, the situation in packaged beer might be different as Noël et al. reported [37, 38]. The authors spiked $^{18}O_2$ into bottled beer and found it incorporated in compounds with antioxidative properties such as sulfites, polyphenols, and isohumulones but not in carbonyls. They concluded that oxidation of lipids (and possibly also other compounds) does not proceed in packaged beers.

Furthermore, Amadori products (such as from phenylalanine and glucose) are known to yield Strecker aldehydes upon direct oxidation. This pathway was described to be catalyzed by transition metals. The key step involves the generation of an imine that is successively hydrolyzed [39].

1.4.2. Maillard reaction

The Maillard reaction is a complex of different reactions and source of a variety of products. From the general educts, reducing sugars and amino groups (from amino acids, proteins and such), for example odor-active heterocyclic compounds such as FUR or 5-hydroxymethyl furfural (HMF) can arise. Recently, also the importance of oligosaccharides for the formation of products of Maillard reaction was highlighted [40].

These resulting compounds occur usually well below their odor thresholds but are known to be ideal indicators for thermal intake during the brewing process and also aging [30]. Furthermore, as its other name – “non-enzymatic browning” reaction – suggests, it is responsible for increases in color intensity by melanoidin formation during aging [41]. The reaction is typically favored in pH ranges of 4–7 and temperatures above 50 °C [12].

In the early stage of Maillard reaction, a nucleophile amino group attacks the carbonyl group of an open-chain reducing sugar resulting in an imine (Schiff base). This imine can isomerize to an Amadori rearrangement product, such as fructosyllysine, favored by higher pH values [42]. Yet, the low pH values in beer (4.2–4.5) lead to the formation of 3-desoxyglucoson (3-DG), making it the most abundant α-dicarbonyl in beer [12].

3-DG has a central role in beer aging due to the fact that it is only partly stable in beer. It can participate in a variety of reactions such as isomerization to

3-desoxygalactosone (3-DGal), formation of HMF, formation of melanoidins, Strecker Degradation, and formation of glyoxal, methylglyoxal and advanced glycation end products [43]. Thus, it is discussed as an indicator of natural beer aging [43]. Through subsequent losses of water, the afore-mentioned heterocyclic compounds, FUR or HMF, can arise. The reaction is favored by higher temperatures, low water activity, and long storage times [44]. Yet, it was found that the concentrations of furfural were not influenced by beer color [41].

In the later-stage of Maillard reaction, so called advanced glycation end products occur. One of the most important representatives is pyrraline, a product of 3-DG and lysine, which can occur in a free and in a protein-bound state. In different beer styles, 0.16–1.6 mg/L free pyrraline and 55–400 mg/kg protein in a bound state were detected, highlighting the reactiveness of carbonyl compounds in the malt, wort and beer matrix. Furthermore, a correlation between free pyrraline and the color intensity was observed [42].

1.4.3. Strecker degradation

In this late-stage of the Maillard reaction, the amino group of an amino acid can attack a dicarbonyl. In beer, the predominant α-dicarbonyl is 3-DG but diacetyl [36] and polyphenol-derived *o*-quinone structures are also potential reaction partners [36, 45]. After a transamination and a subsequent decarboxylation, which takes place regardless of the high CO_2 levels in beer, an aldehyde results. The aroma active so-called Strecker aldehydes in beer are 2MP from valine, 2MB from isoleucine, 3MB from leucine, METH from methionine, PA from phenylalanine [30]. Strecker aldehydes have the highest impact on the formation of aging flavors [46].

The rate-limiting step of this reaction is the addition of the amino group to the carbonyl group. It is favored by higher pH values and temperatures [47, 48]. Lermusieau et al. [49] found that an imine formation between lysine and T2N did not occur at temperatures below 40 °C. Other authors reported that the optimal pH value for the formation phenylacetaldehyde in model systems 100 °C of phenylalanine and different carbohydrates resp. dicarbonyls is 5 [50]. Furthermore, oxygen-dependency is also widely accepted since studies with increased oxygen levels lead to higher amounts of Strecker aldehydes [27]. Therefore, the Strecker degradation is strongly temperature, oxygen and pH dependent.

It is also known from brewing trials that higher concentrations of Strecker aldehydes are found in dark beers. Likely, this is due to the fact that higher concentrations of dicarbonyls are found in those malts and beers, since other precursors such as amino acids and higher alcohols did not differ [41]. Thus, from the sole point of instability, Maillard Reaction should be avoided entirely in brewing. Yet, these findings disregard the different aroma situations and the resulting masking effects in darker beers.

1.4.4. Bound state aldehydes

Aldehydes are a quite reactive substance class. In literature, it is assumed that most aging-relevant aldehydes could be produced up-stream and are already present in the fresh beer [51].Yet, they are not present in a free- but in a bound state. These compounds are hypothesized to release aldehydes during the course of aging and thus cause an aged aroma [12]. In this thesis, the term "**release of bound state aldehydes**" comprises all reactions in which bound state precursors react to free aldehydes. Drivers of this release are not unraveled yet but oxygen, temperature, and pH value are the most plausible parameters [49]. In addition, the status of the equilibrium and potential competitive reactions can set free aldehydes [52].

Bound state aldehydes are masked from human perception, most analytical techniques, and technological processes such as evaporation during wort boiling or reduction by yeast during fermentation [12]. This binding can be either reversible or irreversible and depends on aldehyde and the resp. nucleophile. While bound state aldehydes bound at the carbonyl group can be set free relatively easy by hydration, the same was not observed when being bound on the double bond of an unsaturated aldehyde [53]. The sum of reversibly bound-aldehydes in fresh beer is defined as the **bound potential of aging** of beer.

Lermusieau et al. studied the interaction of albumin and T2N. They found that albumin retained 60 % of the initial aldehyde concentration and released only 50 % of the retained amount upon heating [49]. In experiments with ^{13}C-labelled amino acids it was shown that 85 % of Strecker aldehydes in the final aged beer are produced up-stream (mainly in wort boiling) and then bound. In that study, only 15 % were formed *de novo* [54].

In literature, there are a number of described possible bound state aldehydes, such as bisulfite adducts, imines, cysteinylated aldehydes, acetals, and glycosides [47, 55–59]. From these, especially bisulfites and amino group-related adducts appear to be the

ones with the highest impact on flavor instability. They will be introduced in the following lines. Additionally, figure 1 gives an overview of chosen representatives of bound state aldehydes.

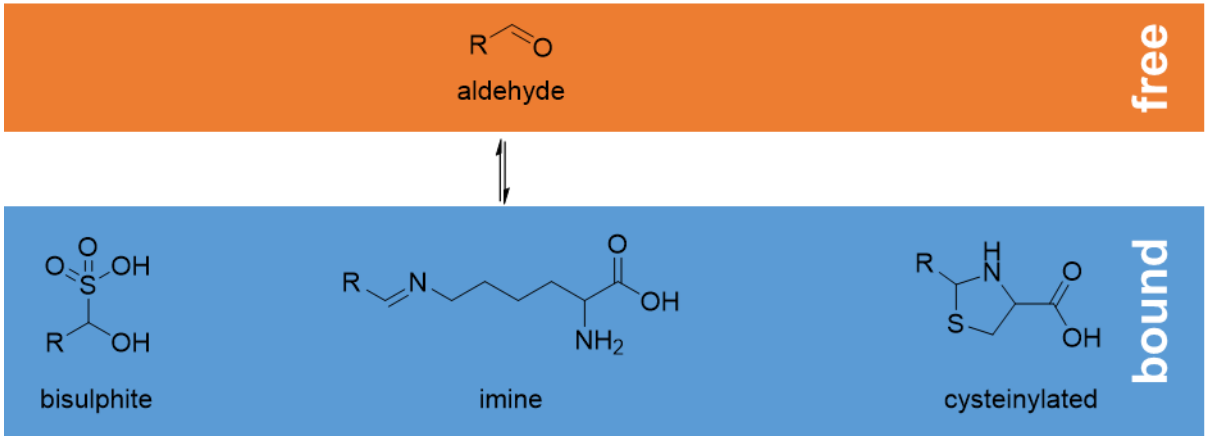


Figure 1: Occurrence of aldehydes in beer; free (aroma active) and bound state (non-aroma active)

Sulfite adducts

Bottom-fermenting yeasts produce sulfur dioxide (SO_2) in the early stage of fermentation (growth phase) as an intermediate of its sulfate metabolism [60]. SO_2 in beer is known to be able to delay oxygen-mediated aging reactions by acting as an antioxidant. Furthermore, it can also reversibly attack aldehydes and ketones in the form of bisulfite (HSO_3^-), the form in which SO_2 is mostly present in beer in a pH range from 3–6 [55].

The resulting α-hydroxysulfonate of acetaldehyde has been described in beer in literature reaching concentration of up to 54 µM [61]. In another study, the bisulfite adduct of glyceraldehyde, representing an intermediate of Maillard reaction, was found in wine. In this way, reactive potential can occur in the final beverage matrix [62]. Interestingly, these adducts show the same antioxidative properties as bisulfite itself [63].

Levels of SO_2 typically decrease during aging which would in turn release aldehydes from the corresponding bisulfites due to a shift in the chemical equilibrium [64]. Reasons for the decrease of SO_2 are direct oxidation to sulfate [65], but also the formation of irreversible adducts such as diadducts (on double-bounds) [53] or

sulfonated polyphenols like epicatechin [66]. In wine, the latter pathway explains about 10 % of the loss of SO_2 during aging [67].

Amino group-related adducts

The main source of amino compounds in brewing is barley. Its raw proteins are broken down during germination in malting (proteolysis). Brewers can influence the degree of proteolysis –among other technological parameters during mashing – with the steeping degree during malt production; the higher it is, the more soluble nitrogen is in the malt [68]. Generally, low Kolbach indices, a calculated, practical measure for the degree of proteolysis, in the malt lead to lower flavor instability in the final beer [69].

Amino groups are nucleophile and can attack aldehyde groups followed by an elimination of water. The resulting structures are called imines or Schiff bases [32]. The attack of the amino group – the rate-limiting step – is favored at higher pH values whereas lower pH values and ΔT can (partly) reverse the reaction [49]. According to other sources, the cleavage of imines is favored at pH values of 4 [70]. Thus, aldehydes would be gradually set free from their bound states via acidic hydrolysis. The occurrence of imines was described by several authors and the resulting imines of lysine appear to be the most studied compounds due its second amino group [32, 49, 71]. Imine adducts are described for T2N, HMF, 2MP but not for FUR or PA [32, 49].

Yet other authors did not report on the occurrence of imines for different amino acids with certain aldehydes [58]. The same authors rather suggested that cysteinylated aldehydes (2-substituted 1,3-thiazolidine-4-carboxylic acids) play are more important role due to the high nucleophilicity of cysteine. Their findings were based on model systems and HPLC-UV measurements for the matrix beer [58]. The behavior of these compounds such as their higher pH-stability in wort in comparison to beer was hypothesized to make these compounds to plausible precursors [72, 73]. Yet, recently it was demonstrated that after fermentation these compounds do not occur in relevant concentrations except for methional [74]. Cysteinylated aldehydes only occur in relevant concentrations for METH but the concentrations of the adduct increase during aging [74]. This suggests that METH arises from other pathways and was partly bound due to the chemical equilibrium, thus being rather the dead end than its source in the sense of a bound pool that is depleted over time.

1.5. Assessment of flavor instability

Next to understanding flavor instability holistically, it is arguably even more important for brewers to have tools for the assessment of flavor instability. They provide the basis for critical decision making, and driver for raw material-based and technological innovation. The status of beer aging, as it would occur during natural aging at 20 °C can be predicted by different approaches, each having their benefits and drawbacks. The most common ways are described in this chapter.

1.5.1. Singular approaches

During malt and beer production, and aging, there are varieties of singular parameters brewers can use for an estimation of the flavor instability of a finished beer.

The most common parameter to judge the thermal intake is the thiobarbituric acid index (TBI). It is a sum-parameter, primarily influenced by the amount of HMF and can be assessed already in malt, over the process and in the final beer [75]. The course of pH value over the process also allows for an estimation of critical steps during production. Thus, enzymatic processes during mashing and fermentation can be judged [75]. Furthermore, the amount of O_2 and SO_2 after filling can give insights into flavor instability [75].

Additionally, the “nonenal potential” is a possibility to assess the capacity of wort to produce T2N in acidic conditions. This provides an assumption how much of this aging indicator could be formed in extreme conditions [76]. Recently, the method was extended with the assessment of hexanal [77].

1.5.2. Antioxidative capacity

The antioxidative capacity of beer can be assessed by electron spin resonance spectroscopy (ESR). This method tracks the formation of unpaired electrons of primary intermediates of oxidation at 50 °C under atmospheric conditions by capturing them with a so-called spin trap reagent. The resulting stabilized spin adducts are formed after a lag-phase during which prooxidative and antioxidative processes compete [63]. ESR is widely used and regarded as the state-of-the-art method to investigate the oxidative instability [78].

There are also high-throughput approaches available. The ferric reducing antioxidant power test (FRAP) determines the prooxidant activity. This is achieved by reduction of

Fe^{3+} to Fe^{2+}. In the oxygen radical absorbance capacity test (ORAC), peroxy radicals are scavenged by the transfer of hydrogen atoms [31].

1.5.3. Prediction by forced aging

Processes that occur during aging at ambient temperatures are usually referred to as natural aging. In order to get insights into the flavor instability of a finished beer faster, elevated temperatures are applied to speed up aging processes and thermally stress the product. These approaches are defined as forced aging. Regimens can range from 28–60 °C for 3 days to several weeks with or without shaking. The most popular regimen appears to be 1 day of shaking at 100 rpm followed by 4 days at 40 °C, which should predict the changes during 3–6 months of natural aging [79]. Afterwards, the forced aged beer is tasted and volatile aging indicators according to Lustig (Table 1) are analyzed.

Obviously, due to the described mechanisms, forced aging does not just speed up the aging processes but further alters them in a significant way. By the law of Arrhenius, an increase in temperature of 10 K results in a 2–3-fold change of reaction rate [30] but due to different activation energies, reactions rates would increase at different rates. This was observed for the formation of Meth and PA whose reaction rates increased with temperature [80].

In fact, reactions might also appear that would not occur normally. One example might be the fact, that heat induces the back reaction from an imine to the initial aldehyde [32]. Other authors also stated that caution should be exercised when using forced aging due to a changed sensory and analytical pattern [81]. Eger et al. concluded that an increase in the applied temperature also increased the sensory differences between natural and forced aging [82]. Consequently, in forced aging, there is a dilemma between the speed of the prediction and the agreement with reality during natural aging.

1.6. Determination of aroma active volatiles

The assessment of volatile aging compounds is mostly performed with gas chromatographic (GC) methods. They are the most established aging indicators since their concentrations in malt, wort (boiled and unboiled) and fresh beer correlate well with tasting results of aged beers [46].

The numerous extraction techniques available for GC can be grouped into solvent extraction, destillative, headspace and sorptive approaches [83]. Due to the high complexity of the sample and the low concentrations of aging compounds, non-invasive and selective techniques are most desirable. The most commonly used extraction techniques are steam distillation (SD), solvent-assisted flavor evaporation (SAFE) and headspace solid-phase microextraction (HS-SPME). Each of these have their specific advantages and disadvantages as described in the discussion section. There are multiple comparisons between those methods in literature and the results usually depend on the sample and the chromatographic problem [84–87].

1.7. Motivation and thesis outline

In the previous sections, the mechanisms of natural and forced beer aging and aroma active compounds involved have been described in detail. Based on this, the initial situation and motivation of this thesis can be summarized as follows:

- In principal, the aging relevant aroma compounds and their formation including critical parameters are known. Yet, on one hand, a big part of this knowledge is based on model systems with reduced complexity in comparison to beer matrix. On the other hand, the most widely used method to predict flavor instability – forced aging – is mostly based on empirical data and provides unrealistic reaction conditions. Therefore, it resembles rather an estimation than a prediction.

- Aroma profiles of natural and forced aging deviate from another, suggesting that the responsible aroma active compounds are formed to different extents. These differences in aroma profiles and compounds involved are unclear. Furthermore, aging compounds are thought to increase linearly during aging. Different stages of reactions as well as respective velocities are not taken into account.

- The concept of bound state aldehydes – its formation and interdependency of competing reactions – and its impact on beer aging is not understood. It is unclear to which extents different aldehydes are present in which bound states as well as if and how they are released during aging. Bound state aldehydes are not used for the early-stage, targeted and multifaceted prediction of flavor instability.

- The parameters affecting the depletion of a bound state pool of aldehydes – especially the impact of thermal intake as well as O_2 – are unclear. Therefore, the behavior of bound state aldehydes during treatment at higher temperature (during forced aging and steam distillation) is unclear. An involuntary release of the compounds is likely to happen, resulting in qualitative and quantitative differences of aldehydes.

Based on this initial situation, the main objective of this thesis is formulated as follows:

Elucidation of the role of bound state aldehydes on beer flavor instability and exploration of their potential as alternative early-stage indicators

In order to achieve this objective, the following steps were taken.

1. Differences between natural and forced aging were elucidated with sensory (aroma quality and profile) and analytical methods (aging indicators by GC-MS)
2. Extraction methods for the analysis of volatile aging compounds were compared qualitatively and quantitatively with a special focus on the heat-induced formation, release, and breakdown of aging compounds
3. Possible bound state forms of aldehydes were critically reviewed and possibilities to analyze them in order to predict the flavor instability of beer were compiled and assessed in regard to their feasibility
4. Systematic variations in precursor compounds were undertaken to elucidate the formation and degradation of free and bound state aldehydes over the course of the wort boiling process
5. The fate of free and bound state aldehydes in beers with systematic variations in precursor compounds was studied over the course of natural and forced aging to investigate the impact amino group adducts on beer flavor instability

2 Methods

The used commercial beers (I, III) and research beers that were produced in a 50 L scale (V) were subjected to natural aging (dark, 20 °C, duration as indicated) and forced aging (24 h of shaking at 100 rpm; 4 d at 40 °C) as indicated. Wort samples were frozen immediately and stored at -20 °C until analysis (IV).

Sensory Analysis was performed in various publications (I, IV, V). In all cases, the beers were tasted according to the DLG 5-point scheme, the Eichhorn aging scheme, and a descriptive scheme. For publication I, a quantitative descriptive analysis (QDA) approach of chosen aging attributes was chosen, while for publications IV and V, a check-all-that-apply (CATA) approach was used.

Gas chromatography-olfactometry (GC-O) coupled to a mass spectrometer (MS) was used for the semi-quantitative detection of aroma active compounds. In publication I, headspace solid-phase micro extraction (HS-SPME) was used for extraction. In publication III, HS-SPME as well as solvent-assisted flavor evaporation (SAFE) and steam distillation (SD) were applied. Quantitation of aging compounds was achieved with an optimized HS-SPME-GC-MS method based on a method of Saison et al. [88] in the publications I, III, IV, V. Additionally, in publication III, an adjusted MEBAK method (MEBAK 2.23.4) was used for the quantitation of these compounds on the same instrument setup. For the release of bound state aldehydes, release agents (4VP resp. ACA) were added into the headspace vial, incubated as indicated and detected via HS-SPME-GC-MS (in publications IV, V). Unless otherwise stated, all analyses were performed in triplicates.

Non-volatile precursors of free and bound state aldehydes were investigated in the publications (IV, V). Amino acids were analyzed by HPLC-MS/MS in multiple reaction monitoring mode as described in Nobis et al. [89]. 3-DG was determined by HPLC-UV as published in Nobis et al. [43].

Where stated, in publications I, III, IV, V the data was analyzed if it followed a normal distribution using the Shapiro-Wilk W test. For normally distributed data, one-way ANOVA were used to uncover statistical differences within a sample set. The post-hoc test, Tukey-Kramer HSD test was the used for pairwise comparisons. In the case of non-normally distributed data, the Kruskal-Wallis test in combination with the post-hoc Wilcoxon each pair method were applied. For all tests, an α-level of 0.05 was used.

Using the Ward method hierarchical clustering was performed in publication I. Normalized z-scores were used for cluster analyses, principal component analyses, and heat maps. All data analyses were performed in JMP Pro 13 resp. 14 (SAS Institute Inc., Cary, NC, USA).

3 Results

3.1 Summary of main results

Part 1: Prediction Power and Accuracy of Forced Ageing - Matching Sensory and Analytical Results for Lager Beer

Pages 25–34

The aim of this study was to evaluate the prediction power and accuracy of flavor instability of pale lager beers with forced aging and to elucidate possible discrepancies between natural and forced aging. This was based on the hypothesis that through different temperatures different aging mechanisms apply to different extents.

Therefore, commercial beers were systematically aged and assessed with sensory (DLG, acceptancy, QDA) and analytical methods (HS-SPME-GC-MS-O, HS-SPME-GC-MS after derivatisation with PFBHA).

The paradigm is that forced aging simulates the natural aging at 20 °C of 3 – 5 months. In this study, it could be shown in sensory experiments that this only holds true for global approaches such as the DLG overall score or acceptancy according to Eichhorn. The former is a quality approach based on a relatively broadly interpreted scale, while the latter is hedonic. With QDA the aroma profiles of the different beers could be described. Principal component analysis and hierarchical clustering of the resulting data revealed 4 different groups. Group 1 was comprised of fresh until 3 month naturally aged beers, group 2 of forced aged samples (2–8 days), group 3 of medium naturally aged beers (5 to 9 months), and group 4 of heavily naturally aged beers (11–17 months). Group 1 was associated with the absence of aging attributes and group 2 with the attributes *cardboard, dull, bready*. Group 4 was rated with high intensities for the attributes *sweetish, fruity, sherry, berry, honey* while group 3 ranged between group 2 and 4.

The quantification of aging compounds of the same samples showed that ethyl 2-methylbutanoate, 3-methylbutanoate, β-damascenone, ethyl nicotinate, ethyl 2-phenylacetate, furfuryl ethyl ether, 2MP, 2MB, PA, FUR, METH, and T2N increased linearly during forced aging. In contrast, during natural aging, only ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, ethyl 2-phenylacetate, furfuryl ethyl

ether and FUR increased linearly. No linear increase for aldehydes except for furfural was observed among these samples.

These results show clearly that when observed in detail, forced ageing does not represent the changes during natural aging satisfactorily despite its widespread use. They also direct to the presence of different underlying mechanisms of aging, especially for the detected aldehydes.

Authors´ contribution:

Florian Lehnhardt designed the working hypothesis, research project and experimental approach after critical discussion with the co-authors. He developed the applied methodology and conducted the main experiments. Furthermore, he evaluated the resulting data. As the principal author of this publication, he wrote the manuscript and submitted it. All authors reviewed and approved the final manuscript.

Part 2: Flavor stability assessment of lager beer: what we can learn by comparing established methods

Pages 35–48

The aim of this study was to compare the qualitative and quantitative differences in aroma active aging compounds amongst three established sample preparation methods on the same analytical instrument. Further, the elucidation of heat-promoted changes in the volatile profiles during the analysis, especially due to *de novo* formation and release of bound state aldehyde was a goal.

The considered methods were headspace solid–phase microextraction (HS-SPME), solvent-assisted flavor evaporation (SAFE), and steam distillation (SD), each in combination with GC-MS-O.

The qualitative comparison of these three methods was performed with GC-MS-O. In SD, the highest number of aroma active compounds and aroma intensities were observed, followed by HS-SPME and SAFE. Since that for SAFE, only 11 aging compounds were identified confidently and aroma intensities were low, it was excluded from further experiments.

A quantitative comparison of SD and HS-SPME showed that both approaches could be validated satisfactorily based on relative standard deviations and recoveries for all compounds, and allow for a robust determination. Anyhow, significant differences in

concentrations were observed for certain aging compounds (for example 3MB, FUR, PA) presumably due to the heat intake during SD in comparison to HS-SPME.

Thus, model systems were designed to elucidate the impact of isolated aging-relevant mechanism and precursors during distillation. It was demonstrated, that the applied elevated temperatures led to the *de novo* formation and also release of bound state aldehydes. Therefore, the matrices and precursors should be approached as cautiously as possible suggesting the least invasive method of analysis.

Authors´ contribution:

Florian Lehnhardt designed the working hypothesis, research project and experimental approach after critical discussion with the co-authors. He developed the applied methodology and conducted the main experiments. Furthermore, he evaluated the resulting data. As the principal author of this publication, he wrote the manuscript and submitted it. All authors reviewed and approved the final manuscript.

Part 3: Forced into aging: Analytical prediction of the flavor-stability of lager beer. A review

Pages 49–60

In order to investigate the known aging mechanism and the available methods for describing them more closely, a literature review was conducted. The focus was set on aldehydes due to their high aroma activity.

It was found that aldehydes can be formed *de novo* during the complete malting and brewing process in the respective known pathways, such as Maillard reaction, Strecker degradation, or lipid oxidation. Yet, as recent research indicates, aldehydes can be bound by nucleophiles and are hypothesized to be released during beer aging. Thus, an aged aroma should arise.

A variety of possible bound state aldehydes could be identified from literature. Hereby, adducts with sulfur dioxide (bisulfites) and amino groups or especially cysteine (imines and thiazolidine acids, resp.) were judged to be the most relevant in beer.

The concept of bound state aldehydes was until then not considered in the method of forced aging and it appears likely to be a cause of the mismatches between natural and forced aging. The heat-promoted release of bound state aldehydes due to different activation energies was discussed.

Also, it was described that the applied forced-aging regimes and methods for the analysis of volatile aging compounds vary drastically within the literature. Each protocol using different extraction parameters, especially variations in temperature led to a certain inconclusiveness in literature.

Furthermore, the direct and indirect analysis of these bound state aldehydes was hypothesized to give a more realistic prediction of the flavor instability of pale lager beers. Therefore, literature was searched for different analytical methods for their determination. It was found that in theory LC-MS techniques allow for the direct and GC-MS techniques after cleavage of bound states for the indirect analysis were promising. Yet, there was a clear gap of knowledge in these areas despite the potential benefits in regard of non-invasiveness and meaningfulness of indicators.

Authors´ contribution:

Florian Lehnhardt conducted the literature research, designed the hypotheses and wrote and submitted the manuscript. All authors reviewed and approved the final manuscript.

Part 4: The Influence of Proteolytic Malt Modification on the Aging Potential of Final Wort

Pages 61–78

Partly, the final aging potential of a beer is already set in the wort. A majority of the relevant compounds are formed during the wort production. Since, this process step offers the suitable reaction conditions for aldehyde formation (abundance of educts, high temperatures, favorable pH for imine formation), it was investigated in detail.
The aim of the study was to investigate the influence of variations of aldehyde precursors (amino acids and α-dicarbonyls) on the aging potential of wort. This was achieved through varying the proteolytic malt modification levels.

Therefore, 12 worts were produced from 6 different malts. These malts were produced from 2 different barley varieties with distinct genetically determined proteolytic modification levels at 3 steeping degrees each. Especially Strecker-relevant amino acids and 3DG increased with higher proteolytic malt modification levels.

The concentrations of free and bound state aldehydes were highest at the on-set of boiling, decreased with boiling time and showed a slight increase towards the end of

boiling. Free and bound forms of 2MP, 2MB and 3MB showed a strong dependency towards the proteolytic modification level. Cysteinylated forms of few aldehydes such as 3MB and PA were detected during the course of wort boiling but were found at negligible amounts.

In general, at the end of boiling the amount of free and bound aldehydes and therefore the aging potential increased with the proteolytic malt modification. Furthermore, it can be concluded that a major part of the total aging potential of beer is set during wort boiling. In conclusion, even though higher malt modifications within the ISO 65 °C specifications are regarded as beneficial for certain aspects of brewing, they also lead to an increased aging potential.

Authors´ contribution:

Florian Lehnhardt designed the working hypothesis, research project and experimental approach after critical discussion with the co-authors. He developed the applied methodology and conducted the main brewing trials and experiments. Furthermore, he evaluated the resulting data. As the shared principal author of this publication, he wrote the manuscript. All authors reviewed and approved the final manuscript.

Part 5: A comprehensive evaluation of flavor instability of beer (part 1): Influence of release of bound state aldehydes

Pages 79–94

The aging potential of fresh beer comprises free aldehydes, their precursors and bound state aldehydes. Since a major part of the aging potential is related to amino compounds, their impact was investigated comprehensively by variations of reactants during malting. Amino compounds can influence the aging potential on the one hand due to increased *de novo* formation and on the other hand due to binding of aldehydes. In this publication, the focus was on the impact of bound state aldehydes and their potential release during aging.

It was shown by sensory analysis that beers produced from malts with higher amounts of reactants (quantified as soluble N and amino acids) have an increased aging instability. This behavior could be correlated with the increase in aroma active free aldehydes, especially Strecker aldehydes such as 2MP, 3MB and PA.

To elucidate the origin of these free aldehydes, the release of bound state aldehydes was investigated with two different release agents. For both methods (4VP and ACA), it was discovered that the amount of free aldehydes increased upon addition of the agents. This release from bound state aldehydes was especially observed in fresh and less in aged samples, indicating that the equilibrium between free and bound state aldehydes was shifted towards the free form during aging. In this study especially Strecker aldehydes were present in a bound state.

In conclusion, this study confirmed the existence of a bound state pool of aldehydes that is depleted during natural and partly during forced aging. This pool is amino group-dependent. The released aldehydes are aroma active and thus cause an aged aroma. The release of the bound state aldehydes has a major impact on the early and medium stage of aging (up to 6 months), whereas afterwards *de novo* formation gains more importance. Therefore, the analysis of bound state aldehydes after release can be used as a tool to assess flavor instability in fresh conditions.

Authors´ contribution:

Florian Lehnhardt designed the working hypothesis, research project and experimental approach after critical discussion with the co-authors. He developed the applied methodology and conducted the main brewing trials and experiments. Furthermore, he evaluated the resulting data. As the shared principal author of this publication, he wrote the manuscript and submitted it. All authors reviewed and approved the final manuscript.

3.2 Thesis Publications

3.2.1 Prediction Power and Accuracy of Forced Ageing - Matching Sensory and Analytical Results for Lager Beer

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F. Lehnhardt, J. Steiner, M. Gastl and T. Becker

Prediction Power and Accuracy of Forced Ageing – Matching Sensory and Analytical Results for Lager Beer

The most common way to predict the shelf life and sensory stability of lager beer is via forced ageing at elevated temperature (40 °C). However, practical results often indicate that forced ageing alters the flavour profile unlike natural ageing. To assess the prediction power of forced ageing using both sensorial and analytical approaches, a lager and a pilsner beer were stored for up to 17 months at 20 °C (natural ageing) and at 40 °C for up to 9 days (forced ageing). The beers were tested by sensory analyses (DLG 5-Point Scheme [Deutsche Landwirtschafts–Gesellschaft e. V], acceptancy, and ageing descriptors). In addition, volatile compounds were measured by solid-phase microextraction (SPME) after on-fibre derivatization with *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA). Based on (i) sensory analyses (DLG rating and acceptancy test) and (ii) the sum of analytical ageing indicators, it was shown that forced ageing over 4 days at 40 °C was able to well predict natural ageing of 3–5 months. Quantitative descriptive analysis (QDA) revealed a difference in the aroma profile of the two ageing processes. Furthermore, it was found that gas chromatography–olfactometry (GC-O) was able to determine whether samples were aged; although it was not suitable for the prediction of the degree of ageing. Between natural and forced ageing, no clear correlation between the detected aroma active indicators was found. Principal components analysis (PCA) of chosen ageing compounds (i.e., their tendency to increase linearly with ageing) revealed that 4 days of forced ageing was not able to satisfactorily predict all ageing indicators. Nevertheless, some indicators increased linearly in both ageing processes and so could be used for prediction. Therefore, breweries should be aware of the sensory and analytical discrepancies between forced and natural ageing and should critically reconcile the different prediction methods.

Descriptors: beer ageing, prediction of flavour stability, forced-ageing, beer flavour

1 Introduction

With increased globalization, the beverage industry and especially breweries, face the challenge of ensuring product quality over long shipping distances and storage times. Since beer is inherently prone to sensory deterioration (appearance of aged flavours), consumers may complain about products shipped over long distances. It has been shown that during cargo shipping, samples experience temperatures of greater than 40 °C [1]. This promotes flavour changes due to accelerated ageing, as described by *Dalgliesh* et al. [2] and later by *Zufall* et al. [3]. However, both plots should be regarded rather as an indication of possible ageing flavours that greatly depend on beer style, temperature, and oxygen levels. On the one hand, undesirable attributes arise due to chemical reactions during production and ageing, such as the Maillard reaction, Strecker degradation, lipid peroxidation, degradation of hop bitter compounds, formation of ethers and esters, and release from adducts [4, 5]. On the other hand, degradation of desirable aroma attributes (e.g., acetate esters) leads to a loss of masking effects, resulting in aged or stale flavours [6]. Additionally, most ageing compounds are already present at low concentrations in fresh beer. Even after intense ageing, most indicators do not exceed their thresholds, although interactive effects between ageing compounds have been shown [7]. Thus, beer ageing can be considered a phenomenon driven by quantitative rather than qualitative changes [8].

The sensory stability of lager beer is mainly influenced by the parameters O_2, temperature, time, and pH. Other influences, such as antioxidant protection mechanisms, prooxidant influences, and the tendency of the beer matrix toward oxidation, also play key roles [9]. Moreover, the masking effects of other aroma active compounds influence the aroma profile and can delay the point in time at which an aged aroma is perceived [10].

In 2005, *Eger* showed that beer aged at varying temperatures tends to age to different extents; whereas, e.g., the sherry attribute did not occur in samples aged at 0 °C even after 20 weeks [11]. According

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Authors

Florian Lehnhardt, Julia Steiner, Martina Gastl, Thomas Becker, Chair of Brewing and Beverage Technology, Technische Universität München, Freising, Germany; corresponding author: martina.gastl@tum.de

to Arrhenius' law, reactions proceed faster at higher temperatures; however, the activation energies of certain reactions also have to be overcome. For example, *Lermusieau* found that the formation of an imine, a limiting step during Strecker degradation, from (E)-2-nonenal and lysine did not occur at temperatures below 40 °C [12].

The prediction of sensory stability can be performed in various ways, including N-tert-butyl-α-phenylnitrone assay associated with electron spin resonance (PBN–ESR), thiobarbituric acid assay or the degradation of iso-α-acids [13–15]. Undoubtedly, the method of forced ageing at elevated temperature is the most commonly used when predicting the sensory stability of lager beer. Thereby, beer is typically stored at temperatures between 28 °C [16] and 60 °C [17]. The forced ageing method of *Eichhorn* and *Lustig* remains one of the most used approaches. Hereby, samples are shaken for 1 day to simulate transportation and are then kept at 40 °C for 4 days. This regimen is reported to predict the changes occurring during 3–5 months storage well [18]. Usually, forced ageing is performed in combination with sensory analysis and the determination of ageing indicators by gas chromatography. However, elevated temperatures will lead to an altered reaction potential and ultimately to altered sensory and chemical properties [19]. Recently, certain hop companies have also recognized the sensory discrepancies between natural and forced ageing and have started to naturally age their beer samples to allow for more realistic ageing predictions.

The aim of this work was to study the different behaviours of chosen ageing indicators and the resulting aroma profiles during natural ageing at 20 °C and forced ageing at 40 °C. Moreover, we aimed to provide recommendations as to how prediction by forced ageing should be used.

2 Materials and Methods

2.1 Chemicals and samples

The chemicals, *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (≥ 99 %), ethyl 2-methyl propanoate (99 %), ethyl 2methyl butanoate (99 %), ethyl 4methyl pentanoate (≥ 97 %), β-damascenone (≥ 98 %), γ-nonalactone (98 %), 2-aminoacetophenone (98 %), ethyl 2-phenylacetate (99 %), ethyl nicotinate (99 %), 2-methylpropanal (≥ 99.5 %), 2-methylbutanal (95 %), 3-methylbutanal (97 %), 2-phenylacetaldehyde (≥ 90 %), methional (≥ 97 %), benzaldehyde (≥ 99.5 %), pentanal (≥ 97.5 %), hexanal (98 %), heptanal (95 %), (E)-2-hexenal (98 %), (E)-2-heptenal (≥ 95 %), (E)-2-nonenal (97 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethyl 3-methyl butanoate (Fluka Analytical, ≥99.7 %), dimethyl trisulfide (SAFC, ≥ 98 %), furfuryl ethyl ether (Fluorochem, 95 %), and 2-furfural (Fluka Analytical, ≥ 99.0 %) were purchased as indicated. Samples were purchased in the freshest condition possible. The lager had 4.82 vol. % alcohol, a colour of 6.8 EBC, a pH of 4.57, and 20 IBUs. The pilsner had 4.89 vol. % alcohol, a colour of 6.6 EBC, a pH of 4.55, and 28 IBUs. All samples were brewed according to German purity laws.

The samples underwent two different ageing processes. Natural ageing was performed at room temperature (~20 °C) for up to 17 months. For forced ageing, samples were shaken for 24 hours and then kept for 4 days at 40 °C.

2.2 Sensory analysis

2.2.1 Training of panellists

Panellists underwent weekly training for an extended period, whereas most tasters were certified DLG (Deutsche Landwirtschafts-Gesellschaft e.V.) tasters. Special attention was given to orthonasal training with olfactory samples. In total, 21 aroma substances in beer, together with items such as honey, bread, ribes juice, and cardboard dissolved in water, were used for the training. A variety of forced- and naturally aged beers (up to 29 years old) were tasted by a small group of six people to agree on powerful descriptors for ageing bottom-fermented beers. Special focus of training was on these chosen descriptors and attributes from literature associated with beer ageing.

2.2.2 DLG 5-Point Scheme and acceptancy test (Eichhorn)

Fresh and aged beers were evaluated using the DLG 5-Point Scheme and hedonic acceptancy according to Eichhorn by six to seven trained panellists. The DLG method is designed for the specific rating of defects and ageing impressions. Five categories (purity of odour, purity of taste, palate fullness, freshness, and quality of bitterness) were rated on the following scale: 0 = inadequate (not evaluable); 1 = not satisfactory (strong deviations); 2 = less satisfactory (clear deviations); 3 = satisfactory (perceptible deviations); 4 = good (slight deviations); 5 = very good (quality expectations reached in full). Acceptancy according to Eichhorn was evaluated on a scale from 100 % to 0 %, where100 % represented full acceptancy and 0 % no acceptancy.

2.2.3 Quantitative descriptive analysis (QDA) of ageing attributes

For quantitative descriptive analysis (QDA), the agreed-upon ageing attributes, i.e., fruity, sweetish, honey, berry, sherry, bready, dull, and cardboard were rated on a scale from 0 (not perceivable) to 5 (very intense). Each sample was tasted by six or seven trained panelists [20].

2.3 Analysis

2.3.1 Gas chromatography-olfactometry (GC-O)

Five millilitres of unfiltered sample was placed in a 20-mL headspace vial and incubated at 40 °C for 5 min. SPME extraction was performed for 30 min at 40 °C with a CAR–PDMS–DVB fibre. The fibre was injected splitless at 250 °C by an autosampler (TriPlus RSH, Thermo Scientific Inc., Waltham, MA, USA) into a gas chromatograph (GC) (Trace 1300 Gas Chromatograph, Thermo Scientific Inc., Waltham, MA, USA) coupled to a single quad mass spectrometer (ISQ QD, Thermo Scientific Inc., Waltham, MA, USA) and a olfactory detection port (ODP 3, Gerstel, Mühlheim an der Ruhr, Germany). The GC was equipped with a DB-5 column (length: 60 m, inner diameter: 0.25 mm, film thickness: 0.25 µm). Helium was used as carrier gas (flowrate: 1.85 mL/min). The initial

temperature was 60 °C, held for 4 min. Heating at 5 K/min was undertaken until a final temperature of 250 °C was reached and held for 3 min. Each sample was analysed once by one panellist to assess whether the method worked well for the rapid determination of ageing degree. Peak detection was performed in Xcalibur 3.1.66.10 (Thermo Scientific Inc., Waltham, MA, USA) and identification was performed via the addition of pure compounds and using the NIST database. Odor intensity was rated on a scale from 0 to 3 (0 = not detected, 1 = weak intensity, 2 = medium intensity, 3 = strong intensity).

2.3.2 Gas chromatography–mass spectrometry for volatile ageing indicators

Five millilitres of unfiltered sample was placed in a 20-mL headspace vial together with 5 µL of internal standard (~1 mg/L of ethyl 2-methyl pentanoate and p-fluorobenzaldehyde). Ethyl 2-methyl pentanoate was used for quantification of underivatised compounds, while p-fluorobenzaldehyde was used for quantification of derivatised compounds.

SPME extraction was performed as described by Saison et al. [21]. A CAR–PDMS–DVB fibre was loaded with PFBHA for 10 minutes in the headspace of a vial with PFBHA solution (1 mg/L). Extraction was performed at 40 °C for 30 minutes under agitation (600 s shaking, 5 s no shaking). The fibre was injected splitless at 270 °C into a GC (GC-Ultra 1300, Thermo Scientific Inc., Waltham, MA, USA) coupled to a single quad mass spectrometer (ISQ, Thermo Scientific Inc., Waltham, MA, USA). The GC was equipped with a DB-5 column (length: 60 m, inner diameter: 0.25 mm, film thickness: 0.25 µm). Helium was used as carrier gas (flowrate: 1.85 mL/min). The initial temperature was 60 °C, held for 4 min. Heating at 5 K/min was undertaken until a final temperature of 250 °C was reached and held for 3 min. A selected ion monitoring mode with a dwell time of 0.02 s was applied for the analysis. The following ions (m/z) were monitored: 0 min: 81, 88, 102, 116; 12.0 min: 102; 12.9 min: 88, 126; 20.8 min: 78, 85, 135, 164, 239, 250: 25.8 min: 103, 190, 239, 250, 291; 30.3 min: 250, 252, 276, 291, 319; 33.6 min: 315; 35.0 min: 152, 250. Each sample was analysed in triplicate. Peak detection was performed in Xcalibur 3.1.66.10 (Thermo Scientific Inc., Waltham, MA, USA) and identification was performed via the addition of pure compounds and using the NIST database. Calibration was performed by adding ten standard solutions of differing concentration to standard beer samples. For each compound, a calibrated range was determined with $R^2 > 0.99$ and ≤ 20 % deviation between the added and calculated concentrations.

2.3.3 Calculation of odour activity values

To determine the direct aroma contribution of individual compounds, odour activity values (OAVs) were calculated by division of determined concentrations in the samples thresholds obtained from the literature [6, 22, 23].

2.4 Data analysis

The presence of a normal distribution was assessed using the Shapiro-Wilk W test. For non-normally distributed sensory data, the Kruskal-Wallis test and Wilcoxon each pair method were used. Clustering of data was achieved using the Ward method for hierarchical clusters. Together with principal components analysis (PCA), all data analysis was performed in JMP Pro 13 (SAS Institute Inc., Cary, NC, USA). For cluster analysis, PCA, and the display of indicators in heat maps, normalized z-scores were used.

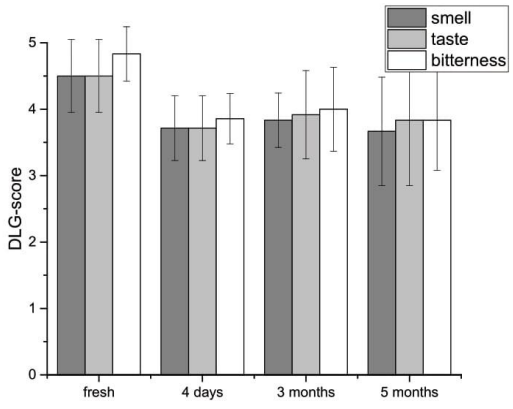

Fig. 1 DLG scores for fresh, 3-month naturally aged, 5-month naturally aged, and 4-day forced-aged lager beer (n = 7)

3 Results and Discussion

3.1 Sensory analyses – DLG 5-Point Scheme and acceptancy test

In order to predict natural ageing for lager beer, sensory analysis according to the DLG method was performed after forced ageing (see Fig. 1). In particular, the attributes smell, taste, and bitterness showed decreases during the course of ageing, whereas the attributes palate fullness and freshness showed no significant changes. Acceptancy according to Eichhorn was assessed using the same samples (see Fig. 2).

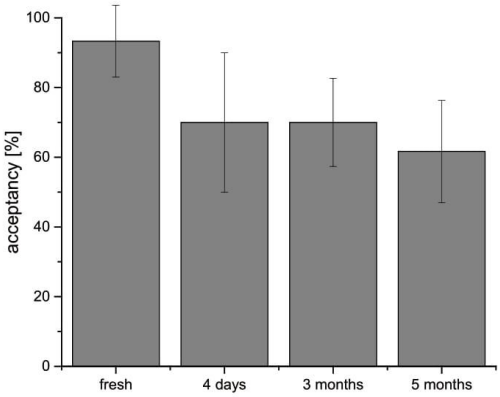

Fig. 2 Acceptancy test for fresh, 3 month naturally aged, 5-month naturally aged, and 4-day forced-aged lager beer (n = 7)

Table 1 GC–O analysis of fresh, 3-month naturally aged, 6-month (resp. 5-month) naturally aged, and 4-day forced-aged pilsner and lager; intensity scale: blank = not detected, x = weak, xx = medium, xxx = strong intensity

RI	compound	aroma description	pilsner fresh	3 months naturally	6 months naturally	4 days forced	lager fresh	3 months naturally	5 months naturally	4 days forced
730	3-methyl butanol	malty	x	xx	xx	xx	xx	xx		xx
759	ethyl 2-methyl propanoate	fruity, estery		x	x	x				x
800	ethyl butanoate	fruity, estery	x	xx	x	x	x	xx	xx	xx
827	3-methylbut-2-enthiol	dump, skunky				xx		xx	x	xx
850	ethyl 2/3-methyl butanoate	fruity, estery		x		x		x	xx	
875	3-metylbutyl acetate	fruity (banana)	xx	xx		xx		x	xx	xx
904	furfuryl ethyl ether	sweetish						xx		
908	methional	potatoes, vegetable soup		xx	x	x				
965	ethyl 4-methyl pentanoate	fruity, estery	x	x	x	x				xx
998	ethyl hexanoate	fruity, estery	xx	xx	xx	x	x	xx	x	xxx
1049	phenylacetaldehyde	roses		xx	xx	xx			x	xx
1058	furaneol	caramell	xx	xx	xx	xx	xx	x	x	xx
1095	n. i.	floral						xx		x
1098	linalool	floral, citric	x	xx	xx	x	xx			xx
1117	2-phenylethanol	roses		xx		x	xx	xx	xx	xx
1162	(E)-2-nonenal	cardboard							x	
1185	n. i.	earthy, mouldy		x				x		x
1262	ethyl 2-phenylacetate	roses	x	x	x	x	x		x	x
1301	n. i.	green banana				x				
1315	2-aminoacetophenone	fruity, floral	x	xx		xx	xx	x	xx	x
1395	β-damascenone	cooked apple	x	xx	xx	x	xx	xx	xx	xx

The obtained data did not follow a normal distribution; therefore, the Kruskal-Wallis test and Wilcoxon method were applied to each pair. The significance level α was set to 0.05.

For the category smell, the Kruskal-Wallis test revealed significant differences within the samples ($\chi^2 = 8.93$). The Wilcoxon method showed statistically significant differences between fresh and 5-month naturally aged samples ($p = 0.024$) and between fresh and 4-day forced-aged ($p = 0.033$) but not between fresh and 3-month naturally aged samples ($p = 0.054$). Among the aged samples, no significant differences were found.

The differences for the category taste followed a similar pattern. The Kruskal-Wallis test did not reveal any significant variance in the data set. However, the Wilcoxon method showed significant differences between the fresh sample and 5-month naturally aged beer ($p = 0.024$), and between the fresh sample and the 4-day forced-aged beer ($p = 0.036$). Only the fresh sample and 3-month naturally aged sample showed no significant difference ($p = 0.138$). No significant differences between the aged samples could be observed.

Bitterness proved to differ significantly within the data set (Kruskal-Wallis test: $\chi^2 = 12.21$). The Wilcoxon method revealed significant differences between fresh samples and 3-month naturally aged samples ($p = 0.036$), fresh and 5-month naturally aged samples ($p = 0.007$), and fresh and 4-day forced-aged samples ($p = 0.005$). Among the aged samples, no significant differences were observed.

For acceptancy, the Kruskal-Wallis test revealed significant differences among the samples ($\chi^2 = 10.76$). Against the fresh sample, the Wilcoxon method showed differences for 3 months of natural ageing ($p = 0.014$), 5 months of natural ageing ($p = 0.010$), and 4 days of forced ageing ($p = 0.021$). Again, there were no significant differences between the aged samples.

Therefore, on the basis of the DLG rating and acceptancy test, forced ageing (4 days at 40 °C) was able to well predict the changes observed during 3 to 5 months of natural ageing. These findings concur with the literature [24]. The most obvious changes were perceived in smell, taste, and bitterness.

3.2 Sensory analyses – gas chromatography–olfaction

GC-O can be used for the identification of aroma active compounds in a sample after GC separation. Thus, it was possible to identify single aroma active compounds without interactive effects or matrix effects.

To assess whether GC-O is a fast and reliable method for determining the state of ageing in any given sample, the same pilsner and lager as used for sensory analysis were used in this analysis. Table 1 shows the compounds detected by GC-O for the fresh, naturally aged, and forced-aged pilsner and lager beers. The

intensity of perceived odours was rated on a scale from 0 (not detected) to 3 (strong intensity). Because this experiment was performed as a pre-trial, each sample was analysed only once. Even though the panelists were trained, the possibility that single compounds remained undetected due to the form of a particular panellist cannot be excluded.

This study highlighted ethyl 2-methyl propanoate (2MP2), ethyl 2/3-methyl butanoate (2MB2/3MB2), phenylacetaldehyde, and (E)-2-nonenal as promising GC-O indicators of beer ageing. None of these compounds were perceived in fresh samples. Methional was detectable only in aged pilsner beers, whereas 3-methyl-but-2-en thiol was detected in aged pilsner and lager samples. Furthermore, furfuryl ethyl ether and three unidentified compounds (RI: 1095, floral; RI: 1185, earthy/mouldy; RI: 1301, green banana) only appeared in a few aged samples. Other compounds, such as 3-methyl butanol, ethyl butanoate, 3-methylbutyl acetate, ethyl hexanoate, furaneol, linalool, 2-phenylethanol, ethyl 2-phenylacetate, 2-aminoacetophenone, and β-damascenone were identified in most of the samples. Thus, GC-O allowed for the distinction between fresh and aged samples. However, the rapid prediction of the state of ageing based on the presence and intensity of aroma active indicators proved not to be convincing. Even so, certain ageing indicators in lager and pilsner beers (e.g., ethyl 2- methyl propanoate, methional, or phenylacetaldehyde) may be present if the sample experienced some degree of ageing.

3.3 Sensory analyses – QDA

In order to describe the aromas that appeared during the ageing of bottom-fermented beers, QDA was performed to identify the attributes fruity, honey, sweetish, berry, sherry, bready, dull, and cardboard. The lager (Fig. 3) and pilsner beers (Fig. 4) were both naturally aged at 20 °C and forced aged at 40 °C.

The fresh lager showed some basic sweetish and fruity notes that were not related to ageing. Between the aged samples, there were clear differences in aroma characteristics. Even though, the sensory data were not statistically significant, clear tendencies were observed. This sample developed a complex aroma profile, where the attributes fruity, sweetish, berry and cardboard after 5 months of natural ageing dominated. In contrast, the forced aged sample developed sweetish, dull, and cardboard notes. During natural ageing, the attribute cardboard increased but then decreased slightly with time. Other attributes such as bready, sherry, berry, sweetish, and fruity showed increasing trends. Honey was only perceived in very old beers. For forced ageing, cardboard and dull notes were the main attributes and these persisted.

In contrast to the lager, fresh pilsner showed no basic sweetish and fruity notes. The naturally aged pilsner developed strong honey, sweetish and berry notes, while also other attributes such as cardboard were observed. The forced aged sample showed strong fruity, sweetish and cardboard notes. During natural ageing, all attributes increased, whereas sweetish was the most prominent one. After 17 months, sherry was the most dominant descriptor. Forced ageing led to an increase in the attributes fruity and sweetish but also to the development of cardboard notes in the pilsner sample.

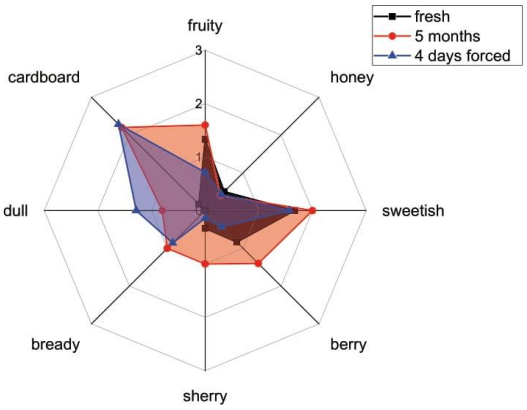

Fig. 3 Quantitative descriptive sensory analysis of fresh, 5-month naturally aged, and 4-day forced-aged lager beer (n = 7)

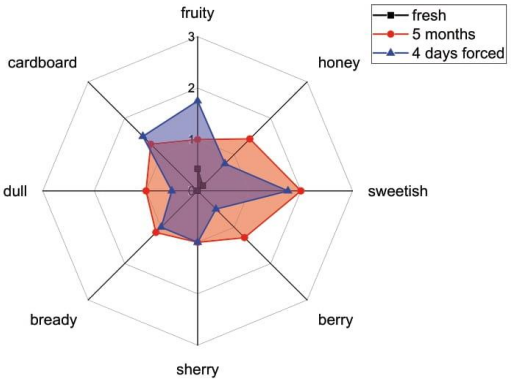

Fig. 4 Quantitative descriptive sensory analysis of fresh, 5-month naturally aged and 4-day forced-aged pilsner beer (n = 7)

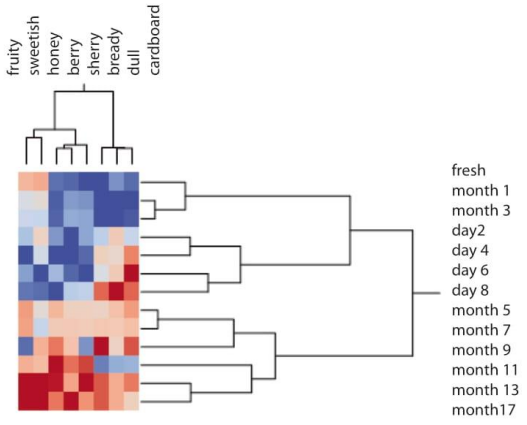

Fig. 5 Two-way hierarchical clustering of sensory ageing attributes

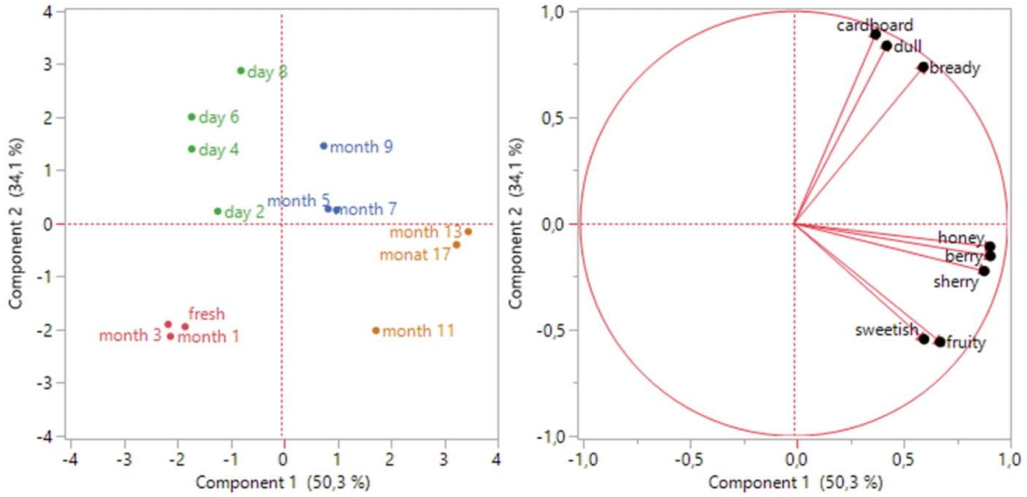

Fig. 6 Principal components analysis of sensory attributes of differently-aged lager beer; left: scores-plot of 13 naturally and forced aged samples; right: loadings-plot of 8 sensory ageing attributes

In both samples, certain attributes (e. g. cardboard) were predicted fairly well by forced ageing at 40 °C, but natural ageing led to a more complex aroma profile. In lager beer, less honey and sherry notes developed compared with the pilsner. It is assumed that the more complex aroma of pilsner can mask certain ageing impressions. Therefore, the lager beer with less masking effects was used for the following experiments.

3.4 Sensory analyses – cluster analysis and PCA

A two-way hierarchical cluster was created for all the investigated samples of aged lager using the Ward method to describe tendencies in the data (Fig. 5). Samples clustered in the second stage into four groups (fresh- and naturally aged beers up to 3 months, forced-aged beers, moderately naturally aged beers, strongly naturally aged beers). The hierarchical cluster of ageing attributes revealed two groups: dull/mouldy (bready, dull, and cardboard) and sweetish (fruity, sweetish, honey, berry, and sherry).

The attributes fruity, honey, sweetish, berry, sherry, bready, dull, and cardboard were then used in PCA (Fig. 6). This multivariate approach was chosen to structure, simplify, and visualize the data set.

Principal Component 1 (PC1, Eigenvalue = 4.02) and PC2 (Eigenvalue = 2.73) showed a cumulative variance of 84.4%. The loading plot separated dull attributes (cardboard, dull, bready) well from fruity/sweetish attributes (fruity, sweetish, sherry, berry, honey). In the score plot, the fresh samples (red) showed greatest distance from the ageing attributes. Forced-aged beers (green) tended toward the dull attributes, whereas strongly naturally aged beers (orange) tended toward the fruity/sweetish attributes. Moderately naturally aged beers lay between the dull and fruity/sweetish attributes.

These findings concur with the time-course trend of aroma during the ageing of conventional beer at 28 °C, as described by Zufall et al. [3]. The attribute cardboard develops after a certain time but disappears at 20 °C resp. 28 °C. Interestingly, we found that at 40 °C, even after 8 days, the cardboard attribute did not disappear.

3.5 Analytical – sum of indicators

Another common way to predict the sensory stability of lager beer is to sum the determined concentrations of indicators that develop due to oxygen, heat, and ageing. Typically, the oxygen indicators (2-methylbutanal (2MB), 3-methylbutanal (3MB), benzaldehyde, and phenylacetaldehyde); heat indicators (furfural and γ-nonalactone); and ageing indicators (3-methylbutanal, furfural, 5-methylfurfural, benzaldehyde, phenylacetaldehyde, diethyl succi-

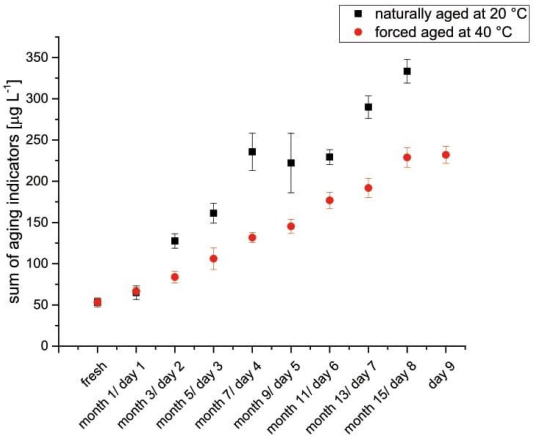

Fig. 7 Sum of ageing indicators after natural ageing at 20 °C and forced ageing at 40 °C

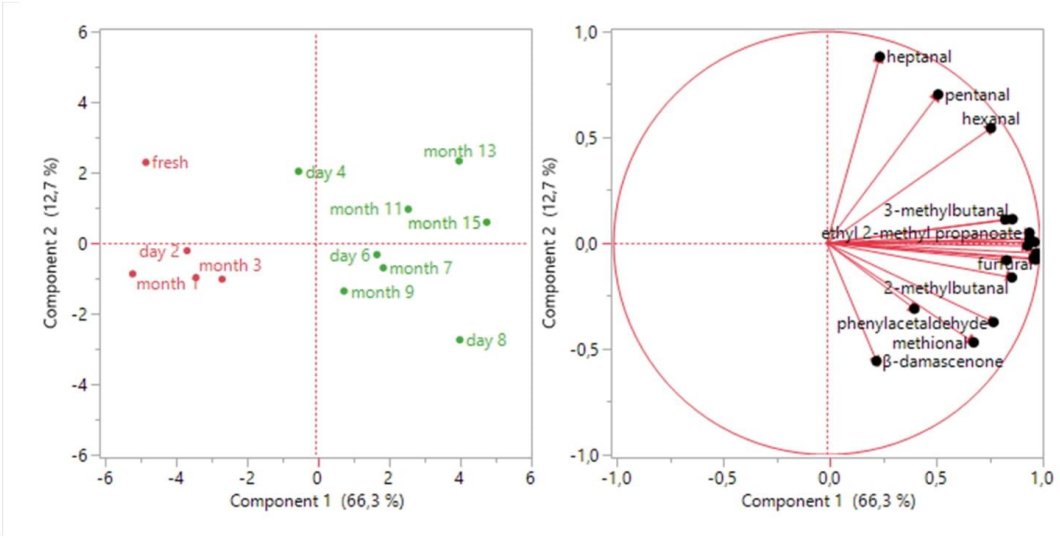

Fig. 8 **Principal components analysis of selected ageing indicators of differently aged lager beer; left: scores-plot of 13 naturally and forced aged samples; right: loadings-plot of 19 chosen ageing indicators**

nate, ethyl 2-phenylacetate, 2-acetyl furan, 2-propionyl furan, and γ-nonalactone) are considered [18]. According to Mitteleuropäische Brautechnische Analysenkommission e. V. (MEBAK) (2.23.4), indicators are analysed after extraction by steam distillation, which might also affect aroma compounds due to heat [25]. Figure 7 shows the sum of all investigated indicators for natural ageing at 20 °C and forced ageing at 40 °C.

For both types of ageing, the sum increased steadily, and 3 to 5 months of natural ageing could be predicted well by 4 days of forced ageing. A few indicators, especially furfural, make up a major portion of the sum and have very high thresholds compared with the other compounds. Therefore, an approach that also takes individual thresholds into account is necessary.

3.6 Analytical part – PCA of ageing indicators

A hierarchical cluster of ageing indicators using the Ward method divided the samples into two groups. The first was made up of fresh and slightly-aged samples (up to 5 months of natural ageing) and the second consisted of moderately- and strongly-aged samples.

PCA of chosen ageing indicators (ethyl 2-methyl propanoate, ethyl 2-methyl butanoate, ethyl 3-methyl butanoate, β-damascenone, γ-nonalactone, ethyl nicotinate, ethyl 2-phenylacetate, furfuryl ethyl ether, 2-methylpropanal (2MP), 2-methylbutanal, 3-methylbutanal, benzaldehyde, phenylacetaldehyde, methional, furfural, pentanal, hexanal, heptanal, and (E)-2-nonenal) was performed (Fig. 8). The resulting groups of cluster analysis were coloured accordingly. Ethyl 4-methyl pentanoate (4MP2), 2-aminoacetophenone, dimethyl trisulfide (DMTS), (E)-2-hexenal, and (E)-2-heptenal were not considered owing to their tendency not to increase during ageing.

PC1 (Eigenvalue = 12.59) and PC2 (Eigenvalue = 2.41) showed a cumulative variance of 79 %. In the loading plot, all indicators pointed toward the right side of the plot, indicating an increase during ageing. In the score plot, the two groups (fresh and slightly-aged samples in red; moderately- and strongly-aged samples in green) were well separated. Interestingly, 4-day forced ageing lay distant from 3- and 5-month natural ageing. Thus, the prediction power of 4-day forced ageing by all analysed indicators is questionable.

3.7 Analytical – time-course trends of selected compounds

As demonstrated above, the naturally aged lager tended to develop fruity/sweetish notes, whereas the forced-aged sample increased in dull attributes like cardboard. To investigate the influence of single compounds on ageing aroma, figure 9 shows the time-

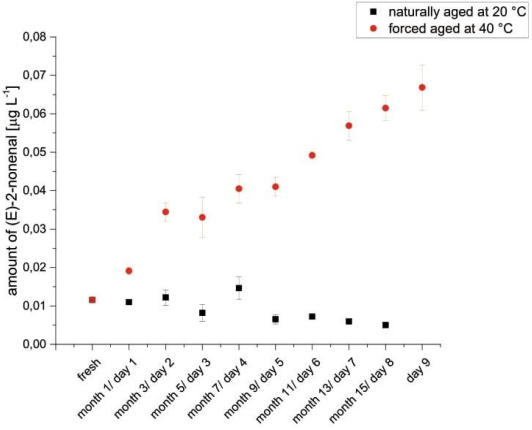

Fig. 9 **Time-course trend of (E)-2-nonenal in lager beer under forced and natural ageing**

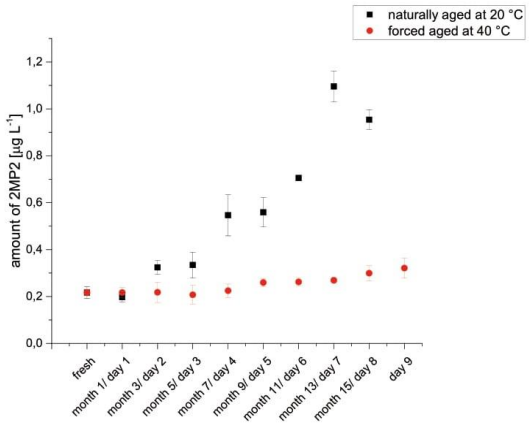

Fig. 10 Time-course trend of ethyl 2-methyl propanoate in lager beer under forced and natural ageing

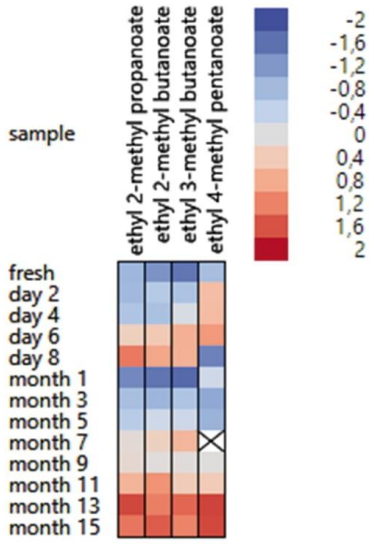

Fig. 11 Analysed volatile humulone degradation products (2MP2, 2MB2, 3MB2, 4 MP2)

course trends in (E)-2-nonenal during natural and forced ageing. Since its discovery in beer [26], this compound alone has been considered to induce a cardboard flavour [27]. However, it has mostly been found under extreme (heated or acidified) conditions [4]. The most prominent determination method for this compound is the nonenal potential method. Drost et al. used a pH of 4.0 to evaluate the potential of pitching wort to generate (E)-2-nonenal at 100 °C over 2 h [27].

In this experiment, it was confirmed that the (E)-2-nonenal concentration kept increasing during forced ageing at 40 °C and even exceeded its threshold concentration of 0.03 ppb. On the other hand, no significant increase was observed during natural ageing. Even so, in some naturally aged beers, cardboard was observed, indicating that (E)-2-nonenal is not solely responsible for the perceived aroma; rather, a complex mixture of aroma compounds is responsible. Therefore, (E)-2-nonenal is not solely responsible for a cardboard flavour in beer and is in no way a good indicator of beer ageing [28]. In the present research, other saturated and unsaturated linear aldehydes derived from lipid oxidation (Fig. 13) also showed no clear tendency to increase during ageing.

The time-course trend of ethyl 2-methyl propanoate during ageing was also investigated (Fig. 10). This compound is derived from (iso)-co-humulones and known to add fruitiness to beers [23]. Under forced ageing conditions, only a slight but steady increase in the concentration of this compound could be observed; however, for natural ageing, there was a more rapid increase. Nevertheless, the flavour threshold concentration of 6.3 ppb was not exceeded [23]. Other esters derived from (iso)-humulones, such as ethyl 2-methylbutanoate and ethyl 3-methyl butanoate, are known to show strong synergistic effects in a mixture [6]. Therefore, this compound is also likely to contribute directly to the aged aroma of naturally aged lager.

3.8 Analytical – time-course trends of all analysed ageing indicators

Time-course trends of all analyzed compounds during ageing were determined. Displayed in the heat maps are the normalized z-scores (Figs. 11–14). Indicators were divided into four groups (humulone degradation products, Strecker aldehydes and Maillard reaction products, saturated and unsaturated linear aldehydes, and other ageing indicators from lactone formation, esterification, and etherification) for clarity.

The humulone-derived esters (ethyl 2-methyl propanoate, ethyl 2-methyl butanoate, ethyl 3-methyl butanoate) showed a continuous increase during forced and natural ageing, although changes in ethyl 3-methyl butanoate were more pronounced. Ethyl 4-methyl pentanoate showed a trend with more fluctuations.

All Strecker aldehydes and furfural showed a continuous increase during forced ageing. During natural ageing, only 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, and furfural kept increasing steadily. Phenylacetaldehyde and methional showed the highest concentrations at 7 months of storage.

Linear saturated and unsaturated aldehydes did not increase during ageing. Only (E)-2-nonenal kept increasing during forced ageing.

The other indicators (β-damascenone, γ-nonalactone, ethyl nicotinate, 2-aminoacetophenone, ethyl 2-phenylacetate, and furfurylethylether [but not dimethyl trisulfide]) also showed continuous increases in concentration during forced ageing. For natural ageing, linear increases were observed for ethyl nicotinate, ethyl 2-phenylacetate, and furfurylethylether.

To assess the "goodness" of the indicators, a linear regression analysis was performed for all analysed compounds. For natural

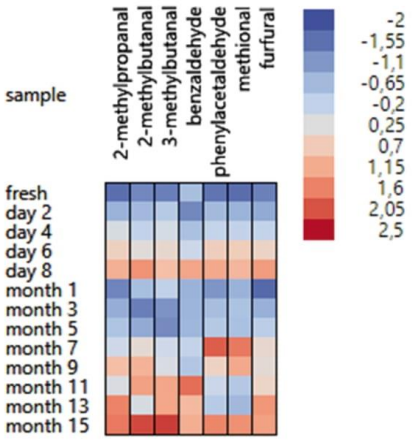

Fig. 12 Analysed Strecker and Maillard reaction products

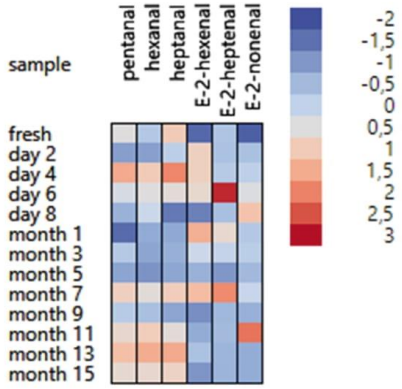

Fig. 13 Saturated and unsaturated linear aldehydes (associated with lipid oxidation)

ageing, ethyl 2-methyl propanoate, ethyl 2-methyl butanoate, ethyl 2-phenylacetate, furfuryl ethyl ether, and furfural showed regression coefficients (R^2 values) greater than 0.9. For forced ageing, R^2 values greater than 0.9 were found for ethyl 2-methyl butanoate, ethyl 3-methyl butanoate, β-damascenone, ethyl nicotinate, ethyl 2-phenylacetate, furfuryl ethyl ether, 2-methylpropanal, 2-methylbutanal, phenylacetaldehyde, furfural, methional, and (E)-2-nonenal. The observation that forced ageing caused more compounds to increase linearly might be because only temperature is elevated in this process; whereas with natural ageing other influences are present.

In order to investigate the direct contribution of a certain compound to the aged aroma in lager, OAVs were calculated by dividing determined concentration by flavour threshold. For forced ageing, only (E)-2-nonenal showed an OAV of > 1. At day 2, an OAV of 1.1 was observed and increased until day 9 (OAV = 2.0). For natural

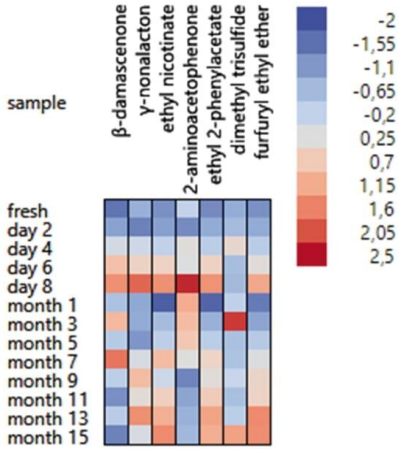

Fig. 14 Other analysed ageing indicators from various reactions (such as lactone formation, esterification, and etherification)

ageing, only methional was found above its threshold, at 7 months (OAV = 1.4) and 9 months (OAV = 1.3).

However, OAVs of < 1 do not imply that other compounds were not aroma active. For instance, ethyl 2-methyl butanoate, ethyl 3-methyl butanoate, 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal must also be considered in the development of an aged aroma during natural ageing. After 15 months, they would show OAVs of ~0.2 to 0.3. Research by Saison et al. showed strong additive effects not only in mixtures of certain esters but also for mixtures of aldehydes (2-methylbutanal) and esters (ethyl 3-methyl butanoate). Furthermore, these mixtures showed combinatory effects as the flavour was described as “winey, candy, caramel, and fruity” [6].

4 Conclusion

In combination with overall ageing sensory analysis (DLG scores and acceptancy) and summary determination of analytical ageing indicators, the prediction of the sensory stability of lager and pilsner beers by forced ageing is a valid method. However, it was demonstrated that the aroma profiles of forced-aged lager and pilsner differ from naturally aged samples. Forced ageing leads to the development of mainly dull notes (cardboard and bready), whereas natural ageing leads to fruity/sweetish attributes (fruity and berry). Repeating the analysis with a greater number of tasters will lead to results that are more significant. The fruity/sweetish notes in natural ageing could be linked to the compounds 2MP2, 2MB2, ethyl 2-phenylacetate, and furfuryl ethyl ether, each of which has a fruity aroma in pure form. However, a lack of compounds such as (E)-2-nonenal will influence the aroma in the same manner.

GC-O as a rapid prediction method proved useful in determining whether the pilsner and lager experienced ageing; although the degree of ageing could not be evaluated. It is also noted that intense panellist training is necessary, especially for rating intensities, as results will be significantly affected by the ability of the panelists.

The assessment of ageing based on the presence of certain compounds such as 2MP2 will also be limited to certain beer styles.

Based on the results of the investigated lager beer, levels of 2MP2, 2MB2, ethyl 2-phenylacetate, furfuryl ethyl ether, and furfural were observed to increase linearly ($R^2 > 0.9$) during natural ageing and were therefore regarded as powerful ageing indicators. In forced ageing, levels of 2MB2, 3MB2, β-damascenone, ethyl nicotinate, ethyl 2-phenylacetate, furfuryl ethyl ether, 2MP, 2MB, phenylacetaldehyde, furfural, methional, and (E)-2-nonenal increased linearly. These results should not be projected to every sample and beer style. Rather, each brewery should be aware that the prediction of sensory stability by forced ageing will lead to significant differences in aroma profile and analytical indicators. The methods should be assessed critically, and sensory and analytical results of natural and forced ageing should be reconciled. Based on the geographical area and average temperature, an appropriate forced ageing temperature should be chosen. As such, the most powerful predictions will be achieved with the most significant indicators.

5 Literature

1. Pankoke, I.: Securing beer and beverage quality during long distance distribution process – Using a special sensor system, 35th EBC Congress, Porto, 2015.
2. Dalgliesh, C. E.: Flavour stability. Proceedings of the European Brewery Convention Congress, Amsterdam, DSW, Dordrecht: The Netherlands (1977), pp. 623-659.
3. Zufall, C.; Racioppi, G.; Gasparri, M. and Franquiz, J.: Flavour stability and ageing characteristics of light-stable beers, Proceedings of the 30th EBC Congress (2005), pp. 617-624.
4. Vanderhaegen, B.; Neven, H.; Verachtert, H. and Derdelinckx, G.: The chemistry of beer aging – a critical review, Food Chemistry, **95** (2006), no. 3, pp. 357-381.
5. Baert, J. J.; Clippeleer, J. de; Hughes, P. S.; Cooman, L. de and Aerts, G.: On the Origin of Free and Bound Staling Aldehydes in Beer, Journal of Agricultural and Food Chemistry, **60** (2012), no. 46, pp. 11449-11472.
6. Saison, D.; Schutter, D. P. de; Uyttenhove, B.; Delvaux, F. and Delvaux, F. R.: Contribution of staling compounds to the aged flavour of lager beer by studying their flavour thresholds, Food Chemistry, **114** (2009), no. 4, pp. 1206-1215.
7. Herrmann, M.; Klotzbücher, B.; Wurzbacher, M.; Hanke, S.; Kattein, U.; Back, W. et al.: A New Validation of Relevant Substances for the Evaluation of Beer Aging Depending on the Employed Boiling System, Journal of the Institute of Brewing, **116** (2010), no. 1, pp. 41-48.
8. Evans, D. J., Schmedding, D. J. M.; Bruijnje, A.; Heideman, T.; King, B. M. and Groesbeek, N. M.: Flavour Impact of Aged Beers, Journal of the Institute of Brewing, **105** (1999), no. 5, pp. 301-307.
9. Wurzbacher, M.: Untersuchungen zum Einfluss antioxidativer Substanzen auf die Geschmacksstabilität des Bieres, Dissertation TU München, 2011.
10. Thiele, F.: Einfluss der Hefevitalität und der Gärparameter auf die Stoffwechselprodukte der Hefe und auf die Geschmacksstabilität (2006).
11. Eger, C.; Habich, N.; Akdokan, N.; Goßling, U. and Bellmer, H.-G.: Profiling of beers during ageing at different temperatures, Proceedings of the 30th EBC Congress (2005).
12. Lermusieau, G.; Noël, S. and Collin, S.: Nonoxidative Mechanism for Development of trans-2-Nonenal in Beer, Journal of the American Society of Brewing Chemists, **57** (1999), no. 1, pp. 29-33.
13. Blanco, C. A.; Nimubona, D. and Caballero, I.: Prediction of the ageing of commercial lager beer during storage based on the degradation of iso-alpha-acids, Journal of the science of food and agriculture, **94** (2014), no. 10, pp. 1988-1993.
14. Lehnhardt, F.; Gastl, M. and Becker, T.: Forced into aging: Analytical prediction of the flavor-stability of lager beer. A review, Critical reviews in food science and nutrition, (2018), pp. 1-35.
15. Li, H.; Liu, F.; He, X.; Cui, Y. and Hao, J.: A study on kinetics of beer ageing and development of methods for predicting the time to detection of flavour changes in beer, Journal of the Institute of Brewing, **121** (2015), no. 1, pp. 38-43.
16. Wietstock, P. C.; Kunz, T. and Methner, F.-J.: The Relevance of Oxygen for the Formation of Strecker Aldehydes during Beer Production and Storage, Journal of Agricultural and Food Chemistry, (2016).
17. Bravo, A.; Herrera, J. C.; Scherer, E.; Ju-Nam, Y.; Rubsam, H.; Madrid, J. et al.: Formation of alpha-dicarbonyl compounds in beer during storage of Pilsner, Journal of Agricultural and Food Chemistry, **56** (2008), no. 11, pp. 4134-4144.
18. Lustig, S.: Das Verhalten flüchtiger Aromastoffe bei der Lagerung von Flaschenbier, 1995.
19. Saison, D.; Vanbeneden, N.; Schutter, D. P. de; Daenen, L.; Mertens, T.; Delvaux, F. et al.: Characterisation of the Flavour and the Chemical Composition of Lager Beer after Ageing in Varying Conditions, BrewingScience – Monatsschrift für Brauwissenschaft, **63** (2010), no. 3/4, pp. 41-53.
20. Back, W.: Ausgewählte Kapitel der Brauereitechnologie, Fachverlag Hans Carl GmbH, Nürnberg, 2005.
21. Saison, D.; Schutter, D. P. de; Delvaux, F. and Delvaux, F. R.: Optimisation of a complete method for the analysis of volatiles involved in the flavour stability of beer by solid-phase microextraction in combination with gas chromatography and mass spectrometry, Journal of chromatography. A, **1190** (2008), no. 1-2, pp. 342-349.
22. Meilgaard, M. C.: Flavor chemistry of beer, part II: flavor and thresholds of 239 aroma volatiles, Tech. Q. Master Brew. Assoc. Am., **12** (1975), no. 3, pp. 151-168.
23. Kishimoto, T.; Wanikawa, A.; Kono, K. and Shibata, K.: Comparison of the odor-active compounds in unhopped beer and beers hopped with different hop varieties, Journal of Agricultural and Food Chemistry, **54** (2006), no. 23, pp. 8855-8861.
24. Eichhorn, P.: Untersuchungen zur Geschmacksstabilität des Bieres, 1991.
25. Jacob, F.: MEBAK (Würze, Bier, Biermischgetränke), MEBAK, Freising-Weihenstephan, 2012.
26. Palamand, S. R. and Hardwick, W. A.: Studies on the relative flavour importance of some beer constituents, Tech. Q. Master Brew. Assoc. Am. (1969), no. 6, pp. 117-128.
27. Drost, B. W.; van den Berg, R.; Freijee, F. J. M.; van der Velde, E. G. and Hollemans, M.: Flavor Stability, Journal of American Society of Brewing Chemists (1990), no. 48, pp. 124-131.
28. Schieberle, P. and Komarek, D.: Staling of beer aroma: a long-known, but still unresolved challenge in brewing science, Proceedings of the 30th EBC Congress (2005).

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3.2.2 Flavor stability assessment of lager beer: what we can learn by comparing established methods

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ORIGINAL PAPER

Flavor stability assessment of lager beer: what we can learn by comparing established methods

Florian Lehnhardt[1] · **Thomas Becker**[1] · **Martina Gastl**[1]

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Abstract
Beer is prone to flavor changes during aging that influence consumer acceptance within shelf life. The shelf life of beer is defined as the period over which flavor changes remain acceptable. Assessment of flavor changes caused by volatiles is typically achieved with a combination of sensory evaluation and gas chromatography–mass spectrometry (GC–MS). Volatile indicators causing flavor changes during beer aging are commonly determined with headspace solid–phase microextraction (HS-SPME), solvent-assisted flavor evaporation (SAFE), or steam distillation (SD). However, discrepancies occur when comparing results from different analytical methods that affect the assessment of the degree of flavor stability. This article discusses the effect of different established analytical methods on flavor stability assessment. Reaction potentials of de novo formation, release from adducts, and degradation are hypothesized to participate in the observed discrepancies, and evidence is verified using model systems. Three extraction methods were qualitatively compared by multiple gas chromatography–olfactometry experiments (GC–O) of a one-year, naturally aged, pale lager beer. SD showed the highest number of detected aroma compounds (41), followed by HS-SPME (33), and SAFE (26). Aroma intensities for SD were more pronounced for most aging indicators than with other methods. With SAFE, only 11 aging compounds could be identified confidently, with weak aroma intensities at GC–O, and this method was thereby excluded from further experiments. Certain aging compounds were calibrated for gas chromatography-mass spectrometry (GC–MS) from HS-SPME and SD, although most compounds were present at the lower limits of detection and quantification. Relative standard deviation and recoveries for all compounds were acceptable for both methods. Quantitative comparison was conducted for four different commercial pale lager beers at different stages of aging at 20 °C (fresh, 5 months, 10 months). Aging-related changes of pale lager beer presented with altered profiles and behavior in SD compared to the non-invasive HS-SPME due to heat intake, and were borne out by GC–O results. Model systems were used to describe the impact of isolated aging-relevant mechanisms and precursors during distillation. Our findings suggest that results from different methods in reactive matrices should be compared cautiously, especially regarding aroma activity, and indicate that the most gentle or non-invasive method should be applied for analysis.

Keywords Beer aging · Flavor stability · Solid-phase microextraction · Steam distillation · Solvent-assisted flavor evaporation · Bound-state aldehydes

Introduction

Sensory or flavor stability of lager beer is gaining importance due to growing consumer awareness in competitive and globalized beer markets [1]. During aging, desirable aroma compounds such as isoamyl acetate decrease [2] while undesirable aroma compounds increase in concentration. The main indicators thereof are aldehydes from Maillard reaction, Strecker degradation, and lipid oxidation. Other aroma compounds, such as hop degradation products, ketones, lactones, and ethyl esters also increase over time and contribute to an aged flavor [3]. The shelf life of beer

✉ Martina Gastl
Martina.Gastl@tum.de

Florian Lehnhardt
florian.lehnhardt@tum.de

[1] Chair of Brewing and Beverage Technology, Technische Universität München, Weihenstephaner Steig 20, 85354 Freising, Germany

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is the length of time it can be stored before flavor changes render the product undesirable and is defined individually by each brewery for each product [4].

Saison et al. investigated the flavor units (FU) of a range of aging compounds in different lager beers. They found that acetaldehyde, (E)-2-nonenal, 3-methylbutanal, methional, diacetyl, furfuryl ethyl ether, and β-damascenone exhibited FUs of more than one in at least one of the three analyzed lager beers after forced aging (3 weeks at 40 °C). Thus, these compounds were discussed to have direct impact on the sensory properties of these products. In the same study interactive effects of aging aldehydes were elucidated, highlighting the sensory impact of Strecker aldehydes. An exception to this was benzaldehyde, which showed FUs lower than 0.005 [5]. In another study, methional was the only compound to show odor activity values above 1 in a naturally aged lager beer [6].

The formation process of volatile aging compounds is highly complex, involving many physicochemical influences, such as temperature, time, pH, and oxygen level, in packaged beer, in the malt, and in the brew house [3]. Furthermore, these mechanisms are interdependent and in equilibrium with non-volatiles throughout the brewing process [7, 8].

Assessment of flavor stability of lager beer is typically done by gas chromatography (GC) and sensory analysis. When assessing sensory stability by GC, different methods are used to determine volatile organic compounds (VOC), each with their own advantages and disadvantages. The ideal method would indiscriminately extract all key aroma compounds without modifying any of the VOCs.

Common techniques thereof used in laboratory practice for raw materials and beer are solvent extraction, distillation, headspace, and sorptive techniques, alone or in combination.

In solvent-assisted flavor evaporation (SAFE), solvent extracts (usually diethyl ether) are distilled under vacuum to separate volatile and non-volatile fractions. The volatiles are trapped using liquid nitrogen and further concentrated at low temperatures. Organic acids can be removed by washing with sodium carbonate and residual water by addition of sodium sulfate. Liquid foodstuffs can be distilled directly, whereas distillates can be extracted with solvents later [9]. This method is used to monitor volatiles during beer aging [10–12] and in flavor dilution assays.

In steam distillation (SD), samples are distilled at 100 °C, and distillates are extracted using organic solvents alkaline pH. Release of VOCs can be enhanced with the addition of sodium chloride. The extract is concentrated under a nitrogen stream and then analyzed via GC. This is also a reference method of MEBAK (Mitteleuropäische Brautechnische Analysenkommission) for the determination of aging compounds in wort and beer [13].

Headspace techniques (dynamic or purge and trap) extract the headspace above a given sample either statically or dynamically, possibly with enrichment on sorptive material. Studies have successfully applied such techniques for hops and aged beer [14, 15].

Solid-phase microextraction (SPME) and stir bar sorptive extraction (SBSE) are sorptive methods with adsorption enrichment of VOCs on a fiber and can be used to supplement headspace and liquid extractions. HS-SPME [16–20] and SBSE [21–23] are frequently used for volatiles in beer and wine, often with derivatization steps, such as *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) for the enhanced determination of aldehydes.

Previous studies detail multiple comparisons of analytical methods, each specific to the given matrix and analyzed compounds. However, no study has yet compared established methods for assessing aging indicators in beer both qualitatively and quantitatively.

Richter et al. compared HSSE (headspace sorptive extraction), SBSE, HS-SPME, and SAFE on hop volatiles in beer and found that SAFE favors more alcohols and acids compared to the sorptive methods. When comparing extraction capacities, HSSE was better suited for esters and aldehydes, and SBSE for acids. HS-SPME extracted fewer compounds overall due to its limited surface area [24].

In another study, SBSE and SD were compared in detecting esters and organic acids in beer samples. Although the methods showed favorable correlation, SBSE was more sensitive to esters, while SD was more sensitive to higher alcohols, such as 2-phenylethanol [25]. The same authors also compared SBSE with SPME and found them to be similar in terms of linearity, recovery, and repeatability. In this study, HS-SPME proofed superior to SBSE due to shorter times of analysis [26].

Thompson-Witrick et al. compared HS-SPME and SAFE for aroma-active compounds using GC–O in lambic beer. SAFE extracted more organic acids, while HS-SPME isolated more esters due to their higher volatility [27].

Thus, it can be concluded that the applied extraction technique influences the obtained results both qualitatively and quantitatively (each method having its advantages and disadvantages). In a complex sample such as beer, where precursors for de novo synthesis as well as bound-state aroma compounds are present, it is also likely that temperature, pH shifts, and solvent extraction affect the extracted VOC profile. Accordingly, this study evaluated the influence of the extraction method in beer aging quantification via aroma-active compounds using GC–O to assess the profile of compounds extracted by each method. Extraction methods were then compared quantitatively over the course of aging and to gain insight into their differences using model distillation systems.

Materials and methods

Chemicals

The chemicals, *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (≥ 99%), ethyl 2-methyl propanoate (99%), ethyl 2-methyl butanoate (99%), ethyl 4-methyl pentanoate (≥ 97%), β-damascenone (≥ 98%), γ-nonalactone (98%), 2-aminoacetophenone (98%), ethyl 2-phenylacetate (99%), ethyl nicotinate (99%), 2-methylpropanal (≥ 99.5%), 2-methylbutanal (95%), 3-methylbutanal (97%), 2-phenylacetaldehyde (≥ 90%), methional (≥ 97%), benzaldehyde (≥ 99.5%), pentanal (≥ 97.5%), hexanal (98%), heptanal (95%), (E)-2-nonenal (97%), acetaldehyde (≥ 99.5%), isovaleric acid (99%), D-(+)-xylose (≥ 99%), L-arginine (≥ 98%), L-lysine (≥ 98%), L-leucine (≥ 98%), 2-(furan-2-yl)-1,3-thiazolidine-4-carboxylic acid (≥ 98%), and methylglyoxal (~ 40% in H_2O) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ethyl 3-methyl butanoate (≥ 99.7%, Fluka Analytical), dimethyl trisulfide (≥ 98%, SAFC), furfuryl ethyl ether (95%, Fluorochem), 2-furfural (≥ 99.0%, Fluka Analytical), ammonia (25%, VWR International S.A.S., Leuven, Belgium), dichloromethane (for HPLC, VWR International S.A.S., Leuven, Belgium), sodium chloride (VWR International S.A.S., Leuven, Belgium), DCHA-Iso ICS-I4 (65.2% w/w; Labor Veritas, Zurich, Switzerland), and diethyl ether (for analysis, Merck, Darmstadt, Germany) were obtained from the indicated manufacturers. 2-(isobutyl)-1,3-thiazolidine-4-carboxylic acid was synthesized as previously described and confirmed by ^{1}H-NMR [28].

Qualitative and quantitative method comparison

Internal standards

An internal standard mixture (~ 1 mg L^{-1} of ethyl 2-methyl pentanoate, methyl undecanoate, and *p*-fluorobenzaldehyde in ethanol) was used for quantification. Ethyl 2-methyl pentanoate was used for quantification of highly volatile, underivatized compounds; methyl undecanoate for moderately volatile, underivatized compounds; and *p*-fluorobenzaldehyde was used for all aldehydes (derivatized compounds). Figure 4 indicates which internal standard was used for the respective compounds. Standards were prepared anew for every measurement.

HS-SPME procedure

Five mL of cooled, unfiltered sample were placed in a 20-mL headspace vial and incubated at 40 °C together with 50 µL of internal standard. HS-SPME extraction was performed as described by Saison et al. [19] using a CAR–PDMS–DVB fiber. The fiber was injected splitless at 270 °C into the GC.

SD procedure

Steam distillation was performed according to MEBAK 2.23.4. Briefly, 2 mL of internal standard mixture together with 5 mL ethanol p. a. were spiked into 200 mL of cooled sample. Using a Büchi K-355 distillation apparatus (BÜCHI Labortechnik AG, Flawil, Switzerland), the sample was distilled for 4.5 min, and 100 mL of distillate were collected. Twenty mL of distillate were then removed. Subsequently, 20.8 g of sodium chloride, 4 mL of ammonia (25%) and 1 mL of dichloromethane were added to the sample. The sample was shaken for 30 min and then centrifuged at 0 °C and 2400 rpm for 15 min. The organic phase was transferred to a vial, concentrated under nitrogen, and 2 µL were injected at a 1/5 split at 250 °C into the GC.

SAFE procedure

The SAFE extraction was performed according to previous reports [9]. Briefly, 200 mL of beer sample, 2 mL of internal standard mixture, and 2 g of sodium chloride were poured into the SAFE apparatus (Glasbläserei Bahr, Manching, Germany). The distillation was performed at 40 °C and under vacuum at $< 9 \times 10^{-6}$ mbar, and the distillate was trapped in a flask cooled with liquid nitrogen. The distillate was then extracted with diethyl ether (3 × 100 mL), organic acids were removed by washing with saturated sodium carbonate solution (2 × 20 mL), and the sample was dried with $NaSO_4$. The extract was concentrated to 1 mL in a Vigreux column at 42 °C, and 2 µL were injected at a 1/5 split at 250 °C into the GC.

GC–MS parameters

The GC (GC-Ultra 1300, Thermo Scientific Inc., Waltham, MA, USA) was equipped with a DB-5 column (length, 60 m; inner diameter, 0.25 mm; film thickness, 0.25 µm; Thermo Scientific Inc., Waltham, MA, USA) and two split/splitless injectors. Helium was used as the carrier gas (flow rate 1.85 mL/min). The initial temperature was maintained at 60 °C for 4 min, followed by heating at 5 K/min up to a final temperature of 250 °C, which was held for 3 min.

The GC was coupled to a single quad mass spectrometer (ISQ QD, Thermo Scientific Inc., Waltham, MA, USA) via a transfer line that was heated to 250 °C. Ionization was achieved in EI mode. A full scan mode (m/z 35–350) with a dwell time of 0.02 s was applied for the analysis. Each sample was analyzed in triplicate. Peak detection was performed in Xcalibur 3.1.66.10 (Thermo Scientific Inc., Waltham,

MA, USA), and identification was performed via the addition of pure compounds and the NIST database.

GC–O

The naturally aged sample (pale lager beer, aged for one year at 20 °C) was extracted by SAFE, SD, and HS-SPME as described above. Olfactometry assessment was performed by two panelists in multiple repetitions ($n = 5$–7), trained specifically on aging aromas using different sniffing samples. The olfactometry system used in this study was an ODP 3 (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany). It was equipped with a temperature controller heated to 250 °C (C200, Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany). The makeup gas was synthetic air that was humidified (10 mL/min. The gas stream was splitted equally using a y-splitter after the GC column, one part going to the MS, the other via a transfer line (heated to 250 °C) to the olfactory port. The lengths of the capillaries were adjusted to compensate any pressure differences between MS and olfactory port (at atmospheric pressure). Time, quality, and intensity of odors (1 = weak; 2 = medium; 3 = high intensity) were recorded manually by another person. The means of the odor intensities that were perceived reproducibly (more than once in 5–7 experiments) are displayed in Figs. 1–3 and summarized in Table 1.

Calibration and validation

Standard addition was used for calibration of SD and HS-SPME to minimize matrix effects and changes during sample preparation. Standard solutions (target compounds in ethanol) were added to fresh pale lager at ten different dilutions and measured as technical duplicates. For each compound, a calibration range was determined with a linearity > 0.99. Relative standard deviation (RSD) was assessed in a five-fold replicate analysis of a 1-year-aged (20 °C) pale lager beer sample. Recoveries were prepared by adding a medium calibration concentration to a beer sample in duplicate, and accepted if the values were between 80 and 110%. Limit of detection (LOD) and limit of quantification (LOQ) were determined as previously described using the standard deviation of the y-intercept (σ; peak area of analytes divided by peak area of internal standard) and the slope (s) of the calibration curve (LOD = 3.3 σ/s; LOQ = 10 σ/s) [29]. The LOD and LOQ determined by visual examination were far lower than the indicated values in Fig. 4 as red and black lines respectively.

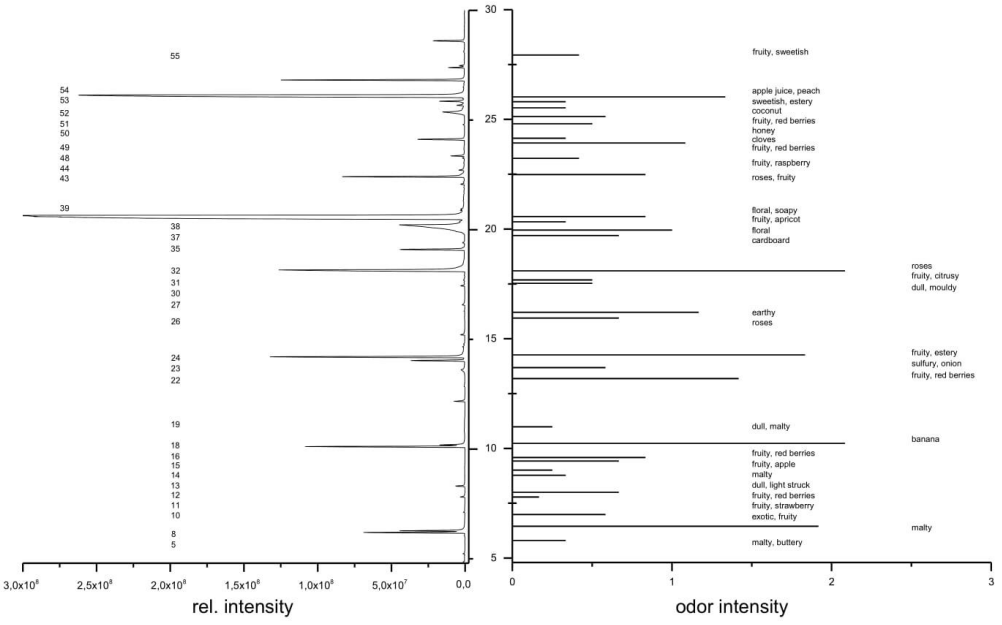

Fig. 1 Aromagram after SPME extraction. Left, GC–MS chromatogram with detected compounds (numbers correspond with compounds in Table 1); right, odor descriptions and intensities for detected compounds ($n = 6$)

Beer samples

All beer samples were purchased in the freshest condition possible. The pale lager beer investigated with GC–O was aged for 12 months at 20 °C prior to analysis. It had 4.82 vol.% alcohol, a color of 6.8 EBC, a pH of 4.57, and 20 IBUs. For quantitative comparison, four commercially available beer samples (pale lager beers) were purchased and aged naturally at 20 °C. All samples were brewed in accordance with German purity laws. Samples were analyzed fresh, at 5 months, and at 10 months.

Breakdown, formation, and release reactions in model systems

Model distillations were carried out in phosphate buffer at pH 4.5 according to the MEBAK method [13]. Blank runs were used to identify fragments from the sample workup. Possible breakdown reactions were investigated by a methional system (0.02 mM). Possible formation reactions were investigated for ethyl 3-methylbutanoate (40 mM isovaleric acid; 0.01 mM DCHA), furfural (1.3 mM xylose; 1.3 mM xylose and 0.4 mM arginine/lysine; 0.1 mM FURFCys), and 3-methylbutanal (2 mM leucine and 3 mM methyl glyoxal; 0.02 mM 3-MB-Cys). Semi-quantitative yield rates were achieved by 1-point calibration in duplicate of the corresponding target compound.

Data analysis

Except as otherwise stated, all samples were analyzed in triplicate and means and standard deviations were calculated. Vapor pressures were obtained from www.pubchem.ncbi.nlm.nih.gov. Two-way ANOVAs were performed to uncover statistical differences within sample sets. Tukey–Kramer's test was used to further divide statistical groups.

Results and discussion

Qualitative comparison using GC–O

Aromagrams for the three extraction techniques (Figs. 1–3) covered in this study are presented individually and then compared with one another subsequently. On the left of each aromagram the GC chromatograms and on the right the aroma descriptors of the detected compounds and aroma intensities of GC–O are indicated. This makes it possible to quickly match the detected aroma compounds with their odor impression. The numbers of the assigned compounds on the left in the aromagrams match those for the compounds in Table 1, wherein all compounds can be compared directly among methods.

HS-SPME

HS-SPME uncovered 33 volatile aroma compounds in the aged sample, 26 of which could be identified by retention index, odor impression, and mass spectrum. Most were typical beer aroma compounds such as 3-methyl butanol, 3-methylbutyl acetate, linalool, and 2-phenylethanol. Thirteen aging related aroma compounds were also detected; seven esters, two aldehydes, and dimethyl trisulfide, 2-aminoacetophenone, γ-nonalactone, and β-damascenone. Figure 1 shows an aromagram of the HS-SPME analysis ($n=6$).

SD

SD uncovered 41 volatile compounds of which 31 were identified, four were tentatively identified, and six remained unknown. Nineteen of the detected aroma compounds are known as aging compounds and comprised four aldehydes, nine esters, 2,4,5-trimethyl-1,3-dioxolane, diethoxyethane, dimethyl trisulfide, 2-aminoacetophenone, γ-nonalactone, and β-damascenone. Figure 2 shows the aromagram of the SD analysis ($n=5$).

SAFE

The SAFE procedure yielded 26 detected aroma compounds in GC–O, but only 11 could be identified by retention index, odor impression, and mass spectrum. Thirteen aging compounds were detected and comprised six esters, three aldehydes, and diethoxyethane, dimethyl trisulfide, 2-aminoacetophenone, and β-damascenone. Figure 3 shows the aromagram of the SAFE analysis (n = 7).

Summary of GC–O

GC–O allows the coupling of gas chromatographic separation and sensory evaluation for a comprehensive analysis of possible aroma-active compounds. In this study, three common extraction methods were compared, with different underlying physicochemical principles. Data comparison was considered valid because peak areas were generally comparable. For the detected compounds, qualitative, and semi-quantitative differences in odor intensity were observed among the methods. HS-SPME results most resembled the actual composition of the headspace above the sample at 40 °C without solvents and were thereby comparable to human orthonasal olfaction. SD (high temperature intake) and SAFE (low temperature intake) reflect volatiles extracted by dichloromethane (DCM) and diethyl ether (DEE), respectively. As such, the most important factor for HS-SPME is volatility, whereas that for SD and SAFE is solubility in the applied solvent. In addition, extraction enrichment occurs during HS-SPME. For

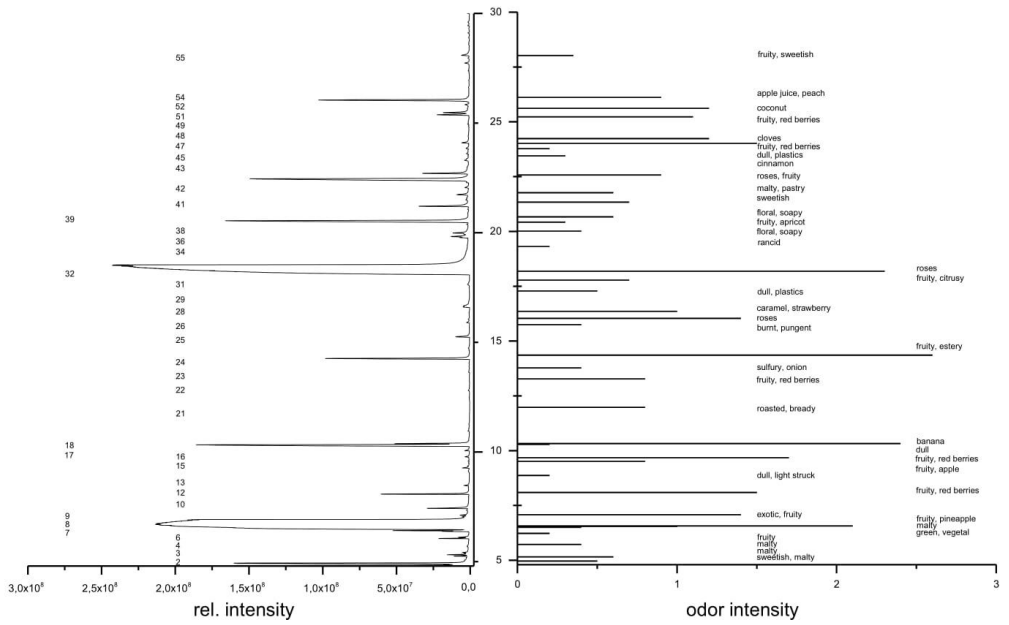

Fig. 2 Aromagram after SD extraction. Left, GC–MS chromatogram with detected compounds (numbers correspond with compounds in Table 1); right, odor descriptions and intensities for detected compounds ($n=5$)

SD and SAFE, no selective enrichment occurs resulting in significant concentration differences among volatiles. This can lead to saturation of certain compounds (see peak shapes of 3-methylbutanol or 2-phenylethanol in Figs. 2, 3) and limitations in concentration of the liquid extract. Different methods can be applied and the results combined to overcome discrepancies among methods and the resulting under- or overestimation of specific aroma contributions [30]. Highly and moderately volatile compounds were favored in HS-SPME, although in a complex matrix, such as beer, some of these compounds might be formed, degraded, or discriminated.

In this study, 3-methylbutanol (0.4 kPa at 20 °C) was detected at the highest intensity by HS-SPME. In contrast, the less polar and more volatile 3-methylbutanal (3-MB; 6.1 kPa at 20 °C) was not detected by HS-SPME, but was detected by SD. These results suggest that 3-MB is formed during thermal distillation at 100 °C, as gentle distillation under vacuum at 40 °C did not result in perceivable amounts of 3-MB.

The two acetals 2,4,5-trimethyl-1,3-dioxolane (acetaldehyde + 2,3-butanediol) and diethoxyethane (acetaldehyde + 2 molecules of ethanol) also apparently formed during distillation at higher temperatures, as these compounds were only detected at low concentrations by HS-SPME and SAFE

Given the fact that ethyl 3-methylbutanoate (3MB2) and ethyl 2-methylpropanoate (2MP2) show higher threshold in literature compared to ethyl 2-methylbutanoate (2MB2) and both former ones were detected more intensely in GC–O it was concluded that 3MB2 and 2MP2 were present in higher concentrations than 2MB2 [31]. This is most likely due to the higher amounts of humulone precursors in finished beer. Degradation thereof appeared to be temperature-dependent.

γ-nonalactone showed the highest odor intensity in SD, which also indicates its formation from precursors during distillation. The same observation was made for acetylfuran, and was tentatively made for benzothiazole in SD.

Conversely, methional was only detected in SAFE, indicating too little sensitivity in HS-SPME for this compound and its degradation at higher temperatures. It was not detected by HS-SPME. (E)-2-nonenal (T2N) was apparently degraded during distillation.

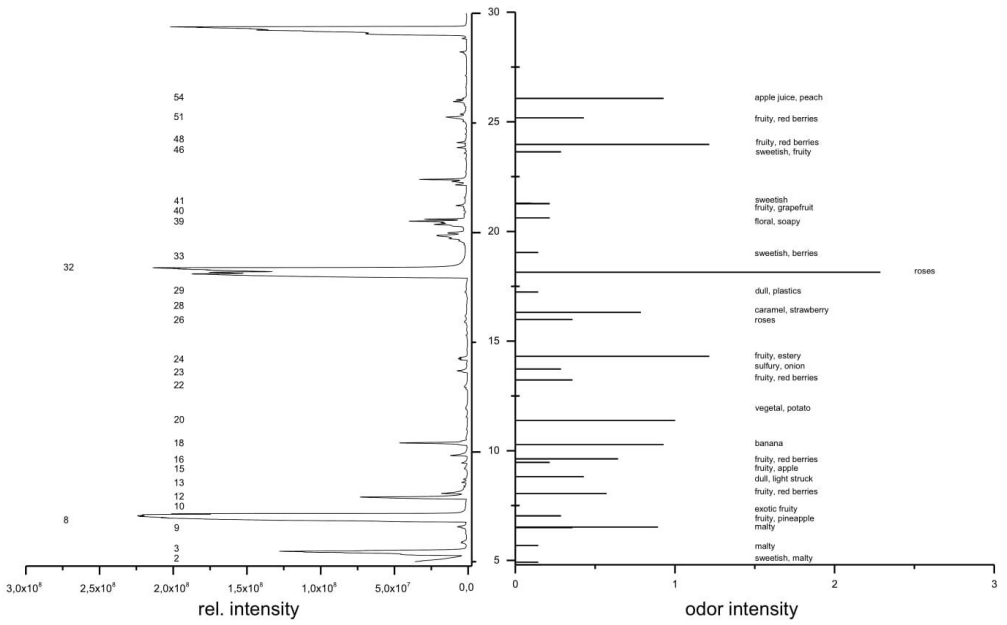

Fig. 3 Aromagram after SAFE extraction. Left, GC–MS chromatogram with detected compounds (numbers correspond with compounds in Table 1); right, odor descriptions and intensities for detected compounds ($n=7$)

Quantitative comparison

Due to low odor intensity and difficulties in identifying aroma compounds, SAFE was further excluded from quantitative comparisons even though it has been used in the quantification of aging compounds in beer in previous work; of note, one such study utilized GC–MS [10].

Below quantitative comparisons of HS-SPME and SD of four different samples, after calibration and validation of each method are displayed.

Calibration and validation

SPME and SD were compared using a 10-point matrix-assisted calibration curve ($y = \text{area}_{\text{analyte}}/\text{area}_{\text{internal standard}}$; $x = \text{concentration}_{\text{analyte}}$ [µg L^{-1}]) prepared for chosen compounds relevant to beer aging [6]. Three internal standards were applied: ethyl 2-methylpentanoate (A), methyl undecanoate (B), and p-fluorobenzaldehyde (C). To further characterize the methods, correlation coefficient of calibration curve (R^2), LOD, LOQ, RSD and recovery rate were assessed.

Figure 4 shows the calibrated ranges for HS-SPME and SD for each compound. LOD (red) and LOQ (black) are represented by vertical lines.

One-way ANOVA showed the means of the lowest points of the calibration curve were not significantly different at $\alpha = 0.05$ (HS-SPME, 0.84 µg L^{-1}; SD, 4.11 µg L^{-1}; $p = 0.094$); the same was found for the highest points of the calibration curve (HS-SPME, 42.3 µg L^{-1}; SD, 40.0 µg L^{-1}; $p = 0.875$), the mean of R^2 (HS-SPME, 0.995; SD, 0.994; $p = 0.721$), and the mean of RSD (HS-SPME, 6.9%; SD, 6.1%; $p = 0.16$). SD tended to yield lower RSD values, likely due to the higher volumes of sample and internal standard and therefore minimized sampling error compared to HS-SPME.

The means of LOD and LOQ also showed no statistical differences (mean LOD HS-SPME, 1.22 µg L^{-1}; mean LOD SD, 2.65 µg L^{-1}; $p = 0.12$). For most compounds, however, HS-SPME yielded lower LODs and LOQs, with the exception of the less volatile ethyl cinnamate, benzaldehyde, and phenylacetaldehyde, among others. This was especially pronounced for highly volatile compounds such as 2MB2 or 2-methylbutanal. Nonetheless, recoveries were acceptable for all calibrated compounds.

Table 1 Compound number, retention index, odor impression, and odor intensity (mean of all experiments); identification by retention index, odor impression, and mass spectrum (compared to NIST database)

Compound number	Retention index	Compound	Odor impression	Odor intensity		
				SPME ($n=6$)	SD ($n=5$)	SAFE ($n=7$)
1	553	Diacetyl	Buttery		0.6	
2	634	2-Methylpropanol	Sweetish, malty		0.5	0.1
3	667	**3-Methylbutanal**	Malty		0.6	
4	678	**2-Methylbutanal**	Malty		0.4	0.1
5	703	2,3-Pentadione	Malty, buttery	0.3		
6	718	Ethyl propanoate	Fruity		0.2[a]	
7	730	**2,4,5-Trimethyl-1,3-dioxolane**	Green, vegetal		0.4	
8	732	3-Methylbutanol	Malty	1.9	1	0.4
9	733	**Diethoxyethane**	Fruity, pineapple		2.1	0.9
10	756	**ethyl 2-Methylpropanoate**	Exotic fruity	0.6	1.4	0.3[a]
11	792	n. i	Fruity, strawberry	0.2		
12	802	Ethyl butanoate	Fruity, red berries	0.7	1.5	0.6
13	827	3-Methylbut-2-en-1-thiol	Dull, light struck	0.3	0.2[a]	0.4[a]
14	835	n. i	Malty	0.3		
15	849	**Ethyl 2-methylbutanoate**	Fruity, apple	0.7	0.8	0.2[a]
16	854	**Ethyl 3-methylbutanoate**	Fruity, red berries	0.8	1.7	0.6[a]
17	874	n. i	Dull		0.2	
18	876	3-Methylbutyl acetate	Banana	2.1	2.4	0.9
19	901	n. i	Dull, malty	0.3		
20	911	**Methional**	Vegetal, potato			1.0[a]
21	928	**2-Acetylfuran**	Roasted, bready		0.8	
22	967	**ethyl 4-Methylpentanoate**	Fruity, red berries	1.4	0.8	0.4[a]
23	982	**Dimethyl trisulfide**	Sulfury, onion	0.6	0.4	0.3[a]
24	999	Ethyl hexanoate	Fruity, estery	1.8	2.6	1.2
25	1039	n. i	Burned, pungent		0.4	
26	1054	**Phenylacetaldehyde**	Roses	0.7	1.4	0.4
27	1058	n. i	Earthy	1.2		
28	1059	4-Hydroxy-2,5-dimethyl-3-Furanone	Caramel, strawberry		1[a]	0.8[a]
29	1088	n. i	Dull, plastics		0.5	0.1
30	1098	n. i	Dull, moldy	0.5		
31	1103	Linalool	Fruity, citrusy	0.5	0.7	
32	1116	2-Phenylethanol	Roses	2.1	2.3	2.3
33	1146	n. i	Sweetish, berries			0.1
34	1154	n. i	Rancid		0.2	
35	1170	**(E)-2-Nonenal**	Cardboard	0.7		
36	1174	n. i	Floral, soapy		0.4	
37	1178	n. i	Floral	1.0		
38	1191	**Diethyl succinate**	Fruity, apricot	0.3	0.3	
39	1199	Ethyl octanoate	Floral, soapy	0.8	0.6	0.2
40	1220	n. i	Fruity, grapefruit			0.2
41	1222	**Ethyl nicotinate**	Sweetish		0.7	0.1[a]
42	1238	Benzothiazole	Malty, pastry		0.6[a]	
43	1266	**2-Phenylethyl acetate**	Roses, fruity	0.8	0.9	
44	1292	n. i	Fruity, raspberry	0.4		
45	1297	**Cinnamaldehyde**	Cinnamon		0.3	

Table 1 (continued)

Compound number	Retention index	Compound	Odor impression	Odor intensity		
				SPME ($n=6$)	SD ($n=5$)	SAFE ($n=7$)
46	1305	n. i	Sweetish, fruity			0.3
47	1309	n. i	Dull, plastics		0.2	
48	1318	**2-Aminoacetophenone**	Fruity, red berries	1.1	1.5	1.2[a]
49	1326	2-Methoxy-4-vinylphenol	Cloves	0.3	1.2	
50	1350	n. i	Honey	0.5		
51	1362	**Ethyl 3-phenylpropionate**	Fruity, red berries	0.6	1.1	0.4[a]
52	1377	**γ-Nonalactone**	Coconut	0.3	1.2	
53	1386	Ethyl (Z)-4-decenoate	Sweetish, estery	0.3		
54	1397	**β-Damascenone**	Apple juice, peach	1.3	0.9	0.9
55	1471	**Ethyl cinnamate**	Fruity, sweetish	0.4	0.4	
	Sum of detected aroma compounds			33	41	26
	Sum of detected aging compounds			13	19	13
	Sum of identified aroma compounds			26	31	11
	Sum of tentatively identified aroma compoundsa			0	4	11
	Sum of non-identified aroma compounds			7	6	4

[a]Tentatively identified by retention index and odor impression

Aging compounds are printed in bold

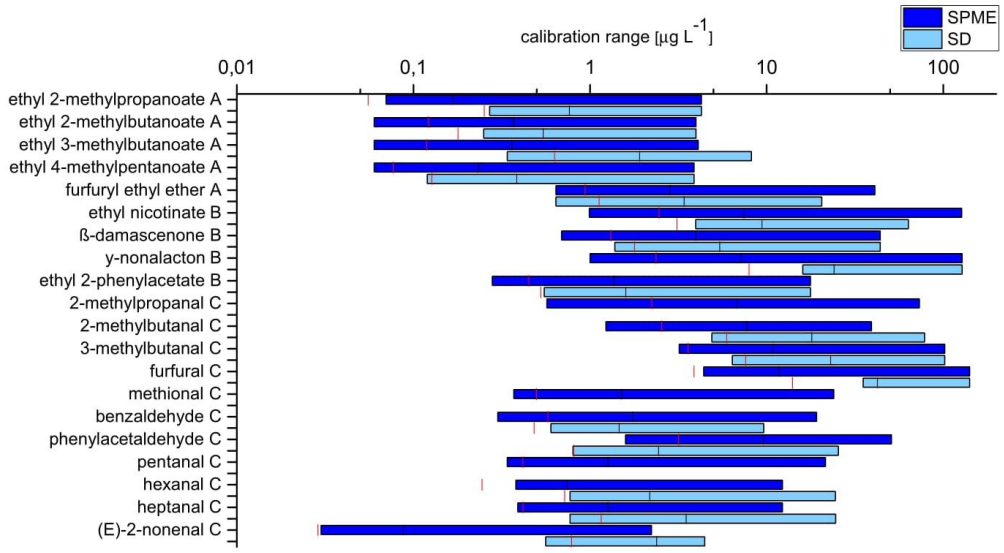

Fig. 4 Calibration ranges. LOD, red marks; LOQ, black marks of volatile aging indicators for SPME and SD. Letters after compound names indicate the standard used for calibration, as follows: A, ethyl-2-methylpentanoate; B, methyl undecanoate; C, p-fluorobenzaldehyde

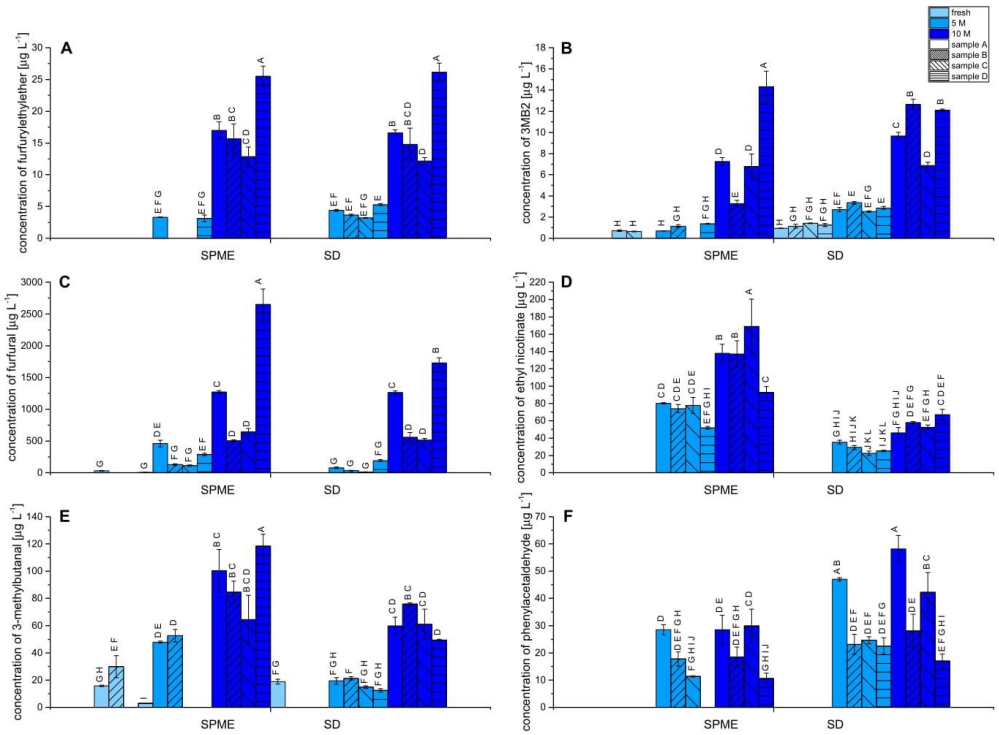

Fig. 5 Selected compound concentrations of fresh, 5 months and 10 months-aged beer from samples **a**, **b**, **c**, and **d**. **a** furfuryl ethyl ether, $p = 0.65$; **b** 3MB2, $p = 0.15$; **c** furfural, $p = 0.86$; **d** ethyl nicotinate, $p = 0.002$; **e** 3-MB, $p = 0.08$; **f** phenylacetaldehyde, $p = 0.07$. Numbers in brackets show p values resulting from ANOVA; letters above bars represent statistical groups as designated by Tukey–Kramer's test

Quantification of aging indicators

The quantification of volatile aging indicators with HS-SPME and SD was performed on four different samples at fresh and aged conditions (5 and 10 months at 20 °C). Methional, pentanal, and 2-methylpropanal were not quantified in SD due to inadequate calibration. Figure 5 shows values for selected compounds over the course of aging. ANOVA at $\alpha = 0.05$ revealed statistical differences between the methods, especially for T2N ($p = 0.0012$), ethyl nicotinate ($p = 0.0015$), and others. The letters above the bars indicate statistical groups resulting from the Tukey–Kramer test.

Only a few compounds appear above their respective LOQ for either method; despite this, most compounds presented with acceptable peaks on the GC–MS. Due to the method used for calculating LOD and LOQ, higher values resulted in comparisons to visual assessment or signal-to-noise ratio [29].

Furfuryl ethyl ether was found to behave similarly for both methods. It was not quantifiable by HS-SPME in samples B and C at 5 months despite a slightly lower LOQ. 3MB2 also appeared to behave similarly between both methods, although a considerable deviation between them was observed in sample B. Furfural and furfuryl ethyl ether behaved linearly over all samples ($R^2 = 0.83$). The concentrations of furfural were well-matched at 10 months for all but sample D. Again, a high amount of precursors that will be partly converted to the resp. analytes is suspected to underlie this result.

3-MB only was comparable after 10 months in samples B and C, and fresh in sample A.

In most compounds, HS-SPME yielded higher concentrations than SD. One explanation might be matrix

influence during calibration, especially in SD. Through the heat intake, fragmentation, de novo formation, and release from adducts may occur and alter the ratios of dosed calibration solution to endogenous volatiles in the sample. One exception to this trend was phenylacetaldehyde, which was present in higher concentrations in samples A, C, and D in SD. In sample B, only a small increase during aging was observed and the obtained concentrations fit well between the two methods. The underlying reasons for this behavior should be investigated further, with special focus on the compound's non-volatile precursors such as imines, cysteine-, or bisulfite-adducts.

The choice of method and analyzed aging indicators remains crucial for the assessment of flavor stability. While for most indicators in HS-SPME sample D appeared highest in concentration, only the assessment of 3-MB and phenylacetaldehyde with SD resulted in the lowest observed concentrations. These results underscore the importance of analyzing a broad range of indicators to cover a diversity of reactions and influences.

This quantitative comparison strongly indicated that the extraction technique influences the obtained results to a greater or lesser degree depending on the aging indicator and matrix or sample. Few indicators were found to behave comparably; many differed significantly. The considerable discrepancies between the methods in this study were ascribed to the large amount of precursors affected during sample workup.

Breakdown, formation, and release reactions in model systems

Model systems were applied to further delineate the observed qualitative and quantitative results and to clarify the influence of sample matrix and thermal intake on detected VOCs. These systems enabled an investigation into possible mechanisms of breakdown, de novo formation, and release of adducts. Sample preparation by SD is accompanied by several invasive steps. First, ethanol is added and the sample is distilled at 100 °C. Then, ammonia, sodium chloride, and dichloromethane are added to the distillate. The model system used in this case was phosphate buffer (pH 4.5) with spiked different precursors representing de novo formation and release reaction from bound-state compounds.

In a blank run of distillation without spiked compounds, multiple chlorinated and non-chlorinated fragments could be identified such as: 1,2-dichloroethene, ethyl acetate, chloroform, 2-methylbutan-2-ol, 3-methylbutan-2-one, 2-chloro-2-methylbutane, diethoxyethane, and others. This might be an underlying cause of the observed higher LODs and LOQs in SD.

Breakdown of methional during distillation

To explore why methional was not detected in SD using GC–O and not possible to calibrate in SD at relevant concentrations, methional was added to the model system and distilled. It was possible to identify dimethyl disulfide, dimethyl trisulfide, methional diethyl acetal, a methional dimer, and a methional trimer in considerable amounts in addition to methional in the model system (Fig. 6). This partly explains the behavior observed in the former experiments.

Formation mechanisms of 3MB2 during distillation

Figure 7a shows possible precursors for 3MB2. Esterification of isovaleric acid with ethanol was observed. Its impact

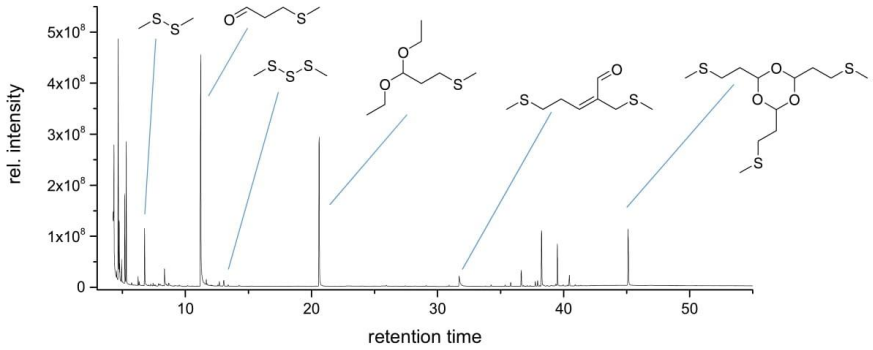

Fig. 6 Fragments of methional in model solution after distillation. Dimethyl disulfide, methional, dimethyl trisulfide, methional diethyl acetal, a methional dimer, and a methional trimer are represented

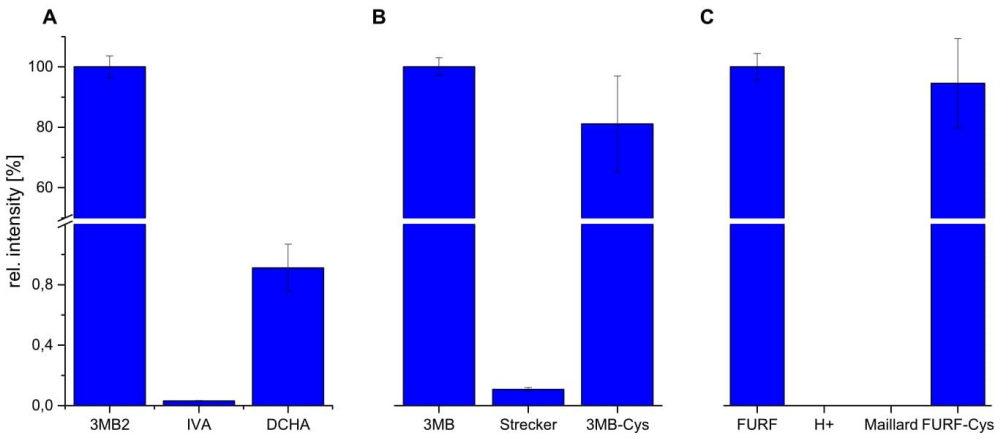

Fig. 7 Possible formation pathways after distillation for 3MB2 (isovaleric acid, DCHA), 3-MB (Strecker reaction bound-state adduct 3-MB-Cys), and FURF (acidic hydrolysis, Maillard reaction bound-state adduct FURF-Cys)

on the analysis, however, seems negligible due to the small amounts of precursors (isovaleric acid) normally present in beer matrices or reaction conditions were not sufficient during distillation. Conversely, isohumulones (in the form of DCHA) were promising precursors of 3MB2 during distillation. A GC–O experiment on DCHA distillate revealed fruity odors for 2MB2 and 3MB2, indicating high aroma activity (data not shown).

Formation mechanisms of 3-MB during distillation

Possible precursors for 3-MB are shown in Fig. 7b. Strecker reaction occurred to a minor extent. Anyhow, due to the high amount of leucine in beer matrices the impact of this pathway has a high impact and yields significant amounts of aldehydes during distillation. The compound 2-(isobutyl)-1,3-thiazolidine-4-carboxylic acid (3-MB-Cys), a promising representative bound-state aldehyde, was almost fully converted to aldehyde form during distillation. So if samples contain this compound or other bound-state forms in reasonable amounts, the analysis will be strongly influenced by this pathway.

Formation mechanisms of furfural during distillation

The formation of furfural was not observed via acidic hydrolysis nor Maillard reaction probably due to the distillation time (Fig. 7c). Presumably, distillation time was too short with only 4.5 min and therefore, reaction conditions were not sufficient. It was once again observed that 2-(furan-2-yl)-1,3-thiazolidine-4-carboxylic acid (FURF-Cys) was almost fully converted to the aldehyde form during distillation, resulting in the same consequences as those discussed for 3-MB-Cys.

Conclusion

This study used GC–O to evaluate qualitative differences between HS-SPME, SD, and SAFE in the detection of VOCs that occurred due to the underlying physicochemical properties (volatility and polarity) of each method. SD detected the most VOCs while SAFE was insensitive to most aging-related compounds. Some discrepancies could be mitigated by tedious solvent fractionation of extracts or more complex instrumental setups such as GCxGC couplings. SPME prevented solvent discrimination, yet fiber discrimination remains possible. Differences in detected compounds might also be explained by harsh distillation conditions (thermal intake), especially for reactive food matrices. Observed and potential breakdown and formation mechanisms were discussed.

Further, quantitative differences were discovered between HS-SPME and SD. Few aging indicators matched well, most showed significant discrepancies in calibration and quantification behavior. Differences among samples were also observed, with one sample in particular differing more severely between the methods than others. These results suggest that in addition to invasive steps during distillation, matrix composition might also be a crucial factor, and special consideration must be given to sensory conclusions.

Observed differences were further delineated using model distillations. The detected chlorinated and non-chlorinated fragments in blank runs might have caused LODs and LOQs to be higher in SD. Fragmentation of methional during distillation did not allow its calibration at adequate concentrations. In this study, bound-state precursors (representably cysteinylated aldehydes) were found to be the most important aldehyde precursors during distillation. Strecker reaction also occurred to a considerable extent; Maillard reaction did not.

Our data indicate that for complex matrices such as beer, an agreement about method and calibration procedures is necessary for comparability between studies. Preferably, the most non-invasive method should be chosen. Furthermore, to assess sensory stability in the most holistic way non-volatile precursors should always play an important role.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

References

1. Jaskula-Goiris B, de Causmaecker B, de Rouck G, Aerts G, Paternoster A, Brae J et al (2019) Influence of transport and storage conditions on beer quality and flavour stability. J Inst Brew 125(1):60–68
2. Vanderhaegen B, Neven H, Coghe S, Verstrepen KJ, Verachtert H, Derdelinckx G (2003) Evolution of chemical and sensory properties during aging of top-fermented beer. J Agric Food Chem 51(23):6782–6790
3. Lehnhardt F, Gastl M, Becker T (2018) Forced into aging: analytical prediction of the flavor-stability of lager beer. Crit Rev Food Sci Nutr Rev 59:1–35
4. Bamforth CW (2011) 125th Anniversary review: the non-biological instability of beer. J Inst Brew 117(4):488–497
5. Saison D, de Schutter DP, UyttenhoveDelvaux BF, Delvaux FR (2009) Contribution of staling compounds to the aged flavour of lager beer by studying their flavour thresholds. Food Chem 114(4):1206–1215
6. Lehnhardt F, Steiner J, Gastl M, Becker T (2018) Prediction power and accuracy of forced ageing—matching sensory and analytical results for lager beer. Brew Sci 71:39–48
7. Ditrych M, Filipowska W, de Rouck G, Jaskula-Goiris B, Aerts G, Andersen ML et al (2019) Investigating the evolution of free staling aldehydes throughout the wort production process. Brew Sci 72:10–17
8. Bustillo Trueba P, Jaskula-Goiris B, de Clippeleer J, Goiris K, Praet T, Sharma UK et al (2019) Validation of an ultra-high performance liquid chromatography-mass spectrometry method for the quantification of cysteinylated aldehydes and application to malt and beer samples. J Chromatogr A 1064:460–467
9. Engel W, Bahr W, Schieberle P (1999) Solvent assisted flavour evaporation—a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. Eur Food Res Technol 209(3–4):237–241
10. Wietstock PC, Kunz T, Methner F-J (2016) The relevance of oxygen for the formation of Strecker aldehydes during beer production and storage. J Agric Food Chem 64:8035–8044
11. Wietstock PC, Kunz T, Methner F-J (2016) Influence of hopping technology on oxidative stability and staling-related carbonyls in pale lager beer. Brew Sci 69:73–84
12. Fritsch HT, Schieberle P (2005) Identification based on quantitative measurements and aroma recombination of the character impact odorants in a Bavarian Pilsner-type beer. J Agric Food Chem 53(19):7544–7551
13. Jacob F (ed) (2012) MEBAK Brautechnische Analysemethoden - Würze, Bier Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission, Selbstverlag der MEBAK. Freising-Weihenstephan, Biermischgetränke
14. Eichhorn P (1991) Untersuchungen zur Geschmacksstabilität des Bieres. Dissertation, TU München
15. Aberl A, Coelhan M (2012) Determination of volatile compounds in different hop varieties by headspace-trap GC/MS—in comparison with conventional hop essential oil analysis. J Agric Food Chem 60:2785–2792
16. Andrés-Iglesias C, Nešpor J, Karabín M, Montero O, Blanco CA, Dostálek P (2016) Comparison of carbonyl profiles from Czech and Spanish lagers: traditional and modern technology. LWT Food Sci Technol 66:390–397
17. Baert JJ, de Clippeleer J, Jaskula-Goiris B, van Opstaele F, de Rouck G, Aerts G et al (2015) Further elucidation of beer flavor instability: the potential role of cysteine-bound aldehydes. J Am Soc Brew Chem 73:243–252
18. Canuti V, Conversano M, Calzi ML, Heymann H, Matthews MA, Ebeler SE (2009) Headspace solid-phase microextraction-gas chromatography-mass spectrometry for profiling free volatile compounds in Cabernet Sauvignon grapes and wines. J Chromatogr A 1216(15):3012–3022
19. Saison D, de Schutter DP, Delvaux F, Delvaux FR (2008) Optimisation of a complete method for the analysis of volatiles involved in the flavour stability of beer by solid-phase microextraction in combination with gas chromatography and mass spectrometry. J Chromatogr A 1190(1–2):342–349
20. Vesely P, Lusk L, Basarova G, Seabrooks J, Ryder D (2003) Analysis of aldehydes in beer using solid-phase microextraction

with on-fiber derivatization and gas chromatography/mass spectrometry. J Agric Food Chem 51(24):6941–6944
21. Castro LF, Ross CF (2015) Determination of flavour compounds in beer using stir-bar sorptive extraction and solid-phase microextraction. J Inst Brew 121(2):197–203
22. Tsuji H, Mizuno A (2010) Volatile compounds and the changes in their concentration levels during storage in beers containing varying malt concentrations. J Food Sci 75(1):C79–84
23. Ochiai N, Sasamoto K, Daishima S, Heiden A, Hoffmann A (2003) Determination of stale-flavor carbonyl compounds in beer by stir bar sorptive extraction with in-situ derivatization and thermal desorption–gas chromatography–mass spectrometry. J Chromatogr A 1:101–110
24. Richter TM, Eyres GT, Silcock P, Bremer PJ (2017) Comparison of four extraction methods for analysis of volatile hop-derived aroma compounds in beer. J Sep Sci 40(22):4366–4376
25. Horák T, Čulík J, Kellner V, Čejka P, Hašková D, Jurková M et al (2011) Determination of selected beer flavours: comparison of a stir bar sorptive extraction and a steam distillation procedure. J Inst Brew 117(4):617–621
26. Horák T, Čulík J, Kellner V, Jurková M, Čejka P, Hašková D et al (2010) Analysis of selected esters in beer: comparison of solid phase microextraction and stir bar sorptive extraction. J Inst Brew 116(1):81–85
27. Thompson-Witrick KA, Rouseff RL, Cadawallader KR, Duncan SE, Eigel WN, Tanko JM et al (2015) Comparison of two extraction techniques, solid-phase microextraction versus continuous liquid-liquid extraction/solvent-assisted flavor evaporation, for the analysis of flavor compounds in gueuze lambic beer. J Food Sci 80(3):C571–C576
28. Bustillo Trueba P, de Clippeleer J, van der Eycken E, Guevara Romero JS, de Rouck G, Aerts G et al (2018) Influence of pH on the stability of 2-substituted 1,3-thiazolidine-4-carboxylic acids in model solutions. J Am Soc Brew Chem 76(4):272–280
29. Shrivastava A, Gupta V (2011) Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chronicles Young Sci 2(1):21
30. Brattoli M, Cisternino E, Dambruoso PR, de Gennaro G, Giungato P, Mazzone A et al (2013) Gas chromatography analysis with olfactometric detection (GC-O) as a useful methodology for chemical characterization of odorous compounds. Sensors 13:16759–16800
31. Kishimoto T, Wanikawa A, Kono K, Shibata K (2006) Comparison of the odor-active compounds in unhopped beer and beers hopped with different hop varieties. J Agric Food Chem 54(23):8855–8861

3.2.3 Forced into aging: Analytical prediction of the flavor-stability of lager beer. A review

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Forced into aging: Analytical prediction of the flavor-stability of lager beer. A review

Florian Lehnhardt, Martina Gastl, and Thomas Becker

Chair of Brewing and Beverage Technology, Technische Universität München, Weihenstephaner Steig 20, Freising, Germany

ABSTRACT

Despite years of research, sensory deterioration during beer aging remains a challenge to brewing chemists. Therefore, sensorial and analytical tools to investigate aging flavors are required. This review aims to summarize the available analytical methods and to highlight the problems associated with addressing the flavor-stability of beer.

Carbonyls are the major contributors to the aroma of aged pale lager beer, which is especially susceptible to deterioration. They are formed via known pathways during storage, but, as recent research indicates, are mainly released from the bound-state during aging. However, most published studies are based on model systems, and thus the formation and breakdown parameters of these adducts are poorly understood. This concept has not been previously considered in previous forced-aging analysis.

Only weak parallels can be drawn between forced and natural aging. This is likely due to the different activation energies of the chemical processes responsible for aging, but may also be due to heat-promoted release of bound aldehydes. Thus, precursors and their binding parameters must be investigated to make appropriate technological adjustments to forced-aging experiments. In combination with sophisticated data analysis, the investigation of volatile indicators and non-volatile precursors can lead to more reliable predictions of flavor stability.

KEYWORDS

Beer aging; flavor stability; bound-state aldehydes; forced-aging; analytical indicators; precursor potential

1. Introduction

1.1. Flavor of fresh and aged beer

The flavor of beer is a complex mixture of a variety of potential flavor agents that are greatly influenced by the raw materials used and the method of production employed. In beer, these agents are present at concentrations in the ng L^{-1} to g L^{-1} range and affect both positively perceived and off-flavor attributes. Even small alterations in their concentrations can have a huge impact on the overall flavor. Despite the great number of potential flavor compounds, it has been shown that only a small number of compounds are able to activate odorant receptors and create aroma perception (Grosch 2001). Meta-analysis undertaken by Dunkel et al. identified only 17–20 key food odorants in all the types of fresh bottom-fermented beer they investigated (Dunkel et al. 2014). Thus, most flavor agents are either non-active or contribute to a background perception, whereas relatively few are considered to be key aroma compounds (Fritsch and Schieberle 2005).

More than 40 years ago, Meilgaard et al. investigated single volatile compounds in beer with respect to their flavor activities for the first time. The odor activity values (OAVs) of 239 compounds in beer were determined, revealing that ethanol, carbon dioxide, several esters (e.g., 3-methylbutyl acetate and ethyl hexanoate), higher alcohols (e.g., 3-methylbutanol), dialkyl sulfides (e.g., dimethyl sulfide (DMS)), and short-chain fatty acids (e.g., butanoic acid) were key aroma compounds in American lager beers (Meilgaard 1975). In sensory omission tests, ethanol, (R)-linalool, ethyl butanoate, DMS, 3-methylbutanol, ethyl hexanoate, 2-phenylethanol, and furaneol were found to be significant for the overall flavor in a synthetic aroma recombination study of pilsner beer (Fritsch et al. 2005).

The chemical composition of beer is a dynamic system and changes throughout storage. This phenomenon, referred as "flavor instability" or "beer staling" is a highly complex process owing to the many different oxidative and non-oxidative reactions that take place. These comprise the Maillard reaction, Strecker degradation, degradation of hop bitter compounds, etherifications, esterifications, glycoside and ester hydrolysis, and the release of molecules from adducts (Aron and Shellhammer 2010; de Clippeleer et al. 2010; Suda et al. 2007; Baert et al. 2015; Rakete, Klaus, and Glomb 2014). Diverse chemical species such as phenols, proteins, amino acids, carbohydrates, isohumulones, alcohols, tannins, lactones, aldehydes, unsaturated carbonyls, vicinal diketones, ionones, esters, fatty acids, essential oils, sulfur compounds, nucleotides, metal ions, and organic acids are involved in these reactions (Verhagen 2010). Thus, no single character-impact compound is responsible for the staling of beer, but rather a variety of products from different reactions (Meilgaard 1972).

The course of aging of lager beer is well documented since it is especially susceptible (Baxter and Hughes 2001). Typically, staling flavors develop after 3–6 months of storage at 18°C (Ilett and Simpson 1995). Dalgliesh et al. (Dalgliesh 1977)

CONTACT Martina Gastl martina.gastl@tum.de Chair of Brewing and Beverage Technology, Technische Universität München, Weihenstephaner Steig 20, 85354 Freising, Germany.

reported in 1977 the changes in aroma during the aging of lager beer in detail, and in 2005 Zufall et al. (Zufall et al. 2005) extended Dagliesh's model using forced-aging at 28°C. During aging, bitterness is reduced, whereas sweetish and caramel notes are amplified. A cardboard flavor as well as ribes-like flavor can develop, but also disappears after time, and even bread, wood, or sherry flavors can be presented after longer storage times. Additionally, fresh aromas and masking effects are diminished (Saison et al. 2009). However, owing to the reasons stated above, the development of a certain flavor cannot be correlated to a single compound.

1.2. Analytically detectable indicators of beer aging

Over years of research, many compounds have been discussed as potential indicators of beer aging. Vanderhaegen et al. (Vanderhaegen et al. 2006) and Baert et al. (Baert et al. 2012) reported critical and comprehensive reviews on the chemistry of beer aging, listing numerous compounds related to beer aging. Table 1 gives a selection of currently known volatile aging indicators and precursors in pale lager beer, their origin, and critical steps during production and storage. The most populated group of indicators is aldehydes, followed by some esters and ketones. In fresh beer, minimal concentrations of aldehydes are present, whereas their concentrations rise during the course of aging. Due to their increase and high aroma potency, they are often linked to typical off-flavors (Saison et al. 2010a).

In 1994, Eichhorn (Eichhorn 1991) and Lustig (Lustig 1995) proposed indicators for heat impact (2-furfural, γ-nonalactone), oxygen uptake (2-methylbutanal, 3-methylbutanal, benzaldehyde, phenyl acetaldehyde), and aging (5-methylfurfural, diethyl succinate, ethyl nicotinate, 2-acetylfuran, 2-propionylfuran). Since then, no comprehensive classification of aging markers has been published, yet raw materials, procedures, and technologies have seen substantial changes. Instead, numerous compounds are currently discussed as indicators.

Due to the different chemical properties (e.g., volatilities and polarities) of these compounds, different methods have been used to monitor them during aging. In most cases, a compound is regarded as relevant if it increases parallel to the sensory perception of aged flavor (Thiele 2006). This does not necessarily imply that these compounds have a sensory impact (aroma relevance), and thus indicators can be divided into analytical and sensory indicators. Analytical indicators occur typically well below their threshold and are very unlikely to have direct aroma relevance. Sensory indicators exceed their thresholds and thus have direct impact on the aroma profile.

Furthermore, some indicators have been criticized and should be used carefully. For instance, since its discovery by Palamand and Hardwick in 1969 (Palamand and Hardwick 1969), (E)-2-nonenal was considered to be a key aging compound for a long time. Due to its extremely low flavor threshold and its characteristic cardboard flavor, the complex phenomenon of beer aging was reduced to the presence of this single compound (Vanderhaegen et al. 2004). However, other studies based on stable isotope dilution assays demonstrated that the increase in (E)-2-nonenal during natural aging is not constant and that it is likely to be degraded or re-trapped. Additionally, its breakthrough value of 0.1 μg L^{-1} was not observed in those studies (Schieberle and Komarek 2003, Schieberle and Komarek 2005). The same authors found in spiking experiments with deuterated (E)-2-nonenal that the free compound is evaporated during wort boiling and entirely reduced to nonenol upon the fermentation of yeast (Schieberle and Komarek 2005). It seems that, under non-extreme aging conditions, the main pathways (e.g., oxidation of higher alcohols and release from adducts) are unlikely to occur. Indeed, it was found that the formation of (E)-2-nonenal is favored by lower pH (Guyot-Declerck et al. 2005) and higher temperatures during storage (Kaneda et al. 1995), suggesting that it only occurs in force-aged beers (Bamforth and Lentini 2009). It was also shown that lower pH enhances cardboard flavor, presumably as it promotes the hydrolysis of alkenal-protein adducts (Noël et al. 1999a; Lermusieau, Noël, and Collin 1999). In addition, cardboard flavor is not necessarily perceived in every beer, mainly in beers brewed with adjuncts. Thus, (E)-2-nonenal is not suitable as an aging marker (Lustig 1995; Eichhorn 1991; Bamforth and Lentini 2009). Nevertheless, with so many compounds involved in the aged flavor of beer, it makes sense to reduce the number of indicators to a manageable minimum as long as they are meaningful and present a strong correlation between analytical and sensory properties.

In 2009 Saison et al. (Saison et al. 2009) confined the list of indicators by re-determining the flavor thresholds of several compounds present in lager beer. They claimed that methional, 3-methylbutanal, 2-furfuryl ethyl ether, β-damascenone, and acetaldehyde, and to a lesser degree (E,E)-2,4-decadienal, phenylacetaldehyde, 2-methylpropanal, diacetyl, and 5-hydroxymethylfurfural, were the key contributors to the aged flavor of beer. The Strecker aldehydes methional and phenylacetaldehyde are generally accepted as indicators for thermal stress, especially during wort boiling (Guedes de Pinho and Silva Ferreira 2006; Soares da Costa et al. 2004). The same is true for 2-furfural, which is thought to derive from xylose species (Antal et al. 1991). Nonetheless, this pathway was not confirmed for packaged beer (Baert 2015) but 3-deoxypentosone was found to be a direct precursor (Rakete, Klaus, and Glomb 2014). In general, Maillard products tend to give caramel or cooked notes but, due to their high flavor thresholds, they seem to have little impact on total flavor. 2-Furfurylethylether is particularly well known to increase in all beers during aging and has been suggested as a potent analytical storage indicator (Eichhorn 1991; Vanderhaegen et al. 2004).

1.3. Flavor-inactive bound-state aldehydes

Evans et al. found that relatively few flavor-active compounds are either newly formed or entirely diminished during aging, while most are already present in fresh beer and somehow masked from sensory and analytical perception (Evans et al. 1999). It has been suggested that, after packaging, non-extreme storage conditions allow the ongoing formation of staling compounds through oxidation and reduction reactions. Furthermore, the contribution of interactive effects between aroma compounds has been discussed (Narziss 1986). It has been found that staling aldehydes exist in two forms. In their **free form**, they directly contribute to flavor, but they can also occur

Table 1. Selection of currently known indicators of beer aging in pale lager beer.

Pathway	Compound name	Origin	Critical steps during process	Source
Strecker degradation	2-Methylpropanal	Valine or oxidative degradation of isohumulones	Oxygen uptake	(Vanderhaegen et al. 2006; Baert et al. 2012; Clippeleer et al. 2010; Eichhorn 1991)
	2-Methylbutanal	Isoleucine or oxidative degradation of isohumulones	Oxygen uptake	(Vanderhaegen et al. 2006; Baert et al. 2012; Clippeleer et al. 2010; Wietstock, Kunz, and Methner 2016b)
	3-Methylbutanal	Leucine or oxidative degradation of isohumulones	Oxygen uptake	(Vanderhaegen et al. 2006; Baert et al. 2012; Clippeleer et al. 2010; Wietstock, Kunz, and Methner 2016b)
	Phenylacetaldehyde	Phenylalanine	Oxygen uptake	(Vanderhaegen et al. 2006; Baert et al. 2012; Wietstock, Kunz, and Methner 2016b)
	Benzaldehyde	Degradation of phenyl acetaldehyde	Oxygen uptake	(Vanderhaegen et al. 2006; Baert et al. 2012; Wietstock, Kunz, and Methner 2016b)
	Methional	Methionine	Oxygen uptake	Baert et al. 2012; Wietstock, Kunz, and Methner 2016b)
	2-Acetyl pyrazine	α-dicarbonyls and amino acids	/	(Vanderhaegen et al. 2006)
Maillard reaction	2-Furfural	Carbohydrates and amino acids	Time and temperature of storage	(Vanderhaegen et al. 2006; Baert et al. 2012; Eichhorn 1991)
	5-Hydroxymethylfurfural	Carbohydrates and amino acids	Time and temperature of storage	(Baert et al. 2012; Rakete, Klaus, and Glomb 2014)
	5-Methylfurfural	Carbohydrates and amino acids	/	(Eichhorn 1991)
	2-Furfuryl ethyl ether	2-Furfural and ethanol	Time of wort boiling	(Vanderhaegen et al. 2004; Schutter 2008)
Lipid oxygenation	Hexanal	Linoleic acid oxidation (LOX and 13-HPL)	Malting and mashing, time and temperature of storage	(Vanderhaegen et al. 2006)
	Heptanal	Oleic acid oxidation	Malting and mashing, time and temperature of storage	(Meilgaard 1975; Lustig 1995; Eichhorn 1991)
	(E)-2-nonenal	Linoleic acid oxidation (LOX and 9-HPL), but also from aldol addition of acetaldehyde and heptanal	Malting and mashing, time and temperature of storage	(Meilgaard 1975; Vanderhaegen et al. 2006; Lustig 1995; Kuroda et al. 2003)
	(ZZ)-2,4-decadienal	Linoleic acid oxidation	Malting	(Lustig 1995)
Hop degradation products	Ethyl-3-methylbutyrate	Degradation of acyl side chain of (iso-)humulones with ethanol	Time and temperature of storage?	(Schnaitter et al. 2016)
	Ethyl-2-methylbutyrate	Degradation of acyl side chain of (iso-)humulones with ethanol	Time and temperature of storage?	(Schnaitter et al. 2016)
Other oxidations	β-damascenone	Carotenoids (neoxanthin), glycoside	Tent. raw material content, pH during mashing and boiling	(Gijs et al. 2002; Chevance et al. 2002)
	Dimethyl trisulphide (DMTS)	Degradation of methional, S-methyl-L-cysteine-S-oxide	/	(Gijs et al. 2002; Thiele 2006)
	γ-nonalactone	Lipids	Time and temperature of storage	(Vanderhaegen et al. 2006; Eichhorn 1991)
	Diethyl succinate	Esterification of succinic acid and ethanol	Time and temperature of storage?	(Eichhorn 1991)
	Ethyl nicotinate	Tryptophan	Time and temperature of storage	(Eichhorn 1991; Lustig 1995, 1995; Eichhorn 1991; Palamand and Grigsby 1974)
	2-aminoacetophenone	Tryptophan	/	(Palamand and Grigsby 1974; Christoph et al. 1999)
Adduct formation	Imines	Reaction with NH_2-groups	Removal with spent grains, hot break, and yeast biomass	(Baert et al. 2012)
	Thiazolidine-4-carboxylic-acids	Reaction with cysteine	Tent. trub separation	(Baert et al. 2015)
	Bisulfites	Reaction with SO_2	Tent. removal with yeast biomass and cold break	(Baert et al. 2012)

in a trapped or **bound state**, both reversibly and irreversibly. In this way, aldehydes are not evaporated during wort production nor reduced to their corresponding alcohols by yeast. Thus, they are obscured from sensory and analytical perception. Then, in the course of aging, these aldehydes will be released from their adducts depending on factors like temperature, pH value, binding strength, and thermodynamic stability, and cause off-flavors (Lermusieau, Noël, and Collin 1999; Liégeois et al. 2002; Noël et al. 1999a). Thus, the determination of these precursors can provide an indication for the endogenous aging potential of a particular beer.

The influence of oxygen, temperature, or other parameters on the formation of bound-state aldehydes has not yet been investigated. However, it may be hypothesized that the more oxygen or temperature intake a beer experiences during production and storage, the more aldehydes are formed (and thus bound to some degree). Consequently, more aldehydes will be released during aging.

Other authors have assumed that oxygen is a release-promoting agent since it is known that oxidative conditions promote staling, and investigations into beer-flavor instability in terms of solely *de novo* formation of compounds were inconclusive and showed contradictions (Wietstock, Kunz, and Methner 2016a; Noël et al. 1999b). This indicates that the chemistry of beer aging has become more nuanced, and that bound-state precursors have attracted increasing research interest. For instance, Suda et al. showed in experiments with ^{13}C-labeled amino acids that 85% of Strecker aldehydes are derived from wort boiling and clarification processes, whereas only 15% are formed *de novo* from the remaining amino acids in bottled beer. They suggested that Strecker aldehydes may be trapped by either amino acids, proteins, or sulfites (Suda et al. 2007). A reactive-oxygen-species (ROS)-induced oxidative degradation pathway for the development of Strecker aldehydes has been previously proposed (Wietstock, Kunz, and Methner 2016b). Noël et al. found that after spiking $^{18}O_2$ to bottled beer and subsequent aging, ^{18}O was incorporated in sulfites, polyphenols, and isohumulones, but not in the carbonyl fraction. Thus, they concluded that lipid peroxidation might not occur in bottled beer (Noël et al. 1999a; Noël et al. 1999b).

The main forms of bound-state aldehydes comprise bisulfite adducts (on SO_2 groups) (Kaneda et al. 1994; Dufour et al. 1999) and imines (on amino groups, e.g., from amino acids, peptides, or proteins) (Baert et al. 2012; Lermusieau, Noël, and Collin 1999; Liégeois et al. 2002). Recently, a new bound-state i.e., reaction of aldehydes with cysteine was found in beer (Baert et al. 2015; Baert et al. 2012; Baert, Clippeleer, and Aerts 2015). Furthermore, glycosidic precursors, as identified for β-damascenone, have to be considered (Chevance et al. 2002). In 1983, Barker et al. reported for the first time the liberation of staling compounds from bisulfite adducts during the course of aging, prompting their inclusion of acetaldehyde in model systems (Barker et al. 1983). Also, acetals have been previously discussed as bound-state aldehydes (C. Liu et al. 2018). Figure 1 shows an overview of several important pathways of adduct formation, and these bound-state precursors will be discussed below.

1.3.1. Bisulfites

Sulfur dioxide can delay the staling of beer by either scavenging ROSs and acting as an antioxidant or forming hydroxysulfonates and stepwise disulfonates with carbonyls through bisulfite

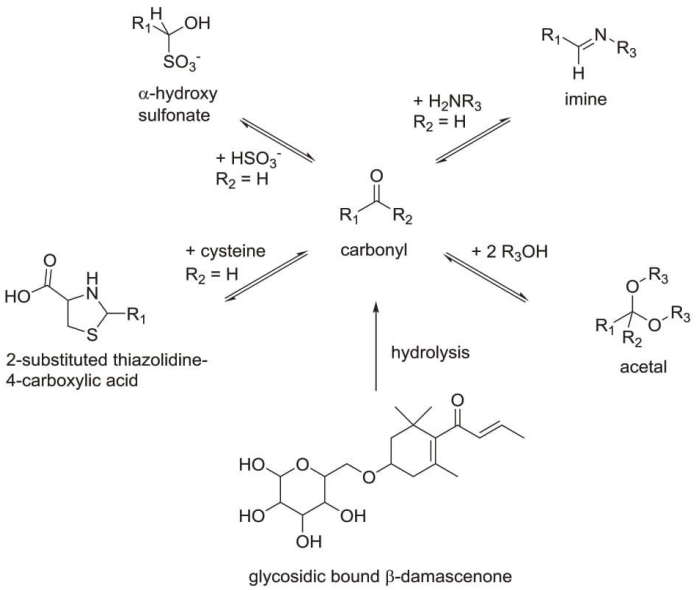

Figure 1. Different pathways for the release of bound-state aldehydes: Bisulfite adduct from Dufour et al. (Dufour et al. 1999), imine adduct from De Schutter et al. (Schutter 2008), 2-substituted thiazolidine-4-carboxylic acid from Baert et al. (Baert et al. 2015), glycosidic bound precursors (here β-damascenone from Chevance et al. (Chevance et al. 2002)), acetal adducts from Vanderhaegen et al. (Vanderhaegen et al. 2006).

ions (HSO_3^-) (Guido 2016). In special cases, e.g., with α-unsaturated aldehydes, irreversibly trapped adducts can be formed where the second addition to the double bond is the rate-limiting step (Dufour et al. 1999). Kaneda et al. (Kaneda et al. 1994) regarded the optimal SO_2 content of beer to be 8–9 mg L^{-1}. In the pH range 3–6, nearly all the sulfites are present as SO_3^- and most carbonyls are bound. However, since acetaldehyde is the most abundant aldehyde in beer (> 95%) (Hashimoto and Eshima 1977; Kaneda, Takashio, and Tamaki 1997) and its concentration rises during aging, other aldehydes may gradually be released from their adducts and thus contribute directly to flavor deterioration. The formation of adducts with acetaldehyde is favored thermodynamically due to its carbon chain length (Barker et al. 1983). For commercial beers, acetaldehyde-bisulfite adduct concentrations of up to 54 μM have been reported (Kaneda et al. 1996). Using results from 10 real wine samples, de Azevedo et al. showed that up to 90% of the acetaldehyde (19 mg L^{-1}) and 50% of the 2-furfural (12 mg L^{-1}), butanal, hexanal, and benzaldehyde are bound as bisulfites (Azevedo et al. 2007). Bueno et al. reported that, due to the depletion of SO_2, bisulfite adducts are cleaved during wine oxidation and thus aldehydes are released (Bueno, Carrascón, and Ferreira 2016).

1.3.2. Imines

Imines or Schiff bases are formed upon the reaction of a carbonyl group with an amino group. The latter can be either an amino acid, a peptide, or protein. In beer, imines can be formed by nucleophilic attack of the nitrogen atom of an amino group with subsequent elimination of water, as reported earlier (Schutter 2008; Pan, Chong, and Pawliszyn 1997). In general, imine formation is more stable at higher pH, while lower pH and heat drive the back reaction to the initial carbonyl (Lermusieau, Noël, and Collin 1999). These parameters have been used previously, e.g., in the "nonenal potential" forcing test (Drost et al. 1990; Lermusieau, Noël, and Collin 1999; Liégeois et al. 2002; Noël et al. 1999a). Lermusieau et al. found that after addition of an excessive amount of an albumin, 60% of the initial (E)-2-nonenal concentration was retained, whereas 50% of the retention could be reversed after application of heat (Lermusieau, Noël, and Collin 1999). For the reaction of hydroxymethylfurfural (HMF) with primary and secondary amino acids, imine formation was observed (Nikolov and Yaylayan 2011). De Schutter found that (E)-2-nonenal, (E)-2-hexenal, (E)-2-heptenal, and 2-methylpropanal react quantitatively with amino acids, but they also suggested that this mechanism is only applicable for certain aldehydes and, for example, not for 2-furfural or phenylacetaldehyde. It has also been stated that interactions between long-chain aldehydes, especially (E)-2-nonenal and (E,E)-2,4-decadienal, and the hydrophobic regions of proteins occur (Schutter 2008). However, experiments under different conditions by Baert et al. failed to demonstrate the formation of imines between several different amino acids and marker aldehydes (Baert 2015).

1.3.3. 2-Substituted 1,3-thiazolidine-4-carboxylic acids

Recently, a novel type of bound-state aldehydes has been reported (Baert, Clippeleer, and Aerts 2015; Baert et al. 2015). In this case, aldehydes undergo nucleophilic attack by either the amino or thiol group of cysteine and subsequent cyclization and elimination of water to yield 2-substituted 1,3-thiazolidine-4-carboxylic acids in the pH range 4.4–6.0. Also, results by Önen Bayram et al. confirmed these findings. In phosphate-buffered saline (PBS) solution (pH 7.4) the benzaldehyde equivalent (2-phenyl-1,3-thiazolidine-4-carboxylic acid) is almost completely converted to the free aldehyde form after 50 min. However, at pH 2.0 an equimolar ratio was observed. They also found that each compound shows a particular pH stability (Önen Bayram et al. 2016).

The thiazolidine-carboxylic acid equivalent of 2-furfural, 2-(furan-2-yl)-1,3-thiazolidine-4-carboxylic acid, was confirmed in commercial lager beers at concentrations of milligrams-per-liter. The presence of equivalents for other staling aldehydes is yet to be proven. However, it is very likely owing to the favored formation parameters during beer production. These findings support the argument that a larger fraction of aldehydes are present in the bound-state and might be released during the course of aging (Baert et al. 2015; Baert et al. 2014).

1.3.4. Glycosides

Additionally, carbonyls can be trapped as glycosides. Daenen et al. suggested a conversion pathway from benzyl-β-D-glucoside or amygdalin to benzaldehyde in which a β-glucosidase is involved. They further demonstrated that re-fermentation of sour cherry-juice-supplemented beer by *B. custersii* released benzaldehyde, linalool, eugenol, trans-2-hexen-1-ol, geraniol, and isoeugenol from their glycosidic precursors. These released benzyl compounds are then further reduced to benzyl alcohols (Daenen et al. 2008). For β-damascenone, a similar behavior was observed. This compound is present at 450 ng g^{-1} in wort and in fresh beer (though below 25 ng g^{-1}). During the aging of beer, the concentration of β-damascenone increases continuously. Chevance et al. suggested that this increase is partly due to acidic hydrolysis of glycosides (Chevance et al. 2002). Thus, it is conceivable that these compounds are formed during beer production. Later, Gijs confirmed that lower pH elevates the levels of β-damascenone during the artificial aging of beer (Gijs et al. 2002).

1.3.5. Acetals

Acetals are the product of a reaction aldehydes with alcohols. These can either be monohydric or polyhydric. The best known example of the former type is diethylacetal from acetaldehyde and ethanol, while the latter one yields cyclic acetals, such as 2,4,5-trimethyl-1,3-dioxolane from acetaldehyde and 2,3-butanediol (Peppard and Halsey 1982). Vanderhaegen et al. found that 2,4,5-trimethyl-1,3-dioxolane increases during aging similarly to the concentration of acetaldehyde while oxygen seemed to have the biggest impact. This suggests that there is an equilibrium between both compounds (Vanderhaegen et al. 2003). Recently, Liu et al. monitored the course of acetaldehyde and diethylacetal during the brewing process and storage. In contrast to the previous study they observed the gradual decline of diethylacetal, while acetaldehyde increased continuously (C. Liu et al. 2018).In the literature, there is a clear lack of data on adducts and precursors, despite several authors being aware of the necessity of their investigation (Kaneda et al. 1995). In addition to the above-mentioned forms, other types of adducts (e.g., with β-glucans) may also occur in beer. The fact that different

types of adducts react differently under the influence of factors such as pH and temperature makes it difficult to determine the degree of release during storage. For instance, while imines are more stable at higher pH, bisulfites tend to be cleaved (Guido 2005). Thus, bound-state aldehydes seem promising for estimating aging potentials in a non-discriminative way.

2. Forced-aging

Forced-aging (formerly "beer punishment") is a pervasive but discriminative way to accelerate the processes that occur during the natural aging of beer and thus predict flavor stability. Since brewers are not able to wait several weeks or months until the first perceivable changes occur, they depend on sensory and analytical tools for the rapid estimation of flavor stability in beer. Therefore, different forcing regimes are used involving changes in parameters such as temperature, time, mechanical action (e.g., shaking), impact of light, oxygen content, and occasionally even alterations in pH value. Table 2 gives an overview of different regimes, the time equivalent for natural aging, the used analytics and sensorics, and the investigated substances in lager/pilsner beers.

2.1. Influence of temperature on forced-aging

Next to oxygen uptake, storage temperature is considered to have the most dramatic impact on flavor stability (Bamforth and Lentini 2009). According to the Arrhenius law, a 10 K elevation of temperature increases the rate of a reaction by a factor of 2–3. However, due to their different activation energies, reactions do not increase equally, and this results in altered aroma profiles (Vanderhaegen et al. 2006). Consequently, the higher the applied temperature, the greater the difference from natural aging at 4 or 20°C. For some individual sensory descriptors, this can work, but it does not provide a holistic picture (Eger et al. 2005). Therefore, prediction methods without temperature change are more favorable.

Cejka et al. recently demonstrated a clear correlation between temperature and the development of stale flavor during aging. Furthermore, by using neural networks they were able to calculate the storage temperature of beers that had been stored for certain times (Cejka et al. 2013). During distribution, e.g., on long distance carriers, beer also experiences a forcing regimen, as observed by Pankoke, who used thermal degradation units (TDUs) to describe flavor changes during transport (Pankoke 2015).

2.2. Influence of pH on forced-aging

In 1990, Drost et al. developed the "nonenal potential" forcing test, which is used to determine the potential of wort to form (E)-2-nonenal under beer conditions (pH 4.0, argon atmosphere) (Drost et al. 1990). During the storage of lager beer, its pH value typically decreases in the 4.5–3.9 range, leading to inferior flavor stability (Bamforth 2001). Grigsby et al. showed that lowering the pH and subsequent forced-aging (1 d, 60 °C) results in the deterioration of flavor stability. At the initial pH of 4.0, a strong oxidized flavor was observed. At higher pH (4.9), grainy aromas were enhanced (Grigsby, Palamand, and Hardwick 1972). This coincides with the findings of Saison et al., who reported decreased aged flavor with increasing pH. It has also been shown that, at lower pH, the appearance of cardboard notes is favored (Saison et al. 2010b).

2.3. Application of forced-aging parameters

Forced-aging typically involves temperatures ranging from 28 to 60°C applied for 3 days to several weeks, as higher temperatures and longer are both impracticable. It is also very common to shake a beer for a certain time prior to heat treatment to simulate transport (Eichhorn 1991). Gas chromatography-olfactometry (GC-O) analysis of force-aged pilsner beer (60°C for 3 d) revealed elevated concentrations of phenylacetaldehyde, furaneol, (E)-2-nonenal, and two unknown compounds (solvent-like resp. aloe-like) (Bravo et al. 2008). In contrast, GC-O analysis of a naturally aged American lager (1 year at 22 °C) with a "foul, musty, burnt, and acid" flavor presented only phenylacetaldehyde, and the other compounds above were not found (Murakami et al. 2003). In another study, (E)-2-nonenal (alongside others) was reported following GC-O analysis of lager beer that was force-aged at 40°C for 5 days at pH 4.2 (Gijs et al. 2002).

Evans et al. reported that a moderately naturally aged pilsner-type lager beer (11 weeks, 20 °C) developed a light papery flavor that increased upon longer storage (> 1 year), where sweetish notes also appeared. Force-aged beer (6 weeks, 40 °C) showed sherry, woody, and honey flavors (Evans et al. 1999). In 2005, Eger et al. monitored pilsner beers at 0, 20, and 30°C for 22 weeks. The intensity of the overall aging attributes were dependent upon temperature. For example, blackcurrant flavor showed no significant difference between temperatures; honey and sherry flavors only increased at 30 °C; and a bready flavor occurred at 20°C after a small delay. Interestingly, a cardboard flavor developed rapidly at 30°C and diminished totally after 22 weeks. For storage at 0°C, only minor flavor changes were observed (Eger et al. 2005).

Taken together, the results of forced-aging tests present a rather unrealistic picture of staleness. Elevated temperatures cause differences in reaction rates (e.g., Strecker aldehydes like methional and phenylacetaldehyde are temperature-dependent and favored at higher temperatures) (Soares da Costa et al. 2004) and also lead to reactions that might not occur at lower temperatures owing to their high activation energies (e.g., release of certain aldehydes from adducts). The same is true for acidification due to the cleavage of adducts and catalysis of reactions. It seems that some authors are well aware of these disadvantages, while others are not, since they do not report on the degree of staling or the flavors developed (Vesely et al. 2003). After taking into account bound-state aldehydes for a wider picture of beer flavor instability, it seems that, in previous tests, adducts were cleaved by chance rather than intention. Thus, staleness engendered in that way should not be compared to that of naturally aged beers. Other non-discriminative approaches to predicting the shelf-lives of beers seem more promising and should be explored further, since they yield a more realistic representation of beer aging.

Table 2. Overview of different forced-aging regimens, analytical and sensory methods, and substances found with elevated concentrations in pale lager beers.

Reference	Forced-aging regimen	Equivalent time of natural aging	Analytical methods used	Sensorial evaluation	Found substances with significantly elevated concentrations/ used indicators
Bravo et al. 2008	3 d, 60 °C	/	SPME HR-GC/MS; GC-O	/	Furaneol, (E)-2-nonenal, phenylacetaldehyde, two unknown compounds ("solvent-like" resp. "aloe-like")
Cejka et al. 2013	6 d, 45 °C	Approximately 6 w, 30 °C or 6 m, 20 °C	HS-GC precolumn derivatisation (PFBHA)	Staleness: 0 (none)–5 (very strong)	3-methylpropanal, 2-methylbutanal, 3-methylbutanal, phenylacetaldehyde, 2-furfural
Back et al. 1997, Eichhorn 1991, Herrmann et al. 2010	1 d shaking + 4 d, 40 °C	3–4 months at 20 °C	Steam distillation	Impression of aging: 0 (none)–4 (very strong)	Ethyl nicotinate, 2-furfural, 2-methylbutanal, 3-methylbutanal, γ-nonalactone, phenylacetaldehyde, benzaldehyde, 3-methylbutan-2-one, heptanal
Depraetere et al. 2008	7 d, 40 °C	/	P&T GC-MS	Staleness: 0 (none)–5 (very strong); Individual aspects (0–8): sweet, bitter, after-bitter, pungent, fruity, solvent, cardboard, Madeira, ribes, caramel, sulfury	2-Furfural, 5-Methyl-2-furfural, furfuryl ethyl ether, 3-methylbutanal, benzaldehyde, hexanal, 4-methyl-pentan-2-one, ethyl-3-methylbutyrate, (decrease in ethyl hexanoate and isoamyl acetate)
Gijs et al. 2002	5 d, 40 °C; pH set to 3, 4.2, 5, 6, and 7	"This accelerated aging mimics very well a natural 20 °C storage."	SPE (Amberlite XAD-2 resin); Dynamic headspace–GC (sulfur chemiluminescence detection GC-O	/	At pH 4.2:, ethyl butyrate, methional, 2-methoxypyratine, DMTS, 2-acetylpyrazine, maltol, (E)-2-nonenal, γ-nonalactone, β-damascenone, ethyl cinnamate, and 5 unknown compounds
Greenhoff and Wheeler 1981	22 h, 60 °C	3 weeks, 37°C or 6 m, 18 °C	HPLC precolumn derivatization (2,4-DNPH)	Staleness: 0 (none)–5 (extreme): papery, oily, caramel, solvent, and rancid but no differences between T	2-butanone, butanal, acrolein, crotonal, pentenal, hexenal, heptenal, octenal, nonenal, decenal
Guedes de Pinho and Silva Ferreira 2006, Soares da Costa et al. 2004	7 d, 37 °C	180 d, 4 °C	LLE (dichloromethane); GC-O	Quality scoring: +1 (no defect) to −3 (major defect)	methional, phenylacetaldehyde, β-damascenone, sotolon
H. Kaneda et al. 1995	5 d, 37 °C	22 d, 30 °C or 42 d, 25 °C	Chemiluminescence; DPPH reducing activity	Staleness: 1 (not present)–5 (very strong)	/
Malfliet et al. 2009	60 d, 30 °C in the dark	/	*On-fibre* derivatisation (PFBHA) SPME	Overall-aging-score: 0 (fresh)–8 (strongly aged, undrinkable)	2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, phenylacetaldehyde, 2-furfural, hexanal, (E)-2-nonenal
Noël et al. 1999a	5 d, 40 °C	/	Steam distillation	/	(E)-2-nonenal
Rakete, Klaus, and Glomb 2014	2 weeks, 50 °C (aerated and unaerated)	/	HPLC-MS	/	2-Furfural, 5-HMF, and certain dextrins
Saison et al. 2008, Saison et al. 2010b	5 d, 60 °C; 3 w, 40 °C; 3 m, 28 °C	/	*On-fibre* derivatisation (PFBHA) SPME	Overall appreciation: 1–9; Single aspects (cardboard, metal, solvent, old hops, ribes, Maillard, stale-sulfury, acetaldehyde, Madeira): 0 (not present)–8 (extremely strong)	Acetaldehyde, Hexanal, (E)-2-nonenal,2-methylpropanal, 2-methylbutanal, 3-methylbutanal, benzaldehyde, phenylacetaldehyde, methional, 2-furfural, 5-HMF, 5-methylfurfural, acetylfuran, diacetyl, ethyl-3-methylbutyrate, ethyl nicotinate, ethyl pyruvate, 2-furfuryl ethyl ether, γ-nonalactone, β-damascenone
Schieberle and Komarek 2005	4 weeks, 45 °C, flushed with O_2	/	SAFE-GC-O (AEDA)	/	2-methylpropanal, 3-methylbutanal, 2-methylbutanal, methional, phenylacetaldehyde
Vesely et al. 2003	12 weeks, 30 °C	/	*On-fibre* derivatisation (PFBHA) SPME	/	2-methylpropanal, 2-methylbutanal, 3-methylbutanal, pentanal, hexanal, 2-furfural, methional, phenylacetaldehyde, (E)-2-nonenal
Wietstock, Kunz, and Methner 2016b	30 weeks, 28 °C	/	SAFE-GC/MS	/	2-methylbutanal, 3-methylbutanal, methional, phenylacetaldehyde, benzaldehyde, 2-furfural, ethyl nicotinate, γ-nonalactone,
Zufall et al. 2005	12 weeks, 28 °C	/	SPME-GC-FPD, SPME-GC/MS	Modified MEBAK method: 0 (none)–10 (very strong)	/

3. Analysis of volatile aroma compounds and non-volatile precursors in beer and aged beer

There are numerous ways to estimate and predict the stability of beer flavor, such as the 1,1′-diphenyl-2-picrylhydrazyl (DPPH) assay, the *N*-tert-butyl-α-phenylnitrone assay associated with electron spin resonance (PBN-ESR), the peroxide challenge test (PCT), total reactive antioxidant potential (TRAP) analysis, the thiobarbituric acid assay (TBA), free-radical index (FRI) analysis, compound index (CI) analysis, and stability index (SI) analysis (J. Liu et al. 2008; Miedl et al. 2011). They can be correlated with aged flavor fairly well, but work unselectively. Only the determination of aroma profiles and precursors will allow us to understand flavor changes during aging and thus technologically reduce them.

Although, in the last 40 years, numerous studies have focused on the volatile compounds responsible for the staling of beer and huge advances have been made in analytical instrumentation, a comprehensive picture of beer flavor instability is still to be developed. This may be in part due to the use of poorly suited analytical techniques and the reliance on forced-aging, which can be performed in different ways, as shown above (Schieberle and Komarek 2005). In the following section, the impacts of different analysis parameters are discussed.

3.1. Selection of extraction technique

Due to each method's particular advantages and disadvantages, there is no absolute way to determine the compounds responsible for beer aging. The choice of extraction method greatly depends on the matrix and analytes (Werkhoff et al. 2002). In general, the most serious analytical errors occur during sample extraction, and numerous studies have stated that the outcome of an analysis is highly dependent on the extraction and analysis techniques used. Thus, a suitable method for extracting volatile compounds has to cover all key aroma compounds without discriminating, decomposing, or modifying them. Additionally, no new aroma compounds should arise, and non-volatiles should be removed, which can interfere with chromatographic separation (Engel, Bahr, and Schieberle 1999). Thus, to detect the compounds of interest in the best way, a suitable method needs to be selected.

Direct analysis (without previous extraction) of volatile aging compounds in beer by GC is not applicable owing to the presence of other more abundant volatiles that will obscure carbonyl compounds and other compounds that can hinder analysis or damage the GC apparatus (Vanderhaegen et al. 2006). Therefore, preconcentration or enrichment steps are required. For the detection and quantification of carbonyl compounds, there are multiple sample-preparation methods available, which can be grouped into solvent extraction techniques such as liquid-liquid extraction (LLE), continuous LLE (CLLE), steam distillation (SD), solvent-assisted flavor evaporation (SAFE), and headspace (dynamic headspace, *purge and trap*) and sorptive techniques, such as solid-phase microextraction (SPME) and stir-bar sorptive extraction (SBSE) (Da Costa and Eri 2005). Of these, SD, CLLE in combination with SAFE, and SPME are the most frequently used methods. In a complex matrix like beer, reducing sugars and free amino acids are present and high temperatures promote Strecker and Maillard reactions and the release of volatiles from aldehydes. In this way, flavor profiles can be altered due to additional and non-genuine aroma compounds or changes in concentrations (Engel, Bahr, and Schieberle 1999). For example, Noël et al. observed that low levels of E-2-nonenal are quickly lost during SD (Noël et al. 1999a). Thus, it might not be the best extraction technique, even though it is a MEBAK standard method.

Thompson-Witrick et al. compared CLLE/SAFE and SPME while extracting aroma compounds from Gueuze lambic beer. They concluded that the two methods performed equally well. SAFE showed clear advantages for the extraction of organic acids, yet was inferior at extracting esters and higher alcohols like decanol. Conversely, SPME did not extract certain short- and medium-chain organic acids but excelled at isolating esters (Thompson-Witrick et al. 2015).

Thus, the extraction method is crucial and can impact the flavor profile drastically. Additionally, extraction parameters like time and temperature affect the analysis. The choice of organic solvent is crucial for solvent extraction. In the same way, the selection of adsorptive materials is key for techniques like SPME or SBSE. Typically, 3-way fibers are used for complex samples to achieve the lowest degree of discrimination. For SPME, Saison et al. optimized these parameters for several beer-aging compounds (Saison et al. 2008).

In certain cases enrichment techniques, such as solid phase extraction (SPE) can enhance yield and reproducibility. It should be noted that the SPME and SBSE techniques are also enriching (Baltussen et al. 1999; Castro and Ross 2015).

3.2. Derivatization

Depending on the substance class, a derivatization step is often used to promote or alter chemical properties and stabilize the compounds of interest. Furthermore, the sensitivity and selectivity of a method can be improved. Derivatization remains not mandatory (Charry-Parra, Dejesus-Echevarria, and Perez 2011), yet it is highly advisable.

For carbonyls, derivatization with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA) is a well-established method. In the reaction, two oximes arise, leading to two peaks in gas chromatography (cf. Figure 2).

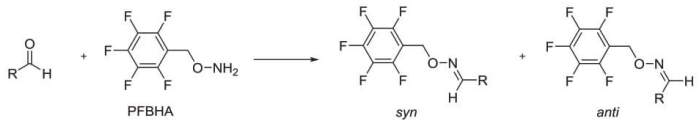

Figure 2. Reaction between carbonyls and PFBHA, yielding two possible oxime isomers (Martos and Pawliszyn 1998).

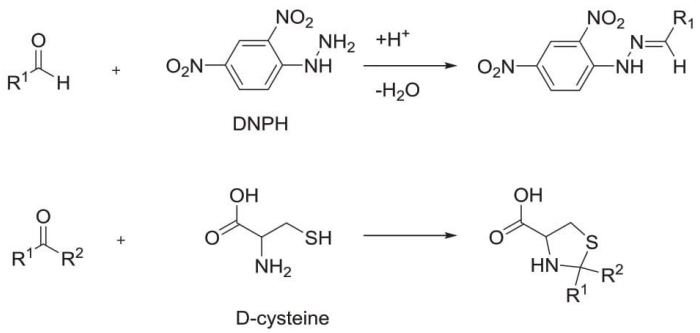

Figure 3. Derivatization with DNPH from Goncalves et al.(Moreira Goncalves et al. 2010) and D-cysteine from Kim et al. (Kim and Shin 2011).

When performing SPME it is easily applied using on-fiber derivatization, which makes it a simple and rapid technique to lower detection limits to the high-ppt range. Saison et al. optimized the method for 32 beer aging compounds (Saison et al. 2008).

Transformation from volatile to non-volatile can be achieved by derivatization with 2,4-dinitrophenylhydrazine (DNPH) or D-cysteine, for example. The former has been used for determination of three aldehydes by gas-diffusion microextraction (GDME) HPLC-diode-array detection (DAD) (Moreira Goncalves et al. 2010). Kim et al. were able to determine several aldehydes with LC-MS/MS after derivatization with d-cysteine (Kim and Shin 2011) (cf. Figure 3). The resulting thiazolidine-4-carboxylic acids are regarded to be aldehyde-precursors in beer aging, as shown above.

3.3. Selection of separation and detection techniques

Depending on the chemical properties of the compounds of interest, a suitable separation technique should be chosen. Volatile compounds are typically analyzed using GC, while non-volatiles are separated using liquid chromatography (LC).

GC is understandably the most common technique for volatile aroma-active compounds (J. L. Goncalves et al. 2014; Andrés-Iglesias et al. 2016; Rossi et al. 2014; Koserske 2016). However, LC can be used to determine volatile compounds after derivatization (as shown above) and non-volatiles like bound-state aldehydes simultaneously.

3.4. Possible strategies for determination of bound-state compounds

Even though the bisulfite and imine adducts of carbonyls have been known for years, methods for their detection remain scarce. Using an HPLC-fluorescent method, Kaneda et al. were able to determine free sulfite and acetaldehyde-bisulfite in beer samples (Kaneda et al. 1996). Furthermore, Nyborg et al. developed an LC-MS method for the quantification of (E)-2-nonenal-bisulfite in model systems (Nyborg, Outtrup, and Dreyer 1999). In addition, De Azevedo et al. demonstrated that the major portion of aldehydes are bound as bisulfite-adducts using a HPLC-UV method (Azevedo et al. 2007).

Liu et al. established a fast headspace (HS) gas chromatography method for acetaldehyde and diethylacetal. Thus, they were able to detect both the free aldehyde and the bound-state acetal simultaneously during fermentation, forced aging and natural aging (C. Liu et al. 2018). Baert et al. developed an UPLC-PDA method for the determination of 2-substituted 1,3-thiazolidine-4-carboxylic acids, and reported that of 2-(furan-2-yl)-1,3-thiazolidine-4-carboxylic acid are present in commercial pale lager beers at concentrations in the range 4.3–7.9 mg L^{-1} (Baert et al. 2015). Furthermore, Al-JaÁfreh et al. were able to determine thiazolidine-4-carboxylic acids in human serum samples after SPE with HPLC-UV (Al-JaÁfreh, Hatrík, and Havránek 1999).

Kim et al. reported an LC-MS/MS method for the determination of linear aldehydes after derivatization with D-cysteine in the form of 2-substituted 1,3-thiazolidine-4-carboxylic acids (Kim and Shin 2011). By determining the specific bound-state aldehydes in the form of 2-substituted 1,3-thiazolidine-4-carboxylic acids in beer, more insight on the parameters of formation, stabilities and behavior during brewing process can be gained.

Another way of indirectly determining bound-state aldehydes is to release the aldehydes from their adducts and quantify the free carbonyls before and after release. A recent study showed that 4-vinylpyridine, a strong base and protein-mapping agent, proved effective for the determination of certain compounds (Baert 2015). Such a rapid method would be valuable to determine the aging potential of a certain sample. On the other hand, 4-vinylpyridine will react with all present nucleophiles in the sample and needs to be used in excess.

These methods indicate the presence of other bound-state aldehydes in beer, with targeted LC-MS methods appearing to be especially promising.

4. Conclusion

Due to years of research, as well as technological and raw-material-related improvements, more flavor-stable beer is produced today. The most common way to estimate flavor stability is forced-aging. Due to the high temperatures commonly employed in force-aging, force-aged beers are not particularly comparable to naturally aged beers since different aroma profiles are obtained. In most samples, no correlation can be drawn

for products aged at 20°C to 60°C. Therefore, both industry and science require non-discriminative methods to study flavor stability.

For such a complex phenomenon as beer aging, one single class of indicators is not sufficient to fully describe and predict flavor changes. Volatile indicators (mainly carbonyls) can be analyzed very well with different methods, with headspace SPME with on-fiber derivatization (often with PFBHA) being the most used. However, how and to what extent these indicators are formed *de novo* or released from the bound-state it is not well understood. Therefore, prior to approaching flavor stability from a technological point, the formation of precursors, the preferred forms of adducts, and the parameters required for release from the bound-state must be understood.

To gain a holistic understanding of beer aging, more focus on bound-state aldehydes is required. Alternatively to forced-aging, a method to assess the pool of bound-state aldehydes involving the use of 4-vinyl pyridine has been developed. Other promising analytical techniques may be LC-MS, UPLC-ToF-MS, MS^2, or assays involving (quantitative) NMR. From the obtained data, an easily manageable number of meaningful indicators (volatile carbonyls and non-volatile precursors) must be defined. This will, in combination with sophisticated data analysis, lead to more reliable predictions of flavor stability that can be tailored to different breweries and beer styles.

ORCID

Florian Lehnhardt http://orcid.org/0000-0002-3699-9149

References

Al-Ja'Afreh, J. Y., S. Hatrík, and E. Havránek. 1999. High-performance liquid chromatography analysis of thiazolidine-4-carboxylic acids in human serum samples. *Chemical Papers* 53 (2):89–92.

Andrés-Iglesias, C., J. Nešpor, M. Karabín, O. Montero, C. A. Blanco, and P. Dostálek. 2016. Comparison of carbonyl profiles from Czech and Spanish lagers: Traditional and modern technology. *LWT – Food Science and Technology* 66:390–97. doi:10.1016/j.lwt.2015.10.066.

Antal, M. J., T. Leesomboon, W. S. Mok, and G. N. Richards. 1991. Mechanism of formation of 2-furaldehyde from d-xylose. *Carbohydrate Research* 217:71–85. doi:10.1016/0008-6215(91)84118-X.

Aron, P. M., and T. H. Shellhammer. 2010. A discussion of polyphenols in beer physical and flavour stability. *Journal of the Institute of Brewing* 116 (4):369–80. doi:10.1002/j.2050-0416.2010.tb00788.x.

De Azevedo, L. C., M. M. Reis, L. F. Motta, G. O. da Rocha, L. A. Silva, and J. B. de Andrade. 2007. Evaluation of the formation and stability of hydroxyalkylsulfonic acids in wines. *Journal of Agricultural and Food Chemistry* 55 (21):8670–80. doi:10.1021/jf0709653.

Back, W., C. Forster, M. Krottenthaler, J. Lehmann, B. Sacher, and B. Thum. 1997. Neue forschungserkenntnisse zur verbesserung der geschmacksstabilität. *Brauwelt* 137 (38):1677–1682, 1684.

Baert, J. 2015. *Unravelling the role of free and bound-state aldehydes in beer flavour instablity*. Dissertation KU Leuven.

Baert, J. J., J. de Clippeleer, and G. Aerts. 2015. Exploring the binding behavior of beer staling aldehydes in model systems. *ASBC* 73 (1):100–108.

Baert, J. J., J. de Clippeleer, L. de Cooman, and G. Aerts. 2014. From concept to functional tool: How bound-state aldehydes can influence beer flavor instability. 4th International Young Scientists Symposium on Malting, Brewing, and Distilling, 2014.

Baert, J. J., J. de Clippeleer, P. S. Hughes, L. de Cooman, and G. Aerts. 2012. On the origin of free and bound staling aldehydes in beer. *Journal of Agricultural and Food Chemistry* 2012 (60):11449–72. doi:10.1021/jf303670z.

Baert, J. J., J. de Clippeleer, B. Jaskula-Goiris, F. van Opstaele, G. de Rouck, G. Aerts, and L. de Cooman. 2015. Further elucidation of beer flavor instability: The potential role of cysteine-bound aldehydes. *ASBC* 73 (3):243–252.

Baltussen, E., P. Sandra, F. David, and C. Cramers. 1999. Stir bar sorptive extraction (SBSE), a novel extraction technique for aqueous samples: Theory and principles. *Journal Microcolumn Separations* 11 (10):737–47. doi:10.1002/(SICI)1520-667X(1999)11:10%3c737::AID-MCS7%3e3.0.CO;2-4.

Bamforth, C., W. and Lentini, A. 2009. The flavor instability of beer. Beer: A Quality Perspective. A volume in Handbook of Alcoholic Beverages. p. 85–109. Edited by: Charles W. Bamforth, Inge Russell and Graham Stewart. Elsevier. 30 Corporate Drive, Suite 400, Burlington, MA 01803, USA. ISBN: 978-0-12-669201-3

Bamforth, C. W. 2001. pH in brewing: An Overview. *MBAA Technical Quarterly* 38 (1):1–9.

Barker, R. L., D. E. F. Gracey, A. J. Irwin, P. Pipasts, and E. Leiska. 1983. Liberation of staling aldehydes during storage of beer. *Journal of the Institute of Brewing* 89 (6):411–15. doi:10.1002/j.2050-0416.1983.tb04216.x.

Baxter, E. D., and P. S. Hughes. 2001. *Beer: Quality, safety and nutritional aspects*. RSC paperbacks. Cambridge: Royal Society of Chemistry.

Bravo, A., J. C. Herrera, E. Scherer, Y. Ju-Nam, H. Rubsam, J. Madrid, C. Zufall, and R. Rangel-Aldao. 2008. Formation of alpha-dicarbonyl compounds in beer during storage of pilsner. *Journal of Agricultural and Food Chemistry* 56 (11):4134–44. doi:10.1021/jf703696p.

Bueno, M., V. Carrascón, and V. Ferreira. 2016. Release and formation of oxidation-related aldehydes during wine oxidation. *Journal of Agricultural and Food Chemistry* 64 (3):608–17. doi:10.1021/acs.jafc.5b04634.

Castro, L. F., and C. F. Ross. 2015. Determination of flavour compounds in beer using stir-bar sorptive extraction and solid-phase microextraction. *Journal of the Institute of Brewing* 121 (2):197–203. doi:10.1002/jib.219.

Cejka, P., J. Culik, T. Horak, M. Jurkova, and J. Olsovska. 2013. Use of chemical indicators of beer aging for ex-post checking of storage conditions and prediction of the sensory stability of beer. *Journal of Agricultural and Food Chemistry* 61 (51):12670–75. doi:10.1021/jf403361h.

Charry-Parra, G., M. Dejesus-Echevarria, and F. J. Perez. 2011. Beer volatile analysis: Optimization of HS/SPME coupled to GC/MS/FID. *Journal of food science* 76 (2):C205–11. doi:10.1111/j.1750-3841.2010.01979.x.

Chevance, F., C. Guyot-Declerck, J. Dupont, and S. Collin. 2002. Investigation of the β-Damascenone level in fresh and aged commercial beers. *Journal of Agricultural and Food Chemistry* 50 (13):3818–21. doi:10.1021/jf020085i.

Christoph, N., M. Gessner, T. J. Simat, and K. Hoenicke. 1999. Off-flavor compounds in wine and other food products formed by enzymatical, physical, and chemical degradation of tryptophan and its metabolites. *Advances in Experimental Medicine and Biology* 467:659–69. doi:10.1007/978-1-4615-4709-9_85.

De Clippeleer, J., G. de Rouck, L. de Cooman, and G. Aerts. 2010. Influence of the hopping technology on the storage-induced appearance of staling aldehydes in beer. *Journal of the Institute of Brewing* 116 (4):381–98. doi:10.1002/j.2050-0416.2010.tb00789.x.

Da Costa, N. C., and S. Eri. 2005. *Chemistry and technology of flavors and fragrances*. Oxford, DQ, UK: Blackwell Publishing Ltd.

Daenen, L., F. Sterckx, F. R. Delvaux, H. Verachtert, and G. Derdelinckx. 2008. Evaluation of the glycoside hydrolase activity of a brettanomyces strain on glycosides from Sour Cherry (Prunus Cerasus L.) used in the production of special fruit beers. *FEMS Yeast Research* 8 (7):1103–14. doi:10.1111/j.1567-1364.2008.00421.x.

Dalgliesh, C. E. 1977. Flavour stability. Proceedings of the 16th european brewery convention congress. *Amsterdam, DSW, Dordrecht: The Netherlands*, 623–59.

Depraetere, S. A., F. Delvaux, D. De Schutter, I. S. Williams, J. Winderickx, and F. R. Delvaux. 2008. The influence of wort aeration and yeast preoxygenation on beer staling processes. *Food Chemistry* 107 (1):242–49. doi:10.1016/j.foodchem.2007.08.023.

Drost, B. W., R. van den Berg, F. J. M. Freijee, E. G. van der Velde, and M. Hollemans. 1990. Flavor stability. *Journal of American Society of Brewing Chemists* 48 (4):124–31.

Dufour, J.-P., M. Leus, A. J. Baxter, and A. R. Hayman. 1999. Characterization of the reaction of bisulfite with unsaturated aldehydes in a beer model system using nuclear magnetic resonance spectroscopy. *ASBC* 57 (4):138–44.

Dunkel, A., M. Steinhaus, M. Kotthoff, B. Nowak, D. Krautwurst, P. Schieberle, and T. Hofmann. 2014. Nature's chemical signatures in human Olfaction: A foodborne perspective for future biotechnology. *Angewandte Chemie, International Edition* 53 (28):7124–43. doi:10.1002/anie.201309508.

Eger, C., N. Habich, N. Akdokan, U. Goßling, and H.-G. Bellmer. 2005. Profiling of beers during ageing at different temperatures. Proceedings of the 30th EBC Congress.

Eichhorn, P. 1991. *Untersuchungen zur Geschmacksstabilität des Bieres*.

Engel, W., W. Bahr, and P. Schieberle. 1999. Solvent assisted flavour evaporation – a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. *European Food Research and Technology* 209 (3–4):237–41. doi:10.1007/s002170050486.

Evans, D. J., D. J. M. Schmedding, A. Bruijnje, T. Heideman, B. M. King, and N. M. Groesbeek. 1999. Flavour impact of aged beers. *Journal of the Institute of Brewing* 105 (5):301–307. doi:10.1002/j.2050-0416.1999.tb00524.x.

Fritsch, H. T., and P. Schieberle. 2005. Identification based on quantitative measurements and aroma recombination of the character impact odorants in a bavarian pilsner-type beer. *Journal of Agricultural and Food Chemistry* 53 (19):7544–51. doi:10.1021/jf051167k.

Fritsch, H. T., A. Stephan, G. Stettner, F.-J. Methner, and P. Schieberle. 2005. Total mapping and sensory simulation of beer aroma. Proceedings of the 30 th EBC Congress.

Gijs, L., F. Chevance, V. Jerkovic, and S. Collin. 2002. How low pH can intensify β-Damascenone and dimethyl trisulfide production through beer aging. *Journal of Agricultural and Food Chemistry* 50 (20):5612–16. doi:10.1021/jf020563p.

Goncalves, J. L., J. A. Figueira, F. P. Rodrigues, L. P. Ornelas, R. N. Branco, C. L. Silva, and J. S. Camara. 2014. A powerful methodological approach combining headspace solid phase microextraction, mass spectrometry and multivariate analysis for profiling the volatile metabolomic pattern of beer starting raw materials. *Food Chemistry* 160:266–80. doi:10.1016/j.foodchem.2014.03.065.

Goncalves, L. M., P. J. Magalhaes, I. M. Valente, J. G. Pacheco, P. Dostalek, D. Sykora, J. A. Rodrigues, and A. A. Barros. 2010. Analysis of aldehydes in beer by gas-diffusion microextraction: Characterization by high-performance liquid chromatography-diode-array detection-atmospheric pressure chemical ionization-mass spectrometry. *Journal of Chromatography A* 1217 (24):3717–22. doi:10.1016/j.chroma.2010.04.002.

Greenhoff, K., and R. E. Wheeler. 1981. Evaluation of stale flavour and beer carbonyl development during normal and accelerated aging utilizing liquid and high pressure liquid chromatography. *Proceedings of the EBC Congress* 1981 (18):405–12.

Grigsby, J. H., S. R. Palamand, and W. A. Hardwick. 1972. Studies on the reactions involved in the oxidation of beer. *Journal of the American Society of Brewing Chemists* (30):87–92.

Grosch, W. 2001. Evaluation of the key odorants of foods by dilution experiments, aroma models and omission. *Chemical Senses* 26 (5):533–45. doi:10.1093/chemse/26.5.533.

Guedes de Pinho, P., and A. C. Silva Ferreira. 2006. Role of strecker Aldehydes on beer flavour stability. In *Flavour Science: Recent advances and trends*. ed. W. L. P. Bredie and M. A. Petersen. Vol. 43, 1st ed., 529–32. Developments in Food Science 43. Amsterdam, Boston: Elsevier.

Guido, L. F. 2005. How do sulfites help to control beer ageing? *Cerevisia* 30 (2):132–38.

Guido, L. F. 2016. Sulfites in beer: Reviewing regulation, analysis and role. *Sciences and Agriculture (Piracicaba, Braz.)* 73 (2):189–97. doi:10.1590/0103-9016-2015-0290.

Guyot-Declerck, C., N. François, C. Ritter, B. Govaerts, and S. Collin. 2005. Influence of pH and ageing on beer organoleptic properties. A sensory analysis based on AEDA data. *Food Quality Prefer.* 16 (2):157–62. doi:10.1016/j.foodqual.2004.04.007.

Hashimoto, N., and T. Eshima. 1977. Composition and pathway of formation of stale aldehydes in bottled beer. *ASBCJ* 35:145–150.

Herrmann, M., B. Klotzbücher, M. Wurzbacher, S. Hanke, U. Kattein, W. Back, T. Becker, and M. Krottenthaler. 2010. A new validation of relevant substances for the evaluation of beer aging depending on the employed boiling system. *Journal of the Institute of Brewing* 116 (1):41–48. doi:10.1002/j.2050-0416.2010.tb00396.x.

Ilett, D. R., and W. J. Simpson. 1995. Loss of sulphur dioxide during storage of bottled and canned beers. *Food Research International* 28 (4):393–96. doi:10.1016/0963-9969(95)00012-B.

Kaneda, H., N. Kobayashi, S. Furusho, H. Sahara, and S. Koshino. 1995. Chemical evaluation of beer flavour stability. *Technical Quarterly - Master Brewers Association of the Americas* 32 (2):76–80.

Kaneda, H., T. Osawa, S. Kawakishi, M. Munekata, and S. Koshino. 1994. Contribution of carbonyl-bisulfite adducts to beer stability. *Journal of Agricultural and Food Chemistry* 42 (11):2428–32. doi:10.1021/jf00047a012.

Kaneda, H., M. Takashio, T. Osawa, S. Kawakishi, S. Koshino, and T. Tamaki. 1996. Analysis of Aldehyde-bisulfites in Beer by HPLC-Fluorescence detection with post-column derivatization. *Journal of Food Science* 61 (1):105–108. doi:10.1111/j.1365-2621.1996.tb14736.x.

Kaneda, H., M. Takashio, and T. Tamaki. 1997. Influence of pH on flavour staling during beer storage. *Journal of the Institute of Brewing* 103:21–23. doi:10.1002/j.2050-0416.1997.tb00932.x.

Kim, H.-J., and H.-S. Shin. 2011. Simple derivatization of aldehydes with D-Cysteine and their determination in beverages by liquid chromatography-tandem mass spectrometry. *Analytica Chimica Acta* 702 (2):225–32. doi:10.1016/j.aca.2011.07.006.

Koserske, J. 2016. Chasing volatiles – Gas chromatography tandem mass spectrometry (GC-MS/MS) in beer flavor analysis. 5th International Young Scientists Symposium on Malting, Brewing and Distilling, Chicho, USA, April 21.

Kuroda, H., S. Furusho, H. Maeba, and M. Takashio. 2003. Characterization of factors involved in the production of 2(E)-nonenal during mashing. *Bioscience, Biotechnology, and Biochemistry* 67 (4):691–97. doi:10.1271/bbb.67.691.

Lermusieau, G., S. Noël, and S. Collin. 1999. Nonoxidative mechanism for development of trans-2-Nonenal in beer. *ASBC* 57 (1):29–33.

Liégeois, C., N. Meurens, C. Badot, and S. Collin. 2002. Release of Deuterated (E)-2-Nonenal during beer aging from labeled precursors synthesized before boiling. *Journal of Agricultural and Food Chemistry* 50 (26):7634–38. doi:10.1021/jf020617v.

Liu, C., Q. Li, C. Niu, F. Zheng, and Y. Zhao. 2018. Simultaneous determination of diethylacetal and acetaldehyde during beer fermentation and storage process. *Journal of the Science of Food and Agriculture*. doi:10.1002/jsfa.9008.

Liu, J., J. Dong, Q. Li, J. Chen, and G. Gu. 2008. Investigation of new indexes to evaluate aging of bottled lager beer. *Journal of American Society of Brewing Chemists* 66 (3):167–73.

Lustig, S. 1995. *Das Verhalten flüchtiger Aromastoffe bei der Lagerung von Flaschenbier*.

Malfliet, S., K. Goiris, G. Aerts, and L. Cooman. 2009. Analytical-sensory determination of potential flavour deficiencies of light beers. *Journal of the Institute of Brewing* 115 (1):49–63. doi:10.1002/j.2050-0416.2009.tb00344.x.

Martos, P. A., and J. Pawliszyn. 1998. Sampling and Determination of formaldehyde using solid-phase microextraction with on-fiber derivatization. *Analytical Chemistry* 70 (11):2311–20. doi:10.1021/ac9711394.

Meilgaard, M. C. 1972. Stale flavor carbonyls in brewing. *Brewers Dig* 47:48–57.

Meilgaard, M. C. 1975. Flavor chemistry of beer, part II: flavor and thresholds of 239 aroma volatiles. *Technical Quarterly - Master Brewers Association of the Americas* 12 (3):151–68.

Miedl, M., P. Rogers, G. A. Day, F. M. Clarke, and G. G. Stewart. 2011. The peroxide challenge test: A novel method for holistic near-real time measurement of beer flavour stability. *Journal of the Institute of Brewing* 117 (2):166–75. doi:10.1002/j.2050-0416.2011.tb00456.x.

Murakami, A. A., H. Goldstein, A. Navarro, J. R. Seabrooks, and D. S. Ryder. 2003. Investigation of beer flavor by gas chromatography-Olfactometry. *ASBC* 61 (1):23–32.

Narziss, L. 1986. Centenary review: Technological factors of flavour stability. *Journal of the Institute of Brewing* 92:346–53. doi:10.1002/j.2050-0416.1986.tb04421.x.

Nikolov, P. Y., and V. A. Yaylayan. 2011. Reversible and covalent binding of 5-(Hydroxymethyl)-2-Furaldehyde (HMF) with Lysine and selected amino acids. *Journal of Agricultural and Food Chemistry* 59 (11):6099–107. doi:10.1021/jf200735c.

Noël, S., C. Liégeois, G. Lermusieau, E. Bodart, C. Badot, and S. Collin. 1999a. Release of deuterated nonenal during beer aging from labeled precursors synthesized in the boiling kettle. *Journal of Agricultural and Food Chemistry* 47 (10):4323–26. doi:10.1021/jf990211r.

Noël, S., N. Metais, S. Bonte, E. Bodart, F. Peladan, S. Dupire, and S. Collin. 1999b. The use of Oxygen 18 in appraising the impact of oxidation process during beer storage. *Journal of the Institute of Brewing* 105 (5):269–74. doi:10.1002/j.2050-0416.1999.tb00519.x.

Nyborg, M., H. Outtrup, and T. Dreyer. 1999. Investigations of the protective mechanism of sulfite against beer staling and formation of adducts with tras-2-Nonenal. *Journal of the American Society of Brewing Chemists* 57 (1):24–28.

Önen Bayram, F. E., H. Sipahi, E. T. Acar, R. K. Ulugöl, K. Buran, and H. Akgün. 2016. The cysteine releasing pattern of some antioxidant Thiazolidine-4-Carboxylic acids. *European Journal of Medicinal Chemistry* 114:337–44. doi:10.1016/j.ejmech.2016.03.019.

Palamand, S. R., and J. H. Grigsby. 1974. Stale flavors in beer – Identification of o-aminoacetophenone and ethyl nicotinate in beer. *Brewers Dig.* 49 (9):58–60.

Palamand, S. R., and W. A. Hardwick. 1969. Studies on the relative flavour importance of some beer constituents. *Technical Quarterly - Master Brewers Association of the Americas* 6 (2):117–28.

Pan, L., J. M. Chong, and J. Pawliszyn. 1997. Determination of amines in air and water using derivatization combined with solid-phase microextraction. *Journal of Chromatography A* 773 (1–2):249–60. doi:10.1016/S0021-9673(97)00179-9.

Pankoke, I. 2015. Securing beer and beverage quality during long distance distribution process – Using a special sensor system. 35th EBC Congress, Porto, 2015.

Peppard, T. L., and S. A. Halsey. 1982. The Occurence of two geometrical isomers of 2,4,5-trimethyl-1,3-dioxolane in beer. *Journal of the Institute of Brewing* 88 (5):309–12. doi:10.1002/j.2050-0416.1982.tb04113.x.

Rakete, S., A. Klaus, and M. A. Glomb. 2014. Investigations on the maillard reaction of dextrins during aging of pilsner type beer. *Journal of Agricultural and Food Chemistry* 62 (40):9876–84. doi:10.1021/jf503038c.

Rossi, S., V. Sileoni, G. Perretti, and O. Marconi. 2014. Characterization of the volatile profiles of beer using headspace solid-phase microextraction and gas chromatography-mass spectrometry. *Journal of the Science of Food and Agriculture* 94 (5):919–28. doi:10.1002/jsfa.6336.

Saison, D., D. P. de Schutter, F. Delvaux, and F. R. Delvaux. 2008. Optimisation of a complete method for the analysis of volatiles involved in the flavour stability of beer by solid-phase microextraction in combination with gas chromatography and mass spectrometry. *Journal of Chromatography A* 1190 (1–2):342–49. doi:10.1016/j.chroma.2008.03.015.

Saison, D., D. P. de Schutter, B. Uyttenhove, F. Delvaux, and F. R. Delvaux. 2009. Contribution of staling compounds to the aged flavour of lager beer by studying their flavour thresholds. *Food Chemistry* 114 (4):1206–15. doi:10.1016/j.foodchem.2008.10.078.

Saison, D., D. P. de Schutter, N. Vanbeneden, L. Daenen, F. Delvaux, and F. R. Delvaux. 2010a. Decrease of aged beer aroma by the reducing activity of brewing yeast. *Journal of Agricultural and Food Chemistry* 58 (5):3107–15. doi:10.1021/jf9037387.

Saison, D., N. Vanbeneden, D. P. de Schutter, L. Daenen, T. Mertens, F. Delvaux, and F. R. Delvaux. 2010b. Characterisation of the flavour and the chemical composition of lager beer after ageing in varying conditions. *Brewing Science* 63:41–53.

Schieberle, P., and D. Komarek. 2003. Changes in key aroma compounds during natural beer aging. *Freshness and Shelf Life of Foods* (Chapter 5):70–79.

Schieberle, P., and D. Komarek. 2005. Staling of beer aroma: a long-known, but still unresolved challenge in brewing science. *Proceedings of the 30 th EBC Congress*.

Schnaitter, M., A. Wimmer, H. Kollmannsberger, M. Gastl, and T. Becker. 2016. Influence of hop harvest date of the 'Mandarina Bavaria' hop variety on the sensory evaluation of dry-hopped top-fermented beer. *Journal of the Institute of Brewing* 122 (4):661–69. doi:10.1002/jib.382.

De Schutter, D. P. 2008. *The influence of thermal load during wort boiling on the flavour stability of beer*. Leuven, Belgium: Dissertation University of Leuven. Accessed January 23, 2017.

Soares da Costa, M., C. Goncalves, A. Ferreira, C. Ibsen, P. Guedes de Pinho, and A. C. Silva Ferreira. 2004. Further insights into the role of methional and phenylacetaldehyde in lager beer flavor stability. *Journal of Agricultural and Food Chemistry* 52 (26):7911–17. doi:10.1021/jf049178l.

Suda, T., Y. Yasuda, T. Imai, and Y. Ogawa. 2007. Mechanisms for the development of Strecker aldehydes during beer aging. Proceedings of the 31 st EBC Congress.

Thiele, F. 2006. Einfluss der Hefevitalität und der Gärparameter auf die Stoffwechselprodukte der Hefe und auf die Geschmacksstabilität.

Thompson-Witrick, K. A., R. L. Rouseff, K. R. Cadawallader, S. E. Duncan, W. N. Eigel, J. M. Tanko, and S. F. O'Keefe. 2015. Comparison of two extraction techniques, solid-phase microextraction versus continuous liquid-liquid extraction/solvent-assisted flavor evaporation, for the analysis of flavor compounds in gueuze lambic beer. *Journal of Food Science* 80 (3):C571–6. doi:10.1111/1750-3841.12795.

Vanderhaegen, B., H. Neven, S. Coghe, K. J. Verstrepen, H. Verachtert, and G. Derdelinckx. 2003. Evolution of chemical and sensory properties during aging of top-fermented beer. *Journal of Agricultural and Food Chemistry* 51 (23):6782–90. doi:10.1021/jf034631z.

Vanderhaegen, B., H. Neven, L. Daenen, K. J. Verstrepen, H. Verachtert, and G. Derdelinckx. 2004. Furfuryl Ethyl Ether: Important aging flavor and a new marker for the storage conditions of beer. *Journal of Agricultural and Food Chemistry* 52 (6):1661–68. doi:10.1021/jf035412g.

Vanderhaegen, B., H. Neven, H. Verachtert, and G. Derdelinckx. 2006. The chemistry of beer aging – a critical review. *Food Chemistry* 95 (3):357–81. doi:10.1016/j.foodchem.2005.01.006.

Verhagen, L. C., ed. 2010. *Beer flavor in comprehensive natural products II: Chemistry and biology*. United Kingdom: Elsevier.

Vesely, P., L. Lusk, G. Basarova, J. Seabrooks, and D. Ryder. 2003. Analysis of aldehydes in beer using solid-phase microextraction with on-fiber derivatization and gas Chromatography/mass spectrometry. *Journal of Agricultural and Food Chemistry* 51 (24):6941–44. doi:10.1021/jf034410t.

Werkhoff, P., S. Brennecke, W. Bretschneider, H.-J. Bertram, and R. Marsili. 2002. *Flavor, fragrance, and odor analysis: Modern methods for isolating and quantifying volatile flavor and fragrance compounds*. 1st ed. New York, Basel: Marcel Dekker Inc.

Wietstock, P. C., T. Kunz, and F.-J. Methner. 2016a. Influence of hopping technology on oxidative stability and staling-related carbonyls in pale lager beer. *Brewing Science* 69:73–84.

Wietstock, P. C., T. Kunz, and F.-J. Methner. 2016b. The 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.

Zufall, C., G. Racioppi, M. Gasparri, and J. Franquiz. 2005. Flavour stability and ageing characteristics of light-stable beers. Proceedings of the 30 th EBC Congress, 617–24.

3.2.4 The Influence of Proteolytic Malt Modification on the Aging Potential of Final Wort

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The Influence of Proteolytic Malt Modification on the Aging Potential of Final Wort

Arndt Nobis †, Florian Lehnhardt †, Marcel Gebauer, Thomas Becker and Martina Gastl *

Brewing and Beverage Technology, TU Munich, Weihenstephaner Steig 20, 85354 Freising, Germany; arndt.nobis@tum.de (A.N.); florian.lehnhardt@tum.de (F.L.); marcel.gebauer@tum.de (M.G.); tb@tum.de (T.B.)
* Correspondence: martina.gastl@tum.de
† All authors contributed equally to this manuscript.

Abstract: The dynamic changes in beer flavor are determined by its aging potential, which comprises of present free and bound-state aldehydes and their precursors. Rising flavor-active aging compounds cause sensory deterioration (flavor instability). These compounds are mainly formed upstream in the brewing process through the Maillard reaction, the Strecker degradation, or lipid oxidation. Wort boiling is an especially critical production step for important reactions due to its high temperature and favorable pH value. Amino acid concentration, as an important aging-relevant precursor, is variable at the beginning of wort boiling, mainly caused by the malt modification level, and can further influence the aging potential aging formation during wort boiling. This study investigated the effect of the proteolytic malt modification level on the formation of precursors (amino acids and dicarbonyls) and free and bound-state aldehydes during wort boiling. Six worts (malt of two malting barley varieties at three proteolytic malt modification levels) were produced. Regarding precursors, especially Strecker, relevant amino acids and dicarbonyls increased significantly with an enhanced malt modification level. Concentrations of free and bound aldehydes were highest at the beginning of boiling and decreased toward the end. A dependency of malt modification level and the degree of free and bound aldehydes was observed for 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal. Generally, a higher proteolytic malt modification level tended to increase free and bound aldehyde content at the end of wort boiling. Conclusively, the aging potential formation during boiling was increased by an intensified malt modification level.

Keywords: beer aging; wort boiling; Maillard reaction; brewing; dicarbonyls; bound-state aldehydes; malt modification

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1. Introduction

During storage (after bottling), beer flavor undergoes dynamics, including concentration decrease or increase in various flavor-active substances [1]. This flavor instability causes sensory beer deterioration and occurs mostly due to rising aldehydes, defined as aging indicators [2]. Longer distribution distances and periods intensified the problem for brewing industries. Figure 1 shows an overview of reactants, precursors, and aging indicators of four selected key aging reactions, the Maillard reaction, caramelization, the Strecker degradation, and lipid oxidation, which contribute to aldehyde formation during beer aging [3,4].

Alongside the final stage of aging indicators, the concentration of precursors, such as dicarbonyls or amino acids, play a key role in flavor instability during beer storage [1]. In the former, for precursor formation, the level of reactants, such as carbohydrates and amino acids, is important, whereby the substance class of amino acids can act in both functionalities (reactant and precursor). The important reactions (Figure 1) are subsequently described in further detail.

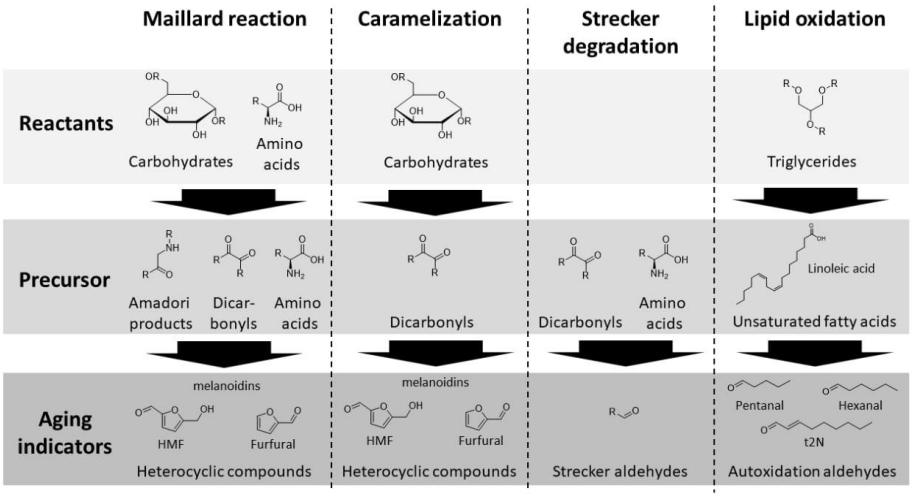

Figure 1. Overview of selected aging-relevant reactions and their key reactants, precursors, and aging indicators.

The oxidation of unsaturated fatty acids occurs enzymatically [5] or by autoxidation [2]. Important precursor compounds include linoleic acid and linolenic acid, which mainly originate from malt. Their oxidation forms aroma-active aging aldehydes, such as (E)-2-nonenal (t2N), pentanal (Pent), hexanal (Hex), and heptanal (Hept) [6,7]. During the Maillard reaction, the nucleophilic amino group of amino acids, as reactants, can react with the carbonyl group of carbohydrates to form Amadori products [8,9]. These compounds can be further degraded to dicarbonyl compounds. The dicarbonyls are a various substance class, with important representatives being 3-desoxypentosone (3-DP), 3-deoxyglucosone (3-DG), or 1-deoxyglucosone (1-DG). Regarding occurring concentrations, 3-DG is the predominant dicarbonyl in beer and wort [10–12]. These dicarbonyls are important precursor compounds [13] and are already formed during malting [14]. The dicarbonyls can also occur directly by carbohydrate dehydration during caramelization [8]. They can further react in several pathways, such as dehydration reactions [15] or the Strecker degradation [16]. In contrast, C5-dicarbonyls mainly result in furfural (Fur), while C6-dicarbonyls produce 5-hydroxymethylfurfural [15] by dicarbonyl dehydration. Regarding the Strecker degradation, amino acids, as precursors, are degraded to Strecker aldehydes. Typical aroma-active compounds are 2-methylpropanal (2MP) from valine, 2-methylbutanal (2MB) from isoleucine, 3-methylbutanal (3MB) from leucine, methional (Meth) from methionine, and phenylacetaldehyde (PA) from phenylalanine [17].

Regarding all reactions, the reactivity of reactants requires high activation energies [3]. Therefore, forming precursors and aldehydes upstream is easier during beer production due to its higher thermal processing when compared to beer storage. Wort boiling, especially, provides the best conditions for forming aging aldehydes and their corresponding precursors due to its intensive thermal load and favorable pH level of 5.4–5.8 for the described aging-relevant reactions, such as dehydration. Alongside its elevated reaction potential, high concentrations of reactants (low molecular carbohydrates and amino acids) at the beginning of boiling induce the best conditions for precursor and aging aldehyde formation [2,9,18]. Thus, aging aldehydes are already formed during wort boiling in high concentrations [19]. These high concentrations in the final wort could be critical for flavor instability; however, it should be considered that yeast partly reduces the aldehydes afterward in the early stage of fermentation [20]. Despite the reduced activity of yeast, the formed aldehyde concentration is important because they can be present in a bound-state due to chemical equilibrium. Here they overcome fermentation in these forms and

get re-released during beer aging [2]. Possible reactions include forming cysteinylated aldehydes [21], bisulfite adducts [22], and imine formation [23]. Suda et al. showed that 85% of the wort aldehydes found in wort were transferred to beer by their bound-state form [24]. Furthermore, Baert et al. pointed out that imine and bisulfite adducts were the most important masking reactions in beer and wort [2]. In particular, imine formation of aldehydes could be important in wort because of the high concentrations of free amino acids [18]. Disadvantageously, imine adducts are more stable at high pH values (pH > 7) [25]. Regarding bisulfite formation, studies showed that sulfur dioxide (SO_2) formation by sulfate reduction in yeast cells occurs mainly in an intermediate stage in the fermentation after yeast cell growth ceases [26,27]. Compared with the imines, the bisulfite adducts show a constant equilibrium at a pH range of 4–6, which is more suitable for wort and beer [28]. Released bisulfite from yeast cells could immediately mask remaining aldehydes, whose reduction rates depend on the strain type, aldehyde type, and fermentation temperature [29]. Alongside the masking effects, the concentration of the formed free aldehydes during boiling could be used as a good indicator for evaluating flavor instability [30].

The formed precursors, free aldehydes, and bound-state aldehydes influence the flavor instability in beer. Therefore, this study defines the sum of the concentration of the three classes as the aging potential. The aging potential is a dynamic result of the brewing process; the ratios of the three classes, and their absolute concentration, changes during the brewing process and aging. Until now, previous studies focused on the investigation of different boiling systems and thermal load [22,31,32], single-substance model-boiling experiments to verify reaction pathways [33,34], or the influence of oxygen [35] on aging potential formation during boiling. However, the influence of a varied reactant concentration, such as amino acid content, was not investigated. Here, the malt modification level by varying the steeping degree is prone to be a suitable parameter because a higher level causes increased nitrogen [36] and peptide contents in malt [37]. This nitrogen potential will be further enzymatically degraded to amino acids during mashing and result in a higher concentration of this substance class at the beginning of wort boiling.

This study hypothesized that a higher content of amino acids, as reactants, directly influences the final wort's aging potential by an increased malt modification level. Thus, the study investigated the influence of a varied reactant concentration on the aging potential formation during wort boiling.

2. Materials and Methods

2.1. Chemicals

All amino acids (L-form), [13C, 15N], labeled amino acids, D-glucose, potassium dihydrogen phosphate, methanol (liquid chromatography–mass spectrometry (LC-MS) grade), o-phenylenediamine (OPD), furfural, pentanal, hexanal, heptanal, (E)-2-nonenal, hydrochloric acid, 4-fluorobenzaldehyde, 2-isobutyl-1,3-thiazolidine-4-carboxylic acid (3MB-CYS; 95%), 2-(2-(methylthio)ethyl)-1,3-thiazolidine-4-carboxylic acid (MET-CYS; 95%), 2-pentyl-1,3-thiazolidine-4-carboxylic acid (HEX-CYS; 95%), caffeine, ethanol (absolute), disodium hydrogen phosphate dihydrate, acetic acid, 2-methylbutanal (2MB), 3-methylbutanal (3MB), 2-methylpropanal (2MP), phenylacetaldehyde (PA), and water (LC-MS grade) were obtained from Merck (Darmstadt, Germany). Acetonitrile used for LC-MS analysis was purchased from VWR (Darmstadt, Germany). Additionally, 2-phenyl-1,3-thiazolidine-4-carboxylic acid (BEN-CYS; 97%), 2-benzyl-1,3-thiazolidine-4-carboxylic acid (PHE-CYS; 97%), and 2-(2-furanyl)-1,3-thiazolidine-4-carboxylic acid (FUR-CYS; 95%) were purchased from Th.Geyer (Berlin, Germany), while the 2-isopropyl-1,3-thiazolidine-4-carboxylic acid (2MP-CYS; 95%) was purchased from ABCR (Karlsruhe, Germany). The 3-DG (>95%) was obtained from Apollo Scientific Ltd. (Cheshire, UK), and the 3-DGal (3-deoxygalactosone; >90%) was purchased from Carbosynth Ltd. (Berkshire, UK). Before use, the water for analytics was purified using a micropore water purification system (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.2. Malt Production

Six malts with different proteolytic modification levels were produced in a pilot-malting system. The malts comprised of two malting barley varieties (harvest year 2019): B1 (Avalon, Nordsaat Saatzucht GmbH, Langenstein, Germany) and B2 (Marthe, Saatzucht Josef Breun GmbH and Co. KG, Herzogenaurach, Germany) with different genetic modification characteristics (high and medium modification levels). The barley varieties had the same protein content of 10% d.m. Both were further targeted and modified, reaching different proteolytic modification levels (P1: low, P2: medium, and P3: high) by varying the steeping degree (Table 1). These variations changed the concentration of reactants (amino acids) and reactant formation potential by protein solubilization (proteolytic enzyme activity) during mashing. All malt samples were malted as standard, according to MEBAK R-110.00.008 (016–03), and standard malt parameters were analyzed on the basis of the isothermal 65 °C laboratory mashing regime R-207.00.002 (2016-03) analogous to common variety evaluation in barley-breeding programs [38]. B1 was germinated at steeping degrees of 38% (P1), 41% (P2), and 44% (P3), while B2 was germinated at steeping degrees of 39% (P1), 43% (P2), and 47% (P3) to obtain the target values of the soluble nitrogen content. Table 1 summarizes the soluble nitrogen targets and the reached values of the malt variations (B1P1, B1P2, B1P3, B2P1, B2P2, and B2P3) in the study.

Table 1. Soluble nitrogen content of brewing malts (n = 3).

Variation	P1 [1]	P2 [1]	P3 [1]
Target value	550 ± 25	625 ± 25	700 ± 25
B1	573 ± 10	601 ± 1	660 ± 1
B2	569 ± 3	620 ± 14	731 ± 1

[1] Data are given in mg/100 g dry weight.

The required specification of soluble nitrogen for brewing purposes of barley malts is 580–680 mg/100 g dry weight (according to isothermal 65 °C mashing procedure) [39,40]. Compared with the target values, the P3 variations of B1 were slightly decreased, and the P3 variation of B2 was slightly increased, but they differed significantly from the P2 samples.

2.3. Wort Production and Sampling

Ten kilograms of produced malts were milled using a type 16/16 two-roller mill from Künzel (Kulmbach, Bayern). The pilot brewhouse (80 L) was used as previously described in [13]. The grist was mashed in with 40 L standardized brewing liquor at 60 °C, and the temperature was raised to 62 °C. Two rests of 30 min each were held at 62 °C and 72 °C, after which the mash was raised to 78 °C and held for 10 min. The heating rate between rests in the mash tun was set to 1.7 °C/min. The mash was then transferred to the lauter-tun, preheated to 78 °C, and a lauter rest of 10 min was held. Lautering was performed with two sparges of 15 L and a third sparge of 14 L with brewing liquor at 78 °C until a target extract content of 10.5° P was reached. A rake was used during the third sparge, and, while collecting the last sparge, the kettle was heated to near-boiling temperatures (95 °C) and brought to a rolling boil after the lautering was finished. Taurus (13.0% α-acids; 33.3 g) was added at the beginning of the 60 min boiling time to reach 15 international bitter units (IBU). The evaporation rate was 10.6% during the 60 min of boiling. All brewhouse steps were standardized for variations in this study. Sampling was done every 10 min up to the end of boiling in a standardized procedure. The sampling containers were 50 and 2 mL plastic tubes used for each of the three sampling times. Sampling was done using a self-manufactured sampling stick. The samples were filtered using a folded filter paper and immediately frozen after filtration. The brewing trials were done in duplicates.

2.4. Quantitation of Free Aldehydes by HS-SPME-GC-MS

The procedure was performed according to Lehnhardt et al. [4], with minor changes. The cooled wort sample (5 mL) was transferred with 50 µL internal standard (2 mg/L

p-fluorobenzaldehyde in ethanol) to a 20 mL headspace vial and stored in a cooled autosampler tray (17 °C). Extraction was performed using a CAR-PDMS-DVB fiber. First, the fiber was loaded with o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) for 10 min at 40 °C. Afterward, the headspace of the sample was extracted for 30 min at 40 °C. Next, the fiber was injected with a 1/5-split at 270 °C into a GC (GC-Ultra 1300, Thermo Scientific Inc., Waltham, MA, USA) coupled to a single quad mass spectrometer (ISQ 7000, Thermo Scientific Inc., Waltham, MA, USA). The GC was equipped with a DB-5 column (length, 60 m; inner diameter, 0.25 mm; and film thickness, 0.25 µm; Thermo Scientific Inc., Waltham, MA, USA). The carrier gas used was helium (flow rate 1.85 mL/min). The starting temperature was held at 60 °C for 4 min, followed by heating at 5 K/min to a final temperature of 250 °C, which was maintained for 3 min. A full scan mode (m/z 35–350), with a dwell time of 0.02 s, was applied to the analysis. Each sample was analyzed in triplicate. Peak detection was performed in Xcalibur 3.1.66.10 (Thermo Scientific Inc., Waltham, MA, USA).

2.5. Quantitation of Bound-State Aldehydes after Release with 4-Vinylpyridine (4-VP)

The procedure was performed as described in the previous section, with one exception. Before adding the internal standard, a 4-VP solution was added (50 µL, 1/1 4-VP/ethanol, *v*/*v*). These samples were incubated in the autosampler tray at 17 °C for at least 6 h before analysis. During the elution of 4-VP from the GC column, mass spectrometric detection was turned off at 13 to 14 min. The concentration of bound-state aldehydes was the difference between the aldehyde contents after 4-VP release and the content of its free form.

2.6. Quantitation of 3-DG and 3-DGal

High-performance liquid chromatography, with ultraviolet detection (HPLC-UV) analytics and sample preparation, were applied as previously performed by Degen, Hellwig, and Henle [11] and modified as published by Nobis et al. [13]. Wort samples were measured directly after derivatization and filtration (0.45 µm).

2.7. Quantitation of Amino Acids

Nineteen amino acids were determined using HPLC-tandem mass spectrometry (MS/MS) in the multiple reaction monitoring mode as previously published by Nobis et al. [14].

2.8. Quantitation of Cysteinylated Aldehydes

UPLC-Q-ToF analysis of cysteinylated aldehydes was performed on a Waters Acquity UHPLC-H system coupled to a Xevo G2-XS Q-TOF (Waters Corporation, Manchester, UK). An Acquity BEH C18 column (2.1 mm × 150 mm, 1.7 µm) was used for chromatographic separation. The samples were kept at 10 °C, and the injection volume was 1 µL. Mobile phase (A) water + 0.1% *v*/*v* formic acid and (B) acetonitrile + 0.1% *v*/*v* formic acid were used with the following gradient: 0–1 min 90% A + 10% B, 1–6 min linear gradient to 48% A + 52% B, 6–6.5 min linear gradient to 100% B, 6.5–7.5 min 100% B, 7.5–7.6 min linear gradient to 90% A + 10% B, and 7.6–8.5 min 90% A + 10% B. The flow rate was kept constant at 0.4 µL/min, and the column temperature was set to 40 °C. Analytes were ionized using an electrospray ionizer in positive mode. Capillary and sample cone voltages were 1 kV and 40 V, respectively. The ion source was kept at 120 °C, and the desolvation temperature was 450 °C. The cone and desolvation gas flow (N2) were 50 and 500 L/h, respectively. The mass range was m/z 50–1200 with a scan time of 0.15 s using resolution mode.

The molecular structures of the seven cysteinylated aldehydes and caffeine, and the m/z of the molecular ion $[M + H]^+$, were calculated. The retention times and m/z of the respective molecular ions are shown in Table 2. Additionally, sample and calibration data were searched for respective m/z, and the response values were used for screening analysis or quantification.

Table 2. Retention times (Rt) and m/z of the molecular ions of the seven cysteinylated aldehydes and caffeine.

Analyte	R_t [min]	$[M + H]^+$
2MP-CYS	1.23	176.0740
MET-CYS	1.50	208.0460
FUR-CYS	1.61	200.0376
Caffeine	2.70	195.0877
3MB-CYS	2.94	190.0896
BEN-CYS	3.19	210.0583
PHE-CYS	3.65	224.0740
HEX-CYS	4.23	204.1053

The calibration was done by preparing a stock solution of the seven cysteinylated aldehydes: 2MP-CYS, MET-CYS, FUR-CYS, 3MB-CYS, BEN-CYS, PHE-CYS, and HEX-CYS, with a final concentration of 100 µg/L in LC-MS-grade water with 5% ethanol. This stock solution was then diluted to obtain four calibration points with concentrations of 0.5, 1, 5, and 10 µg/L of each cysteinylated aldehyde. Next, caffeine, which was used as an internal standard, was prepared in LC-MS-grade water with 5% ethanol and a final concentration of 100 µg/L. For calibration, the internal standard was added to each cysteinylated aldehyde mixture at a concentration of 5 µg/L.

The samples were filtered through a polyamide filter (0.2 µm) and then spiked with the internal standard solution to achieve a caffeine concentration of 5 µg/L. Then, samples were thoroughly mixed and used directly for UPLC-Q-ToF analysis.

2.9. Influence of Varied Reactants during Wort Boiling on Aging Potential

Verifying the dynamic changes in the aging potential during wort boiling was performed by boiling experiments on a laboratory scale with spiked reactants and precursor compounds. The B2P3 malt was milled using a DLFU disk mill from Bühler (Braunschweig, Germany) at a disk gap of 0.2 mm. Fifty grams of grist was mashed with 200 mL tempered distilled water in a laboratory mash procedure (30 min, 62 °C; 6 min, 63 °C–72 °C; 30 min, 72 °C; 5 min, 72 °C–78 °C; and 2 min, 78 °C). The produced mash was filtered using a laboratory filter (folded paper filter) from VWR International GmbH (Darmstadt, Germany) and diluted with water to a target extract content of 12.5° P. Several reactants and precursor compounds were spiked by increasing the initial concentration by 100%, 200%, and 400%. The reactants and precursors used were valine (2-MP), isoleucine (2-MB), leucine (3-MB), methionine (methional), phenylalanine (PA), lysine (3-DG, 3-DGal, and Strecker aldehydes), 3-DG (Strecker aldehydes), fructose (3-DG, 3-DGal, and Strecker aldehydes), glucose (3-DG, 3-DGal, and Strecker aldehydes), arabinose (furfural and Strecker aldehydes), and linoleic acid (lipid oxidation aldehydes). The initial concentrations of amino acids and dicarbonyls were determined as previously described. Sugars (glucose: 9 g/L; fructose: 2 g/L; arabinose: 60 mg/L) and linoleic acid (1 mg/L) were defined according to the literature [18]. All variations were boiled in sealed tubes in triplicate and analyzed after 60 min of boiling. Aging aldehydes and dicarbonyls were determined as described in the previous sections.

2.10. Statistical Analysis

Statistical analysis was performed using JMP Pro v.14 (SAS Institute GmbH, Heidelberg, Germany). Results were presented as average ± standard deviation. ANOVA (Tukey test), at a significance level of 0.05, was used for average comparisons.

3. Results and Discussion

Upon beer aging, aldehyde levels in beer increased and caused undesired flavor changes. Wort boiling plays a major role in flavor instability of beer, as essential aging aldehyde precursors, such as amino acids and aging aldehydes, might be formed. The sum

of the investigated precursor amino acids and dicarbonyls and free and bound-state aldehydes, formed during wort boiling, contributed to the aging potential for beer originating from wort.

3.1. Amino Acids in Wort Boiling Process

In industrial brewing, wort amino acid concentrations are influenced by multiple factors, such as raw material choice, grist load, and malt modification. Amino acids can contribute to aging potential either as reactants in the Maillard reaction or directly as precursors in the Strecker degradation. Therefore, this study monitored them during wort boiling due to different malt modification levels (P1, P2, and P3). Supplementary Materials showed all amino acid concentrations during wort boiling at all malt variations in this study. The concentration of almost all amino acids remained constant during wort boiling. Associating the evaporation rate during boiling, the amino acids were mainly degraded during wort boiling. This behavior indicates their reactivity in aging-relevant reactions, such as the Strecker degradation [41] or the Maillard reaction [8]. Alternatively, the amino acids react in oxidative pathways [8] or form imine adducts with carbonyls [23]. Glutamine, as an exception, was degraded more strongly than the other amino acids. This amino acid showed a linear degradation during wort boiling at all variations (B1P1, B1P2, B1P3, B2P1, B2P2, and B2P3). Presumably, the enhanced reduction was caused by the thermal catalyzed reaction of pyrrolidonecarboxylic acid. The amid function of glutamine, especially, is prone to undergoing internal cyclization, forming the pyrrolidone function [42,43].

Alongside the behavior during wort boiling, this study focused on investigating the influence of the proteolytic malt modification level on the amino acid concentration. Figure 1 shows the content of selected Strecker active amino acids (valine, isoleucine, leucine, phenylalanine, and methionine) at the beginning (0 min) and end (60 min) of wort boiling for different malt modification levels (P1 < P2 < P3). The content of the selected amino acids provides a higher aging potential because they can form Strecker aldehydes in the final product beer [44] and, therefore, influence flavor instability.

According to the described behavior during wort boiling, the concentrations at the beginning and end of boiling were comparable. Despite leucine at B1, the Strecker active amino acids showed increased concentrations by an enhanced malt modification level. In particular, the highest malt modification level (P3) demonstrated increased levels of the presented amino acids (Figure 1). The observed effects could be explained in two ways. First, an increased proteolytic malt modification already caused higher amino acid levels [14]. Suppose the possible formation or degradation reactions during mashing were equal because of standardized mashing procedures for all malt variations; in that case, the ratio of the single amino acid contents between the varied proteolytic malt levels remains constant during wort boiling. Thus, the described effect results in higher amino acid concentrations at the beginning and end of boiling caused by malts with higher proteolytic malt modification. Second, the accelerated protease activity of malts with higher malt modification levels [45] causes higher amino acid contents during mashing. Presumably, the increased contents of amino acids at the P3 variation were a combined effect of the two described ways. Investigating a calculatable influence of the proteolytic malt modification on amino acid concentration in the final wort, the soluble nitrogen content (Table 1), as a representative parameter for the malt modification level, was correlated with the concentrations of amino acids in the final wort at all variations. Table 3 shows the resulting correlation coefficients.

Table 3. Correlation coefficients of amino acid concentration in the final wort and soluble nitrogen content.

Amino Acid	Correlation Coefficient	Amino Acid	Correlation Coefficient
Valine	0.97	Asparagine	0.64
Isoleucine	0.90	Methionine	0.52
Phenylalanine	0.87	Glutamic acid	0.46
Leucine	0.82	Proline	0.42
Threonine	0.78	Tyrosine	0.40
Lysine	0.74	Glycine	0.29
Tryptophan	0.69	Aspartic acid	0.25
Alanine	0.65	Glutamine	0.05
Arginine	0.64	Serine	0.01

A high correlation coefficient at the end of wort boiling indicated that the reactivity of the amino acids during wort boiling was mainly affected by the proteolytic malt modification level. The Strecker active amino acids, valine, isoleucine, leucine, and phenylalanine, showed high correlation factors (Table 3). This result indicated that the intensity of the Strecker reaction was affected by the proteolytic malt modification level. Surprisingly, methionine, as another well-known Strecker active amino acid, showed a different behavior. Presumably, here, alternative reactions were advantaged, such as the oxidation of the sulfur atom to the sulfone group [46]. Regarding other amino acids, it can be assumed that they react more in non-proteolytic modification-influenced pathways, such as oxidation, esterification, or cleavage reactions. It could be indicated that proteolytic malt modification influenced the Strecker reactivity because it strongly influences the chemical reaction pathways of Strecker active amino acids.

3.2. Dicarbonyl Formation during Wort Boiling

Aside from the amino acids, another important group of precursors is the dicarbonyl compounds. They play a key role in aging aldehyde formation during the Strecker degradation and the Maillard reaction. Thus, they directly contribute to aging potential because of their precursor activity. Therefore, the main dicarbonyls, 3-DG and 3-DGal, were monitored during wort boiling due to different malt modification levels (P1 < P2 < P3). Figure 2 shows the concentrations of 3-DG and 3-DGal during wort boiling at all proteolytic variations.

Comparing both compounds, it could be confirmed that 3-DG was the major dicarbonyl [10] because it occurred in higher concentrations during wort boiling. The 3-DGal was formed from 3-DG by interconversion [47] and, therefore, resulted in lower concentrations during wort boiling.

The 3-DG concentration showed a linear increase during wort boiling at all variations. The linearity combined its formation in the Maillard reaction or caramelization and the occurring evaporation effect. The formation of 3-DG indicated that the Maillard reaction was already at its advanced phase during wort boiling [8]. The higher proteolytic malt modification level showed a strong accelerating effect on the initial and final concentrations of the dicarbonyl. Presumably, the enhanced content of amino acids promoted its formation through the Maillard reaction. Additionally, 3-DG precursors, such as Amadori products, are increased by a higher malt modification level in malt [14] and can lead to higher 3-DG concentrations during wort boiling. The 3-DG formation rate was also increased by higher malt modification levels (B1P1: 0.09 mg/(min*L), B1P2: 0.10 mg/(min*L), B1P3: 0.17 mg/(min*L), B2P1: 0.10 mg/(min*L), B2P2: 0.12 mg/(min*L), and B2P3: 0.17 mg/(min*L)). This effect indicates that a higher proteolytic malt modification level forms an increased aging potential for the final beer due to 3-DG during wort boiling. Regarding the calculatable influence (correlation coefficient) of the malt modification level, the initial 3-DG concentration (R^2 = 0.95), final 3-DG concentration (R^2 = 0.97), and 3-DG formation rate (R^2 = 0.81) showed good correlations with the soluble nitrogen content of the malts used (Table 1). Therefore, it could be concluded that the

proteolytic malt modification level strongly influenced the Maillard and caramelization reactivity toward 3-DG formation during wort boiling in this study.

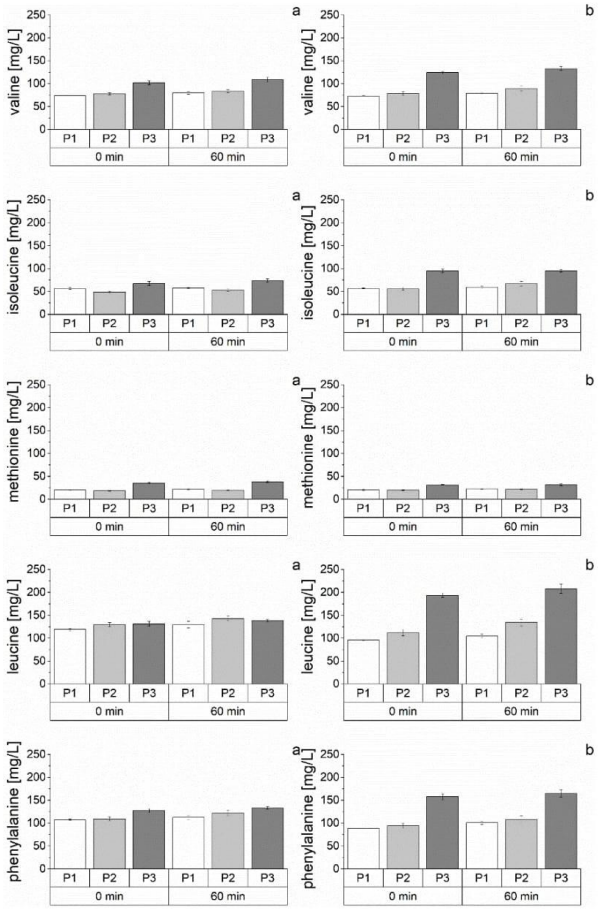

Figure 2. Concentration of selected amino acids at the start (0 min) and end (60 min) of boiling at different malt modification levels (P1 < P2 < P3; $n = 3$); (**a**) = B1; (**b**) = B2.

The second observed dicarbonyl 3-DGal was linearly formed during wort boiling, much like 3-DG. The occurring formation was a combined effect of the 3-DG interconversion and evaporation during boiling. The 3-DGal formation indicated that 3-DG formation and degradation took place simultaneously during wort boiling. The proteolytic P3 level showed the highest initial and final concentrations of 3-DGal. P1 and P2 showed no significant difference in their 3-DGal contents. It could be assumed that a certain level of 3-DG reactivity was needed to promote its interconversion to 3-DGal. The formation rates showed no differences and were calculated to an average of 0.04 mg/(min*L). The 3-DG formation was stronger than the 3-DGal formation, and the effect confirmed the importance of 3-DG as the major dicarbonyl during wort boiling. This study identified that a higher proteolytic modification level enhanced the aging potential toward the 3-DG and 3-DGal formation.

3.3. Formation of Free and 4-VP-Releasable Aging Aldehydes during Wort Boiling

The observed amino acids and dicarbonyls can react as precursors to aging indicators, such as aging aldehydes, which occur during wort boiling in free or bound-state forms. Bound-state aldehydes are releasable by adding 4-VP. After the release, they are detectable as free forms. Therefore, this study investigated the formation of free, 4-VP-releasable, and cysteinylated aging aldehydes during wort boiling. A high concentration of free and bound-state aldehydes contributes to the aging potential because of their functionality as free and releasable aging indicators. Table 4 shows the concentration of aging aldehydes of B1 and B2 at 0, 30, and 60 min of wort boiling. All concentrations during boiling are summarized in the Supplementary Materials section. The analytes comprised of Strecker aldehydes (2MP, 2MB, 3MB, methional, and PA), lipid oxidation aldehydes (t2N, pentanal, hexanal, and heptanal), and aldehydes derived from the Maillard reaction (furfural).

The concentration of all detected aldehydes at all variations (P1, P2, and P3) had a maximum concentration at the beginning of wort boiling and underwent a reduction until approximately 30 min and increased again until the end of boiling. The first decrease was caused by the evaporation effect of the boiling system. In particular, the vacuum pump usage of the pilot brewing system forced evaporation of the highly volatile aldehydes. However, it could be expected that formation reactions took place simultaneously in the first 30 min because of the previously described degradation effects of the amino acids. The first strong decreasing effect was observed in open boiling systems by de Schutter et al. [19]. After 30 min, the formation became more important than degradation or evaporation. The following increase could be caused by ongoing reactions: Strecker degradation, lipid oxidation, Maillard reaction, and caramelization. Alongside 2MP, the other aldehydes showed comparable levels with the literature values [19]. At the P3 level especially, 2MP occurred in strongly increased concentrations in our study.

The aldehydes derived from different reaction types will be discussed separately with regard to the proteolytic malt modification effect during wort boiling. Evaluating the Strecker aldehyde concentrations, the low (P1) and medium (P2) malt modification levels showed no significant difference for B1. However, B2 showed a significant increase in Strecker aldehyde concentration according to an enhanced proteolytic modification order (P1 < P2 < P3). Here, it should be considered that the difference in soluble nitrogen contents of the malts between the P1 and P2 levels, at variety B1, was lower than that at variety B2 (Table 1). Presumably, the difference in soluble nitrogen malt content for B1P1 and B1P2 was too low for differentiation within the Strecker aldehyde concentrations during wort boiling. However, the Strecker aldehydes were strongly increased in P3 (high modification) at both barley varieties. However, the strongly increased contents at the P3 level could be caused by the observed enhanced concentrations of amino acids and dicarbonyls as precursors.

Table 4. Formation of wort carbonyls of B1 (Avalon) and B2 (Marthe) during wort boiling, $n = 3$.

Barley Variety	Malt Modification Level	Boiling Time	2MP	2MB	3MB	Meth	PA	t2N	Pent	Hex	Hept	Fur
		min	µg/L	µg/L	µg/L	µg/L	µg/L	ng/L	ng/L	ng/L	ng/L	µg/L
B1	P1	0	535.3 ± 206.7	166.9 ± 49.7	128.6 ± 49.9	15.4 ± 3.6	64.4 ± 20.7	64.1 ± 23.2	3851.2 ± 630.5	14853.6 ± 3501.9	412.6 ± 94.1	26.7 ± 11.2
		30	59.6 ± 3.4	16.1 ± 0.6	17.8 ± 1.1	2.6 ± 0.6	10.5 ± 3.1	<LOQ	146.5 ± 14.2	709.7 ± 24.3	55.4 ± 15.8	10.3 ± 1.1
		60	120.3 ± 8.2	33.9 ± 2.3	34.0 ± 4.8	2.7 ± 0.4	11.2 ± 0.6	<LOQ	245.6 ± 32.6	1207.8 ± 202.7	88.7 ± 5.6	15.4 ± 2.2
	P2	0	496.5 ± 58.8	140.5 ± 44.5	148.2 ± 49.3	14.5 ± 3.8	46.9 ± 16.7	85.0 ± 68.7	3008.3 ± 1261.5	14429.2 ± 9114.5	230.5 ± 162.8	24.6 ± 4.3
		30	71.7 ± 15.7	15.4 ± 2.4	16.8 ± 1.1	3.9 ± 0.5	12.0 ± 0.7	<LOQ	93.8 ± 8.2	266.4 ± 19.0	14.4 ± 3.4	14.2 ± 2.8
		60	110.5 ± 6.0	26.6 ± 1.8	34.3 ± 3.8	3.0 ± 0.5	10.7 ± 1.6	<LOQ	112.8 ± 10.4	338.2 ± 74.8	20.4 ± 0.1	18.4 ± 3.3
	P3	0	995.8 ± 90.8	348.5 ± 21.7	287.8 ± 41.1	35.7 ± 6.2	108.1 ± 16.2	76.3 ± 8.5	5735.7 ± 457.5	31413.9 ± 4538.9	425.0 ± 87.7	92.22 ± 17.7
		30	91.2 ± 6.1	20.3 ± 1.0	23.9 ± 3.2	7.6 ± 1.2	22.2 ± 1.5	<LOQ	105.5 ± 4.4	283.6 ± 17.4	16.2 ± 2.6	29.2 ± 3.8
		60	155.9 ± 9.3	43.6 ± 7.0	41.8 ± 8.4	7.0 ± 1.7	20.1 ± 1.8	13.7 ± 2.0	147.3 ± 25.3	449.9 ± 83.3	21.3 ± 3.7	43.2 ± 2.2
B2	P1	0	371.9 ± 18.8	115.6 ± 5.5	112.2 ± 7.5	14.5 ± 0.4	54.7 ± 5.4	82.0 ± 10.7	3964.2 ± 441.6	23203.2 ± 2049.7	393.3 ± 28.0	32.0 ± 2.7
		30	47.2 ± 1.1	9.7 ± 0.1	8.7 ± 0.1	2.2 ± 0.4	6.9 ± 0.1	<LOQ	68.8 ± 2.1	208.0 ± 1.7	<LOQ	7.9 ± 0.3
		60	82.0 ± 3.9	17.9 ± 0.7	18.4 ± 0.4	1.9 ± 0.4	5.4 ± 0.7	<LOQ	79.8 ± 2.7	218.5 ± 11.0	<LOQ	8.4 ± 0.4
	P2	0	773.0 ± 128.4	237.3 ± 63.3	159.1 ± 28.8	38.0 ± 11.8	121.8 ± 38.0	128.7 ± 33.7	6276.1 ± 1508.0	24463.7 ± 2805.9	422.0 ± 131.5	163.7 ± 34.5
		30	137.5 ± 7.0	20.8 ± 0.6	24.6 ± 5.5	5.9 ± 1.6	15.8 ± 3.9	<LOQ	197.5 ± 26.1	476.8 ± 21.77	19.9 ± 3.7	108.5 ± 3.8
		60	191.4 ± 6.9	34.4 ± 1.4	40.3 ± 5.7	4.8 ± 0.8	13.4 ± 2.3	<LOQ	254.5 ± 2.3	340.7 ± 4.0	15.7 ± 0.6	121.5 ± 3.6
	P3	0	716.8 ± 55.1	275.8 ± 22.3	190.9 ± 22.6	49.2 ± 9.1	138.8 ± 10.9	133.8 ± 33.2	6399.0 ± 828.5	35685.8 ± 7538.9	759.0 ± 168.7	136.1 ± 30.1
		30	195.9 ± 7.7	47.5 ± 2.3	39.1 ± 1.2	15.2 ± 0.5	13.7 ± 0.4	17.0 ± 0.3	262.5 ± 8.9	306.1 ± 7.3	11.6 ± 0.8	148.9 ± 10.6
		60	233.9 ± 9.9	76.5 ± 2.9	40.7 ± 3.5	20.0 ± 1.7	61.7 ± 3.9	21.7 ± 1.2	315.4 ± 6.7	385.4 ± 19.6	14.2 ± 0.7	181.0 ± 0.9

The increased precursor pool accelerated the Strecker degradation during wort boiling. Furthermore, regarding the correlation with the soluble nitrogen content (Table 1) of the Strecker aldehyde concentration at the end of boiling, 2MP (R^2 = 0.77), 2MB (R^2 = 0.89), methional (R^2 = 0.90), and PA (R^2 = 0.87) showed a good correlation. Therefore, their formation was mainly influenced by the proteolytic malt modification level. Regarding the Maillard reaction, the highest contents for furfural were observed at P3 variations in this study at both barley varieties. The correlation coefficient of the furfural content to the soluble nitrogen content of the used malts (Table 1) was 0.70. This indicated that an increased proteolytic malt modification level accelerated the Maillard reaction during wort boiling due to the enhanced contents of amino acids and dicarbonyls as precursors. Lipid-oxidized aldehydes showed no consistent influence on malt modification. Contrary to the Strecker aldehydes, the highest values were observed at P1 for B1. Surprisingly, B2 showed an inverse effect with the highest levels at P3 variation. Presumably, the formation of lipid-oxidized aldehydes was independent of the malt modification level and was influenced more by barley variety or environmental growing conditions. An increased proteolytic malt modification level enhanced the aging potential originating from final wort toward free aldehyde formation during wort boiling by the Strecker degradation and the Maillard reaction.

Aside from their free form, the formed aldehydes during wort boiling can also be present in bound-state, such as imines or cysteinylated aldehydes [2]. These masked forms also directly contribute to the aging potential. Therefore, this study further investigated the bound-state form of aging aldehydes by release through 4-VP addition during wort boiling due to different malt modification levels (P1, P2, and P3). Figures 3 and 4 show the concentrations of free, 4-VP-releasable, and cysteinylated forms of 3MB, PA, and methional at the end of boiling. Only the cysteine adducts occurred in quantifiable concentrations. The cysteinylated bound aldehyde was the calculated molar equivalent concentration from the determined cysteine adduct. All concentrations of 4-VP-releasable aldehydes are presented in the Supplementary Materials.

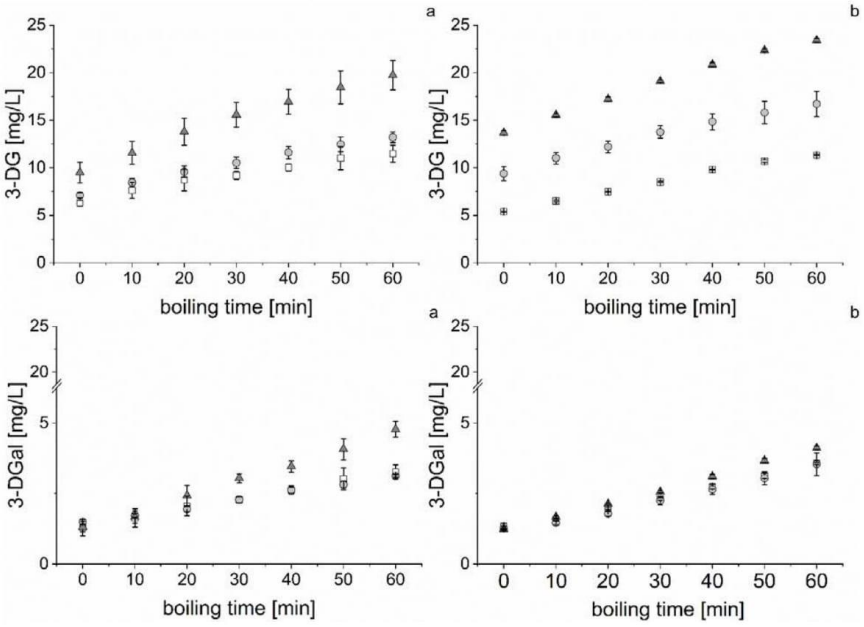

Figure 3. Formation of 3-DG and 3-DGal ((**a**) = B1, (**b**) = B2)) during wort boiling; squares: P1, points: P2, triangles: P3 (n = 3).

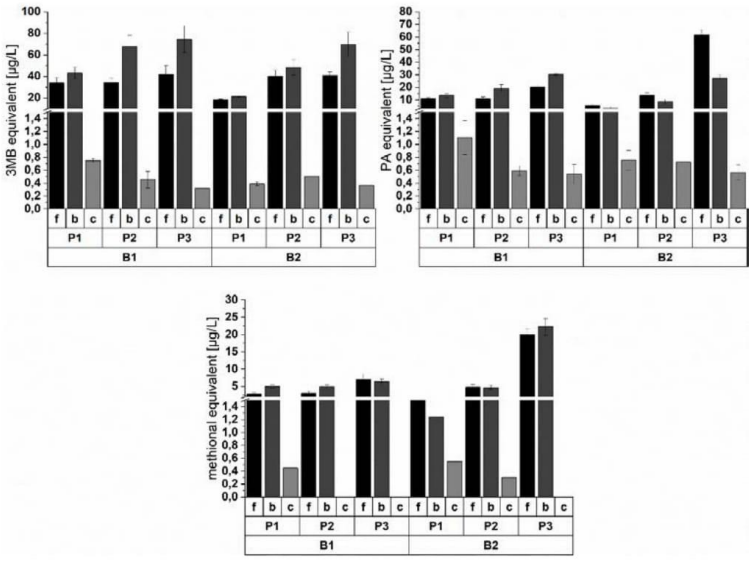

Figure 4. Concentration equivalent of free (f), 4-VP-releasable (b), and cysteinylated (c) forms of 3MB, PA, and methional at the end of boiling (n = 3).

Regarding the 4-VP-releasable aldehydes, Strecker aldehydes and furfural concentrations were increased at the end of boiling by an enhanced malt modification level within a single barley variety. This could be caused by an enhanced imine formation due to higher amino acid contents. A correlation with the soluble nitrogen content of the used malt over both barley varieties could only be observed for 2MB (R^2 = 0.91), methional (R^2 = 0.82), and furfural (R^2 = 0.85). Here, the formation of 4-VP-releasable aldehydes was affected by proteolytic malt modification. Lipid oxidation aldehydes showed no trend in their 4-VP-releasable form due to the malt modification. The ratio of free and 4-VP-releasable forms ranged broadly between 0.19 and 3.66, with an average of 0.95 for all aldehydes at all variations. Furfural and PA showed the highest ratios within the observed aldehydes. The range had a random distribution with no effect on the barley variety or proteolytic malt modification. The formation of imines was acidic-catalyzed and was favored at a pH value of 4–5 (an increase in electrophilicity of C-atom). However, the stability of the imine was increased at higher pH values up to 10 [2,25]. Presumably, the aldehydes underwent a dynamic equilibrium during wort boiling because this study also observed an increase in the 4-VP-releasable form content by higher free aldehyde concentrations.

Baert et al. showed a possible aldehyde release of cysteine and bisulfite aldehyde adducts by 4-VP in beer [48]. The bisulfite adducts could be neglected in the wort samples because they foremost appear during fermentation [2]. However, we could only detect cysteinylated adducts for 3MB, PA, and methional. The formation of the adducts varied for different aldehydes, which indicated various stabilities. There was no consistent trend for different malt modification levels at the end of wort boiling. The percentage of the cysteinylated equivalents of aldehydes within their 4-VP-releasable form varied between different aldehydes. Only 1% for 3MB, 10% for methional, and 7.5% for PA were covered on average by the cysteinylated form. Therefore, presumably, the imine formation was advantaged. According to Bustillo Trueba, the study observed the low importance of cysteinylated aldehydes for the aging potential originating from wort [49].

3.4. Influence of Varied Reactants during Wort Boiling on Aging Potential

To verify the observed dynamic formation of the aging potential (precursors, free, and bound-state aldehydes) during the wort boiling model, boiling experiments were performed. Regarding the aging potential, this study focused on dicarbonyls as representative precursors and free aldehydes as aging indicators in the model-boiling trials. Furthermore, the observed accelerating effect of the proteolytic malt modification level on the aging potential in the final wort was simulated by spiking single substances (precursors and reactants) at the beginning of wort boiling. Thus, the hypothesis could be verified that the proteolytic malt modification level increased the disposable content of reactants and precursors at the beginning of wort boiling, resulting in an increased aging potential at the end of boiling.

Figure 5 shows the relative changes in the aging aldehydes and dicarbonyls as precursors by spiked reactant and precursor compounds. Four groups were added to the wort on a laboratory scale. First, the amino acids (precursor and reactants): valine (Val), leucine (Leu), isoleucine (Ille), phenylalanine (Phe), and methionine (Met) were spiked to verify the Strecker degradation and lysine to verify the Maillard reaction. Second, the dicarbonyl 3-DG (precursor) was spiked to verify the Strecker degradation. The third group were the spiked sugars (reactants): arabinose (Ara), fructose (Fru), and glucose (Glc) as reactants for the Maillard reaction, and the fourth substance was linoleic acid (precursor) to verify lipid oxidation. The addition groups showed different effects on the dicarbonyls as precursors and free aging aldehydes.

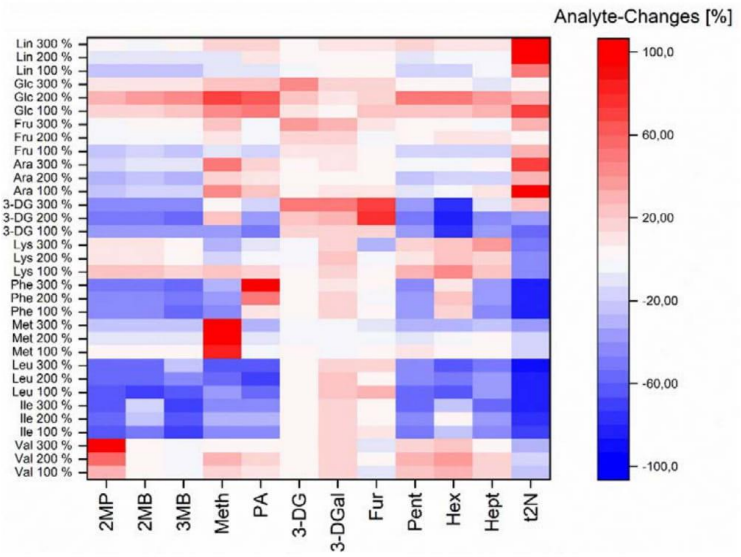

Figure 5. Relative changes [%] in dicarbonyl and aldehyde concentrations by artificially spiked various precursor substances after model wort boiling.

Regarding the formed Strecker aldehydes, it could be shown that their corresponding amino acid (2MP: Val, 2MB: Ile, 3MB: Leu, methional: Met, and PA: Phe) forced a relative increase in the aldehyde within the spiking array (100% < 200% < 300%). That effect confirmed the Strecker degradation of the amino acids during wort boiling from their corresponding amino acid. The noticeable second effect was an absolute decrease in Strecker aldehydes by spiking Phe (despite PA), Ile, Leu, and Met (despite methional). The observed phenomena indicated the formation of imine or proline adducts, such as

oxazolidinones [50], by an increased amino acid content. Therefore, the increased content of 4-VP-releasable aldehydes by higher proteolytic malt modification could be explained by enhanced adduct formation. Surprisingly, 3-DG addition decreased the Strecker aldehyde concentration. Presumably, alternative pathways, such as 5-hydroxymethylfurfrual formation or fragmentation [8], were advantaged, and the formed cleavage products possibly interacted with the Strecker aldehydes. Spiked carbohydrates slightly increased the Strecker aldehyde content. As early precursors in the Maillard reaction, they can promote Strecker degradation.

Further, the Maillard reaction products, 3-DG, 3-DGal, and furfural, were investigated. Amino acid addition showed only slightly enhancing effects on 3-DGal concentration. One explanation could be that the spiked amino acids reacted only to the Amadori compounds and not to the dicarbonyls. Further, the reaction time of 60 min did not provide enough reaction potential for forming dicarbonyls by spiked amino acids. Spiked sugars increased the dicarbonyls and furfural. Glucose and fructose are important precursor compounds and were degraded to 3-DG [14]. An increased 3-DG content was more strongly interconverted to 3-DGal.

The amino acid showed a comparable effect on the Strecker aldehydes regarding the lipid oxidation products (Pent, Hex, Hept, and t2N). Met, Phe, Ile, and Leu forced the formation of imines or oxazolidinones. The linoleic acid addition showed a strong increase in t2N and a slight increase in pentanal, hexanal, and heptanal at the Lin 300% stage. The effect confirmed the formation of these aldehydes from linoleic acid during wort boiling.

These results confirmed the hypothesis by the analytics of single precursors and aging indicators (aging aldehydes and dicarbonyls).

4. Conclusions

The study investigated the influence of a varied precursor concentration (amino acids and dicarbonyls) by different proteolytic malt modification levels on the formation of the aging potential during wort boiling. Summarily, it could be shown that a higher proteolytic malt modification level (calculated by soluble nitrogen content within malt specifications for brewing purposes) increased amino acid content, dicarbonyl concentration, Strecker aldehyde concentration, and the concentration of 4-VP-releasable Strecker aldehydes within malt specifications for brewing purposes. An enhancement of these substance classes lead to an increased aging potential for the final product beer out of final wort. Finally, the model-boiling studies confirmed the hypothesis that a higher amino acid content at the beginning of boiling, by an increased malt modification level, maximized the aging potential originating from the final wort toward the follow-up beer product. Some precursors or substances presented in Table 5 showed a good correlation ($R^2 > 0.8$) to the soluble nitrogen content of the used malt. Table 5 shows their calculated limits in final wort according to the soluble nitrogen specifications for brewing purposes (isothermal 65 °C mashing procedure) [39,40] in this study. Other components observed in this study showed no linear dependency to soluble nitrogen content and could not be used as indicators of the aging potential due to malt modification levels. Although the calculated range was suitable for the brewing industry, the upper limit generally indicated a higher aging potential than the lower malt modification level. These values of single substances could be used as an alignment to evaluate the aging potential originating from wort for the final beer in comparable brewing setups. Further promising analytes to align the aging potential could be Amadori products, such as ε-fructoslylysine (FL), because they act as direct 3-DG precursors. They should be regarded in future studies. Thus, it could be shown that an increased proteolytic malt modification level influences the aging potential formed during wort boiling for pale lager beers.

Table 5. Calculated concentration limits of selected precursors and aging aldehydes in wort according to specifications of pale malts.

Substance Class	Parameter/Substance	Lower Limit	Upper Limit
Malt specifications	Soluble nitrogen [mg/100 g d.m.]	580	680
Amino acid	Valine [mg/L]	79.7	114.1
	Isoleucine [mg/L]	56.5	80.3
	Leucine [mg/L]	119.7	170.5
	Phenylalanine [mg/L]	107.2	142.3
Dicarbonyl compound	3-DG [mg/L]	12.4	20.3
Strecker aldehydes	2MP [µg/L]	112.3	192.7
	2MB [µg/L]	24.6	55.8
	Methional [µg/L]	1.7	12.3
	PA [µg/L]	6.0	37.5
	Furfural [µg/L]	20.9	116.7

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10102320/s1, Supplementary Excel document shows the concentrations of amino acids, free aldehydes, and 4-VP-releasable aldehydes at all sampling points during wort boiling.

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Data Availability Statement: The data presented in this study are available within the article.

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Conflicts of Interest: The authors declare there are no conflict of interest.

References

1. Lehnhardt, F.; Gastl, M.; Becker, T. Forced into aging: Analytical prediction of the flavor-stability of lager beer. A review. *Crit. Rev. Food Sci. Nutr.* **2019**, *59*, 2642–2653. [CrossRef] [PubMed]
2. Baert, J.J.; de Clippeleer, J.; Hughes, S.; de Cooman, L.; Aerts, G. On the Origin of Free and Bound Staling Aldehydes in Beer. *J. Agric. Food Chem.* **2012**, *60*, 11449–11472. [CrossRef] [PubMed]
3. Vanderhaegen, B.; Neven, H.; Verachtert, H.; Derdelinckx, G. The chemistry of beer aging—A critical review. *Food Chem.* **2005**, *95*, 357–381. [CrossRef]
4. Lehnhardt, F.; Steiner, J.; Gastl, M.; Becker, T. Prediction Power and Accuracy of Forced Ageing-Matching Sensory and Analytical Results for Lager Beer. *Brew. Sci.* **2018**, *71*, 39–48.
5. Gardner, H.W. Lipoxygenase as a versatile biocatalyst. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1347–1357. [CrossRef]
6. Garbe, L.-A.; Huebke, H.; Tressl, R. Oxygenated fatty acids and flavour stability—New insights. *Proc. Congr. Eur. Brew. Conv.* **2003**, *29*, 69.
7. Ohloff, G. Recent developments in the field of naturally-occurring aroma components. *Prog. Chem. Org. Nat. Prod.* **1979**, *126*, 431–527.
8. Hellwig, M.; Henle, T. Baking, ageing, diabetes: A short history of the Maillard reaction. *Angew. Chem. Int. Ed. Engl.* **2014**, *53*, 10316–10329. [CrossRef]
9. Baltes, W. The significance of the Maillard reaction on the development of flavor in foods. *Lebensmittelchem. Gerichtl. Chem.* **1980**, *34*, 39–47.
10. Bravo, A.; Herrera, J.C.; Scherer, E.; Yon, N.; Rubsam, H.; Madrid, J.; Zufall, C.; Rangel-Aldao, R. Formation of α-dicarbonyl compounds in beer during storage of pilsner. *J. Agric. Food Chem.* **2008**, *56*, 4134–4144. [CrossRef]
11. Degen, J.; Hellwig, M.; Henle, T. 1,2-Dicarbonyl Compounds in Commonly Consumed Foods. *J. Agric. Food Chem.* **2012**, *60*, 7071–7079. [CrossRef] [PubMed]
12. Hellwig, M.; Nobis, A.; Witte, S.; Henle, T. Occurrence of (Z)-3,4-Dideoxyglucoson-3-ene in Different Types of Beer and Malt Beer as a Result of 3-Deoxyhexosone Interconversion. *J. Agric. Food Chem.* **2016**, *64*, 2746–2753. [CrossRef] [PubMed]

13. Nobis, A.; Kunz, O.S.; Gastl, M.; Hellwig, M.; Henle, T.; Becker, T. Influence of 3-DG as a Key Precursor Compound on Aging of Lager Beers. *J. Agric. Food Chem.* **2021**, *69*, 3732–3740. [CrossRef] [PubMed]
14. Nobis, A.; Roehrig, A.; Hellwig, M.; Henle, T.; Becker, T.; Gastl, M. Formation of 3-deoxyglucosone in the malting process. *Food Chem.* **2019**, *290*, 187–195. [CrossRef]
15. Li, M.; Yang, Z.; Yang, M.; Shan, L.; Dong, J. Determination of furfural in beer by high-performance liquid chromatography with solid-phase extraction. *J. Inst. Brew.* **2009**, *115*, 226–231. [CrossRef]
16. Ghiron, A.F.; Quack, B.; Mawhinney, T.P.; Feather, M.S. Studies on the role of 3-deoxy-D-erythro-glucosulose (3-deoxyglucosone) in nonenzymic browning. Evidence for involvement in a Strecker degradation. *J. Agric. Food Chem.* **1988**, *36*, 677–680. [CrossRef]
17. Hofmann, T.; Muench, P.; Schieberle, P. Quantitative Model Studies on the Formation of Aroma-Active Aldehydes and Acids by Strecker-Type Reactions. *J. Agric. Food Chem.* **2000**, *48*, 434–440. [CrossRef]
18. Krüger, E.; Anger, M.-H. *Kennzahlen zur Betriebskontrolle und Qualitatsbeschreibung in der Brauwirtschaft: Daten uber Roh-und Hilfsstoffe, Halbfertig-und Fertigprodukte bei der Bierbereitung*; Behr: Hamburg, Germany, 1990.
19. De Schutter, D.P.; Saison, D.; Delvaux, F.; Derdelinckx, G.; Rock, J.-M.; Neven, H.; Delvaux, F.R. Release and Evaporation of Volatiles during Boiling of Unhopped Wort. *J. Agric. Food Chem.* **2008**, *56*, 5172–5180. [CrossRef]
20. Perpete, P.; Collin, S. How to improve the enzymatic worty flavour reduction in a cold contact fermentation. *Food Chem.* **2000**, *70*, 457–462. [CrossRef]
21. Baert, J.J.; de Clippeleer, J.; Jaskula-Goiris, B.; van Opstaele, F.; de Rouck, G.; Aerts, G.; de Cooman, L. Further elucidation of Beer flavor instability: The potential role of cysteine-bound aldehydes. *J. Am. Soc. Brew. Chem.* **2015**, *73*, 243–252. [CrossRef]
22. De Schutter, D. *The Influence of Thermal Load During Wort Boiling on the Flavour Stability of Beer*; KU Leuven: Leuven, Belgium, 2008.
23. Dufour, J.-P.; Leus, M.; Baxter, A.J.; Hayman, A.R. Characterization of the reaction of bisulfite with unsaturated aldehydes in a beer model system using nuclear magnetic resonance spectroscopy. *J. Am. Soc. Brew. Chem.* **1999**, *57*, 138–144. [CrossRef]
24. Suda, T.; Yasuda, Y.; Imai, T.; Ogawa, Y. Mechanisms for the development of Strecker aldehydes during beer aging. In *Proceedings of the 31th Congress of the European brewery Convention, Venice, Italy, 6–10 May 2007*; Fachverlag Hans Carl GmbH: Numberg, Germany, 2007.
25. Lermusieau, G.; Noel, S.; Liegeois, C.; Collin, S. Nonoxidative mechanism for development of trans-2-nonenal in beer. *J. Am. Soc. Brew. Chem.* **1999**, *57*, 29–33. [CrossRef]
26. Yoshida, S.; Imoto, J.; Minato, T.; Oouchi, R.; Kamada, Y.; Tomita, M.; Soga, T.; Yoshimoto, H. A novel mechanism regulates H2S and SO2 production in Saccharomyces cerevisiae. *Yeast* **2011**, *28*, 109–121. [CrossRef] [PubMed]
27. Dufour, J.P. Influence of industrial brewing and fermentation working conditions on beer sulfur dioxide level and flavor stability. *Proc. Congr. Eur. Brew. Conv.* **1991**, *23*, 209–216.
28. Guido, L.F. How do sulfites help to control beer ageing? *Cerevisia* **2005**, *30*, 132–138.
29. Vesely, P.; Duncombe, D.; Lusk, L.; Basarova, G.; Seabrooks, J.; Ryder, D. The impact of fermentation temperature on yeast reductase activity. *Tech. Q. MBAA Commun.* **2004**, *41*, 282–292.
30. Gastl, M.; Spieleder, E.; Hermann, M.; Thiele, F.; Burberg, F.; Kogin, A.; Ikeda, H.; Back, W.; Narziss, L. The influence of malt quality and malting technology on the flavor stability of beer. *Monatsschr. Brauwiss.* **2006**, *59*, 163–175.
31. Herrmann, M.; Klotzbuecher, B.; Wurzbacher, M.; Hanke, S.; Kattein, U.; Back, W.; Becker, T.; Krottenthaler, M. A new validation of revelant substances for the evaluation of beer aging depending on the employed boiling system. *J. Inst. Brew.* **2010**, *116*, 41–48. [CrossRef]
32. Buckee, G.K.; Malcolm, T.; Peppard, T.L. Evolution of volatile compounds during wort-boiling. *J. Inst. Brew.* **1982**, *88*, 175–181. [CrossRef]
33. Huang, Y.; Tippmann, J.; Becker, T. A Kinetic Study on the Formation of 2- and 3-Methylbutanal. *J. Food Process Eng.* **2017**, *40*, e12375. [CrossRef]
34. Gallardo, E.; de Schutter, D.P.; Zamora, R.; Derdelinckx, G.; Delvaux, F.R.; Hidalgo, F.J. Influence of lipids in the generation of phenylacetaldehyde in wort-related model systems. *J. Agric. Food Chem.* **2008**, *56*, 3155–3159. [CrossRef] [PubMed]
35. Dupire, S.; Bonte, S.; Eyben, D. Influence of oxygen during wort boiling and clarification. *Monogr. Eur. Brew. Conv.* **1992**, *18*, 45–57.
36. Goode, D.L.; Halbert, C.; Arendt, E.K. Mashing studies with unmalted sorghum and malted barley. *J. Inst. Brew.* **2002**, *108*, 465–473. [CrossRef]
37. Osman, A.M.; Coverdale, S.M.; Onley-Watson, K.; Bell, D.; Healy, P. The gel filtration chromatographic-profiles of proteins and peptides of wort and beer: Effects of processing—Malting, mashing, kettle boiling, fermentation and filtering. *J. Inst. Brew.* **2003**, *109*, 41–50. [CrossRef]
38. Gastl, M.; Kupetz, M.; Becker, T. Determination of Cytolytic Malt Modification—Part I: Influence of Variety Characteristics. *J. Am. Soc. Brew. Chem.* **2021**, *79*, 53–65.
39. Malt analysis: Industry-wide change in analysis approach for barley malt 5 EBC. In *BRAUWELT International 2020*; B. Deutscher Brauer-Bund e.V.: Berlin, Germany; Deutscher Mälzerbund e.V.: Frankfort on the Main, Germany; Braugersten-Gemeinschaft e.V.: Munich, Germany, 2020; Available online: https://brauwelt.com/en/topics/raw-materials/641409-malt-analysis-industry-wide-change-in-analysis-approach-for-barley-malt-5-ebc (accessed on 10 August 2021).
40. Back, W. *Ausgewählte Kapitel der Brautechnologie*; Fachverlag Hans Carl: Nürnberg, Germany, 2005; pp. 249–265.

41. Rizzi, G.P. The Strecker Degradation of Amino Acids: Newer Avenues for Flavor Formation. *Food Rev. Int.* **2008**, *24*, 416–435. [CrossRef]
42. Eichner, K.; Reutter, M.; Wittmann, R. *Detection of Maillard Reaction Intermediates by High-Pressure Liquid Chromatography (HPLC) and Gas Chromatography*; Springer: Berlin, Germany, 1990.
43. Dubourg, J.; Devillers, P. Conversion of glutamic acid and glutamine to pyrrolidonecarboxylic acid and of the latter to glutamic acid. *Bull. Soc. Chim. Fr.* **1956**, *23*, 1351–1355.
44. Lehnhardt, F.; Nobis, A. Something is brewing together. *Nachr. Chem.* **2020**, *68*, 80–83. [CrossRef]
45. Muller, C.; Methner, F.J. An accelerated malting procedure-influences on malt quality and cost savings by reduced energy consumption and malting losses. *J. Inst. Brew.* **2015**, *121*, 181–192. [CrossRef]
46. Chang, K.C.; Kendrick, J.G.; Marshall, H.F.; Satterlee, L.D. Effect of partial methionine oxidation on the nutritional quality of soy isolate and casein. *J. Food Sci.* **1985**, *50*, 849–850. [CrossRef]
47. Hellwig, M.; Degen, J.; Henle, T. 3-Deoxygalactosone, a "New" 1,2-Dicarbonyl Compound in Milk Products. *J. Agric. Food Chem.* **2010**, *58*, 10752–10760. [CrossRef]
48. Baert, J.J.; de Clippeleer, J.; Trueba, B.; Jaskula-Goiris, B.; de Rouck, G.; Aerts, G.; de Cooman, L. Exploring Aldehyde Release in Beer by 4-Vinylpyridine and the Effect of Cysteine Addition on the Beer′s Pool of Bound Aldehydes. *J. Am. Soc. Brew. Chem.* **2018**, *76*, 257–271. [CrossRef]
49. Trueba, B.; Jaskula-Goiris, B.; Ditrych, M.; Filipowska, W.; de Brabanter, J.; de Rouck, G.; Aerts, G.; de Cooman, L.; de Clippeleer, J. Monitoring the evolution of free and cysteinylated aldehydes from malt to fresh and forced aged beer. *Food Res. Int.* **2021**, *140*, 110049. [CrossRef]
50. Schmid, M.B.; Zeitler, K.; Gschwind, R.M. The Elusive Enamine Intermediate in Proline-Catalyzed Aldol Reactions: NMR Detection, Formation Pathway, and Stabilization Trends. *Angew. Chem. Int. Ed.* **2010**, *49*, 4997–5003. [CrossRef] [PubMed]

3.2.5 A comprehensive evaluation of flavor instability of beer (part 1): Influence of release of bound state aldehydes

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A Comprehensive Evaluation of Flavor Instability of Beer (Part 1): Influence of Release of Bound State Aldehydes

Florian Lehnhardt †, Arndt Nobis †, Andreas Skornia, Thomas Becker and Martina Gastl *

Brewing and Beverage Technology, TUM School of Life Sciences, Technische Universität München, Weihenstephaner Steig 20, 85354 Freising, Germany; florian.lehnhardt@tum.de (F.L.); arndt.nobis@tum.de (A.N.); andreas.skornia@tum.de (A.S.); tb@tum.de (T.B.)
* Correspondence: martina.gastl@tum.de
† Both authors contributed equally to this work.

Abstract: Flavor instability of pale lager beer depends decisively on aroma-active aldehydes from the Maillard reaction, Strecker degradation, and lipid oxidation, which are formed in various oxidative and non-oxidative reactions. Therein, aldehydes can be formed *de novo* and be released from bound states to a free, aroma-active form during aging. During malting and brewing, proteolysis affects the amount of soluble nitrogen and thus flavor instability in different ways (e.g., precursors for *de novo* formation and binding agents for bound states). To isolate nitrogen-related aging processes, beers from malts (two barley varieties, three proteolytic malt modifications) were produced on a 50 L scale in part 1 of this study. Sensory analysis revealed increased flavor instability for beers with higher amounts of soluble nitrogen. Especially Strecker aldehydes significantly increased with malt modification. The release of bound state aldehydes revealed most free aldehydes in fresh beers and with higher malt modification. During aging, the equilibrium between free and bound state aldehydes shifted toward the free form. These results reveal a nitrogen-dependent bound pool of aldehydes that is depleted during aging and is responsible for aged aroma, especially in the early and medium stages of aging. Therefore, bound state aldehydes are indicators of the early-stage prediction of flavor instability already in a fresh condition.

Keywords: beer aging; flavor instability; bound state aldehydes

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1. Introduction

The aging of lager beer is one of the key challenges brewers face in a globalized world. During distribution and storage, reactions occur that are detrimental to the quality of beer, especially its sensory aspects [1,2].

In practice, there are various ways to assess the flavor instability of lager beer, the most common being forced aging. The sample is shaken (100 rpm, 24 h) and subjected to an elevated temperature for a certain time (40 °C, 4 day) to simulate transport and aging. After that, its aging status is evaluated using analytical and sensory techniques [3]. However, there are substantial differences in the sensory and analytical aging behaviors of forced-aged beers compared with naturally aged ones because of the elevated temperatures [4]. Because of these limitations, it is crucial to have tools to correctly describe and define the aging status of a beer sample, as well as to simulate its aging potential as early as possible (in fresh beer) [3].

The highly complex aging of pale lager beer has been thoroughly studied for several decades. It is affected by exogenous conditions (time, temperature) and endogenous parameters (pH, O_2 concentration, pro- and anti-oxidant activity, number of precursors of aging compounds) in raw materials, during the brewing process, and in the final product [3]. Beer aging can be considered a combined multi-stage process including all these pathways and reactions [5,6].

The major contributors to aged aroma in beer are aldehydes from the Maillard reaction, Strecker degradation, and lipid oxidation, which prove to be an appropriate class of indicators for evaluating the aging status of a beer sample [7,8]. On the one hand, aldehydes can be formed *de novo* in the respective reactions or radical oxidation reactions [9]. On the other hand, aldehydes can also be released from a bound state [3,8].

De novo formation has been thoroughly studied, yet alone, it cannot explain the observed increases during beer aging. In a finished beer sample, reaction conditions, such as low ambient temperatures and relatively low pH (4.2–4.5), are insufficient for the initiation steps of certain reactions, such as imine formation [10].

In contrast to their free state, aldehydes can also exist in a bound state via various nucleophile additions. In beer, amino groups (amino acids), especially cysteine, and bisulfite (HSO_3^-) appear to be the most apparent reaction partners. The resulting imines, 2-substituted-1,3-thiazolidine-4-carboxylic acids and α-hydroxysulfonates, are hypothesized to degrade during aging and reveal the masked aldehydes [11]. In contrast, according to Bustillo Trueba et al., cysteinylated aldehydes are present in detectable concentrations in the stages from malt to wort, with a maximum at the onset of mashing but not anymore after fermentation, due to the pH instability of these compounds. Only methional (Meth) shows relatively high concentrations in a cysteinylated form [12]. The same results were obtained in worts made from malts with different proteolytic modifications. Therefore, cysteinylated aldehydes can be considered irrelevant for beer flavor instability [13]. Undoubtedly, different aldehydes show different affinities toward amino groups or HSO_3^- because of their molecular structure (inductive and mesomeric effects) and are thus present in a bound state to different degrees. Up to now, this has not yet been investigated in beer.

Since arguably, bound state aldehydes are the main factors for the flavor instability of beer and, in theory, are present at the highest concentrations in fresh beer, they can be used to assess the aging potential of a fresh beer sample. For example, this can be done in the so-called nonenal potential. Hereby, the capacity of a wort to produce (E)-2-nonenal (T2N) and, since recently, also hexanal (Hex) is assessed [14,15]. Using 4-vinyl pyridine (4VP), Baert et al. developed a method to release bound state aldehydes from these bound forms through a pH shift and trapping of cysteine [16,17]. This approach can also be used to assess the aging potential of the final wort [13]. The fact that acetaldehyde (ACA) replaces other (longer-chain) aldehydes from their bound states can, in theory, also be used to predict the flavor instability of fresh beer [18,19].

All these compounds (free aldehydes, bound state aldehydes, and precursors for *de novo* formation) form the so-called aging potential of fresh beer. The totality and distribution within the aging potential vary with raw materials, especially malts, and also with the applied technology [13].

A major influence on the aging potential and thus on the formation and occurrence of aroma-active aldehydes is attributed to amino compounds. They originate mainly from the malt used in the brewing process. The number of reactants (amino compounds) in barley depends on the barley variety and crop year [20]. During germination, barley crude proteins are enzymatically degraded (proteolytic malt modification). The demanded amount of crude proteins should be in the range of 9.5–11.0% [21]. With the modification characteristics of the variety and the technological malting parameter steeping degree, the amount of soluble nitrogen and thus amino acids in the malt can be increased [22]. A practical measure of the degree of proteolysis during malting is the calculated Kolbach index, defined as the ratio of soluble protein in the laboratory wort and the total protein in the malt [21,23]. Lower amounts of soluble nitrogen and a lower Kolbach index (as long as the raw protein content is on the same level) lead to less Strecker aldehydes in the final beer [24]. Likewise, a low soluble nitrogen content provides more flavor-stable beers [25]. The specifications for the soluble nitrogen content vary in the literature. The recommendation for pale barley malt is 580–680 mg/100 g malt d.m. assessed in an ISO 65 °C mashing regime [26].

If these specifications are not met, foam stability and yeast nutrition are negatively affected. If they are exceeded, however, flavor instability increases and turbidity stability decreases [9,21]. Finally, proteolysis can be affected during the mashing procedure. Lund et al. showed that increased protease activity during mashing results in elevated levels of amino acids in final beers. During aging, these beers show significantly higher scores in the fruity aged/vinous attributes but not in papery attribute [27]. Thus, we can hypothesize that by varying reactants by proteolytic malt modification, the aging potential increases in different ways. The amount of precursor for *de novo* formation, followed by the amount of free aldehydes, and, finally, the amount of bound state aldehydes increase.

Therefore, the goal of this study was to comprehensively investigate the aging potential (*de novo* formation and release of aldehydes from bound states) that solely arises from soluble nitrogen compounds, reaction partners in both of these pathways. Furthermore, oxidative and antioxidative effects of O_2 and SO_2 were excluded in the final beer. Proteolytic malt modifications were used as a tool to vary the number of reactants (soluble nitrogen), as described by Nobis and Lehnhardt et al. [13]. The beers produced via a standardized brewing process without promoting further proteolysis during brewing were subjected to forced and natural aging. In part 1 of this study, we focused on (1) the sensory qualities of the respective beers, (2) the influence of soluble nitrogen on the formation of free aldehydes, and (3) the potential release of bound state aldehydes by two different methods, depending on the amount of soluble nitrogen during beer aging and their impact on the sensory qualities of the respective beers. Furthermore, we discussed the application of bound state aldehydes for the early-stage assessment of aging stability directly in fresh beer.

2. Materials and Methods

2.1. Chemicals

O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (≥99%), p-fluorobenzaldehyde (98%), 2-methylpropanal (2 MP) (≥99.5%), 2-methylbutanal (2 MB) (95%), 3-methylbutanal (3 MB) (97%), 2-phenylacetaldehyde (PA) (≥90%), Meth (≥97%), benzaldehyde (Benz) (≥99.5%), pentanal (≥97.5%), Hex (98%), heptanal (95%), T2N (97%), ACA (≥99.5%), and 4-vinylpyridine (95%) were obtained from Merck (Darmstadt, Germany). 2-Furfural (Fur) (≥99.0%) was purchased from Fluka Analytical (Charlotte, NC, USA). Ethanol p.a. was purchased from VWR (Darmstadt, Germany).

2.2. Malt, Wort, and Beer Production

Malts (pilsner style) and worts were produced, as described by Nobis and Lehnhardt [13]. From two barley varieties, B1 (Avalon, Nordsaat Saatzucht GmbH, Langenstein, Germany) and B2 (Marthe, Saatzucht Josef Breun GmbH & Co. KG, Herzogenaurach, Germany), with a different genetically determined modification characteristic, six different malts were produced with different proteolytic modification levels by varying the steeping degree (P1: low; P2: medium; P3: high) (see Table 1).

The 6 malts were processed in a standardized scheme in duplicate, resulting in 12 worts, as previously described [13]. To avoid further proteolysis during brewing, a high-mashing-in procedure at comparable pH values and from 60 °C (mashing-in temperature) to 78 °C was used. Lautering was performed in a preheated (78 °C) lauter tun, with a lauter rest of 10 min. In total, three sparges were performed until a gravity of 10.5 °P was reached. The boiling time was 60 min. The boiled wort was transferred to a whirlpool for a 15 min rest. The cast wort (60 L, 11.5 ± 0.2 °P) was cooled to 10 °C using a plate heat exchanger.

For fermentation, dry yeast (TUM 34/70) (Fermentis, Marcq-en-Barœul, France) was rehydrated in a diluted wort (6 °P) for 6 h. The pitching rate of 15 × 106 living cells/mL was ensured using a Thoma chamber. Open fermentation was performed in cylindro-conical tanks at 15 °C (SO_2 < 2 mg/L in beer). As the extract fell below 3.5 °P, the tanks were closed and maintained at the same temperature for 2 d. The green beers were transferred to 50 L kegs and maintained at 18 °C for 1 d. Lagering was performed at 0 °C for 4 weeks.

Filtration was performed using a Seitz A20Z filter press with Seitz K150 depth filter sheets (Pall, NY, USA). After filtration, the beers were carbonated in 50 L kegs at 4 °C, filled into 500 mL bottles using a semiautomatic back pressure filler (Fillmatic, FH Maschinen und Braumanufaktur Werk II GmbH, Germany), and closed using a pneumatic corking machine (Korkfix, FH Maschinen und Braumanufaktur Werk II GmbH) to guarantee O_2 levels below 0.1 mg/L.

Table 1. Malt and brewing specifications: steeping degree, soluble N (target and real), total amino acids, pH, O_2, and bound SO_2.

Sample	Steeping Degree (%)	Target Soluble N (ISO 65 °C (mg/100 g Malt d.m.)	Soluble N (mg/100 g Malt d.m.)	Total Amino Acids in Fresh Beer (mg/L)	pH (Final Beer)	O_2 (mg/L)	Bound SO_2 (mg/L)
B1/P1	38	550 ± 25	573 ± 10	909	4.57 ± 0.02	0.07 ± 0.04	0.84 ± 0.33
B1/P2	41	625 ± 25	601 ± 1	753	4.44 ± 0.05	0.01 ± 0.00	0.52 ± 0.20
B1/P3	44	700 ± 25	660 ± 1	980	4.52 ± 0.07	0.02 ± 0.00	0 ± 0
B2/P1	39	550 ± 25	569 ± 3	666	4.45 ± 0.07	0.07 ± 0.05	0 ± 0
B2/P2	43	625 ± 25	620 ± 14	784	4.39 ± 0.05	0.08 ± 0.01	0.16 ± 0.18
B2/P3	47	700 ± 25	731 ± 1	1121	4.55 ± 0.02	0.02 ± 0.01	3.16 ± 1.43

2.3. Aging and Sample Treatment

Forced aging was performed, as previously described [4]. The bottles were shaken at 100 rpm for 24 h and then maintained at 40 °C for 4 d. Natural aging of samples was performed in a dark chamber at 20 °C until the indicated sample age was reached (1 to 9 months). At each sampling point, beer samples were filtered, aliquoted into 50 mL tubes, and immediately frozen. Samples for sensory analysis were moved to 0 °C after they reached the respective age and kept there until tasting (maximum—1 week).

2.4. pH, O_2, and SO_2

Prior to alcohol and pH analysis, beer samples were filtered. At each sampling point, the alcohol content was measured using an Anton-Paar Alcolyzer Beer ME (Graz, Austria) and the pH was measured using a pH probe. Oxygen was analyzed using an Anton-Paar CboxQC device (Graz, Austria). The bound sulfur dioxide content was determined via the destillative method (MEBAK 2.21.8.2) [28]. The latter two analyses were performed in duplicate. All other analysis was performed in triplicate.

2.5. Sensory Analysis

All beers were analyzed in a fully randomized setup in a single repetition by, on average, 10 (ranging from 9 to 13) panelists trained and certified by Deutsche Landwirtschafts–Gesellschaft e.V. (DLG). The panelists underwent continuous training (once per week). Three different (two rating and one descriptive) sensory methods were used. In each session, aging-relevant sniffing samples were provided as an introductory exercise and a commercial fresh pale lager beer (not older than 4 weeks) was presented as a control sample. The samples were served in brown glasses at 10 °C ± 2 °C.

First, quality assessment was performed according to the DLG 5-point scheme. Five categories (purity of smell, purity of taste, palate fullness, freshness, and quality of bitterness) were rated on the following monadic scale: 0 = inadequate (not evaluable); 1 = not satisfactory (strong deviation); 2 = less satisfactory (clear deviation); 3 = satisfactory (perceptible deviation); 4 = good (slight deviation); and 5 = very good (quality expectations reached in full). A weighted overall DLG score was calculated as follows:

$$\text{Weighted overall DLG score} = [(2 \times \text{purity of smell}) + (2 \times \text{purity of taste} + \text{palate fullness} + \text{freshness}) + (2 \times \text{quality of bitterness})]/8$$

Second, the aging-specific quality scheme according to Eichhorn was used. Three categories (smell, taste, and bitterness) were rated on the following monadic scale with the possibility of 0.5 steps: 1 = fresh beer, no aging impressions; 2 = slight aging impressions; 3 = strong aging impressions, acceptancy threshold for consumers; and 4 = extreme aging impressions, such as sherry. Only aging-relevant impressions were judged. Acceptance according to the Eichhorn scheme was evaluated on a hedonic scale from 0% (no acceptance) to 100% (full acceptance).

Finally, check all that apply (CATA) was used to describe aging-relevant aromas. The attributes fruity, berry, sweetish, honey, cardboard, bready, sherry, and cooked vegetables were checked if present and not checked if absent. Panelists were also able to provide free comments. The data were obtained as the sum of checks per attribute by the number of panelists.

To evaluate whether the aging character of the produced beers differs between the malt modifications, triangle tests with 16 tasters for all combinations per barley variety at 9 months (M9) were performed according to MEBAK Sensorik 3.1.3 [29]. Therefore, beers from the repeated brews were blended. The samples were presented in a fully randomized setup.

2.6. Quantitation of Free Aldehydes via HS-SPME-GC-MS

Headspace–solid phase microextraction–gas chromatography–mass spectrometry (HS-SPME-GC-MS) was carried out according to Lehnhardt et al. with minor modifications [4]. A cooled beer sample (5 mL) was transferred together with 50 µL of an internal standard (2 mg/L of p-fluorobenzaldehyde in ethanol) to a 20 mL headspace vial and stored in a cooled autosampler tray (17 °C). Extraction was performed using a CAR-PDMS-DVB fiber. First, the fiber was loaded with o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine for 10 min at 40 °C. Then, the headspace of the sample was extracted for 30 min at 40 °C. The fiber was injected with a 1/5 split at 270 °C into a gas chromatography (GC) instrument (GC-Ultra 1300; Thermo Fisher Scientific, Waltham, MA, USA) coupled to a single quad mass spectrometer (ISQ 7000; Thermo Fisher Scientific). The GC instrument was equipped with a DB-5 column (length, 60 m; inner diameter, 0.25 mm; film thickness, 0.25 µm; Thermo Fisher Scientific). The carrier gas was helium (flow rate 1.85 mL/min). The starting temperature was maintained at 60 °C for 4 min, followed by heating at 5 K/min up to a final temperature of 250 °C, which was maintained for 3 min. A full scan mode (m/z 35–350) with a dwell time of 0.02 s was applied for the analysis. Each sample was analyzed in triplicate. Peak detection was performed using Xcalibur 3.1.66.10 (Thermo Fisher Scientific). Quantification was achieved by external calibration. The lowest calibration point was defined as the limit of quantification (LOQ), which was in accordance with previous studies [30,31]. All measurements were performed in biological duplicate and technical triplicate.

2.7. Quantitation of Bound State Aldehydes after Release with 4VP vs. Acetaldehyde (ACA)

The procedure was performed as described in Section 2.6, with one exception. Before adding the internal standard, 50 µL of 4VP solution (1/1 4VP/ethanol, v/v) was added or in the case of competitive release with ACA, 50 µL of ACA stock solution (50 mg/mL) was added. The samples were incubated in an autosampler tray at 17 °C for at least 6 h in the case of 4VP and 12 h in the case of ACA. During 4VP elution from the GC column, mass spectrometric detection was turned off.

The degree of bound state aldehydes was calculated as follows:

$$\text{c(bound)}\ (\%) = (\text{c(released)} - \text{c(free)})/(\text{c(free)}),$$

where c(released) and c(free) are defined as concentrations of released versus free aldehydes.

2.8. Statistical Analysis

Data analysis was performed using JMP Pro 14 (SAS Institute Inc., Cary, NC, USA). From technical and biological multiplicates, means and standard deviations were calculated.

One-way analysis of variance (ANOVA) was performed to determine statistical differences, where indicated. Post hoc testing for the comparison of all pairs was achieved with the Tukey–Kramer honestly significant difference (HSD) test. Unless stated otherwise, $\alpha = 0.05$ was used and each analysis was performed in technical triplicate.

3. Results and Discussion

3.1. Brewing Trials

To comprehensively investigate all aspects of the aging potential, beers were produced from two different barley varieties (B1: Avalon; B2: Marthe) with variations in the proteolytic malt modification level (P1: 550 mg/100 g malt d.m.; P2: 625 mg/100 g malt d.m.; P3: 700 mg/100 g malt d.m.). Table 1 shows these malt specifications as well as pH, O_2, and bound SO_2 amounts of the fresh beers analyzed directly after filling.

Proteolytic malt specifications were achieved through different steeping degrees during malting and goals set to the extrema of specifications, including a medium amount to obtain detectable but still realistic differences. The targets of the individual modification measured as soluble N were satisfactorily reached for all variations. The total amount of amino acids increased with proteolytic malt modification, thus providing more reactants and precursors for aging-relevant reactions. The only exception was B1/P2. During proteolysis, all amino acids increased except for proline. This decrease led to a lower amount of total amino acids in B1/P2 (Table S1).

In malt, the pH values were similar (Table S1). The fresh beers, in contrast, showed significant differences in pH within the acceptable range for pale lager beers. P2 showed the lowest pH in both barley varieties, most likely due to the optimal nutritional value for yeast provided in the wort and the resulting better pH drop during fermentation.

The O_2 levels directly after filling were minimal (<0.1 mg/L) in all samples, as reported earlier [10]. The bound SO_2 target was set to <1 mg/L and was reached for all but one sample (B2/P3 varied from 1.9 to 4.3 mg/L in duplicate beers). However, the bound SO_2 concentration was still low and thus acceptable. The minimal O_2 and SO_2 concentrations were optimal to isolate N-related aging processes. On the one hand, direct oxidation through reactive oxygen species formation is limited, and on the other hand, antioxidative effects and covalent binding of aldehydes are excluded. Therefore, the beers produced in this study were ideal for the isolated investigation of the aging potential provided by N species and were subjected to forced (FO) and natural aging of up to 9 months (M1–M9). Together with fresh samples (FR), all aging points were tasted using a trained sensory panel and analyzed by instrumental analytics, as described next.

3.2. Sensory Analysis

An important part of the evaluation of flavor instability is sensory analysis with ratings (descriptive and discriminative methods). This way, changes in product quality during aging can be assessed and differences in the aging potential unraveled.

3.2.1. Quality Assessment and Descriptive Analysis by DLG, Eichhorn Scheme, and CATA

First, the sensory quality of the produced beers was investigated during aging. Differences in sensory analysis after a certain period of aging indicate an influence of the proteolytic malt modification.

Aging clearly influenced the analyzed samples. A continuous increase of aging impression was observed after M3 in several aspects. The rating in different groups suggested sensory distinguishability. For example, the M1 sample showed the highest DLG overall scores, followed by FR, FO, M2, M3, M4, M5, M6, and M9 samples. Interestingly, fresh and forced-aged samples did not show differences in these attributes. For smell, according to the Eichhorn scheme, the same results were obtained. The M9 sample scored the highest in this attribute, followed by M6, M5, M4, M3, M2, FR, FO, and M1 samples. Hedonic acceptance was the highest for the M1 sample and decreased in FR, FO, M2, M3, M4, M6, M5, and M9 samples. Furthermore, M3–M9 samples showed elevated scores in

the attribute bready: M6, M4, M9, M5, and M3 samples in contrast to M1, FR, M2, and FO samples. The older samples, M6 and M9, showed elevated scores in the attribute honey and the M9 sample also showed elevated scores in the attribute sherry. These results indicate that aging has a perceivable effect on beer quality after M3 (see Table S2).

Furthermore, we investigated the influence of proteolytic malt modification. Figure 1 shows boxplots of chosen attributes of standardized brewed beers by barley variety and malt modification level. One boxplot includes all evaluated aging points (fresh, forced aged, and naturally aged (M1–M9)).

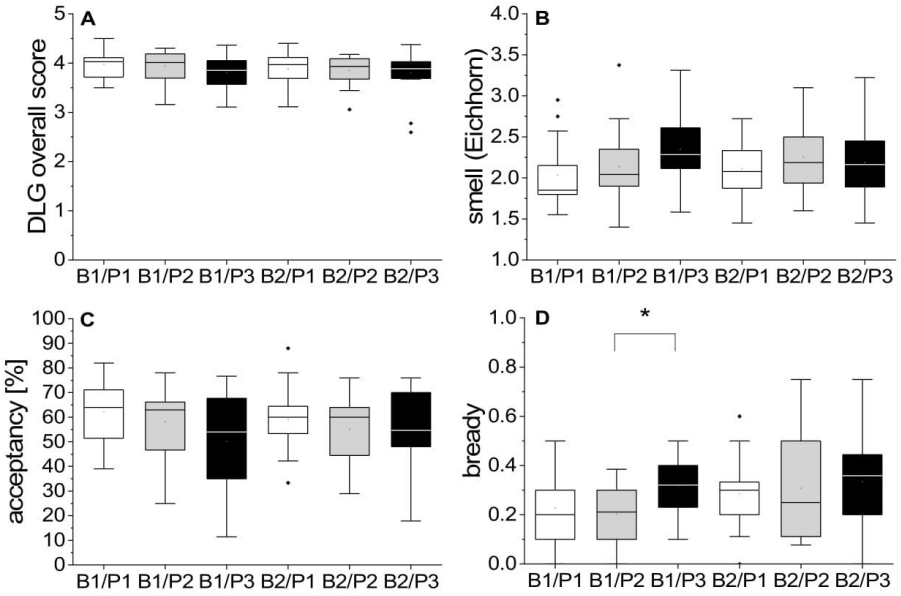

Figure 1. Sensory analysis of beers: (**A**) DLG overall score, (**B**) smell according to the Eichhorn scheme, (**C**) acceptancy (%), and (**D**) attribute bready. Boxplots show values of all aging points (n = 18). Linked boxplots showed a significant difference (*: $p < 0.05$). (B1: barley variety 1; B2: barley variety 2; P1: low proteolytic malt modification; P2: medium proteolytic malt modification; P3: high proteolytic malt modification).

In addition, the malt modification level influenced the aging behavior of the analyzed beer samples. For B1, we observed a stronger aging impression (lower DLG scores, higher Eichhorn scores, higher intensities for aging descriptors) and reduced acceptance with a higher amount of soluble N. Although the results showed no statistical significance, we found strong trends (one-way ANOVA at $\alpha = 0.05$; $p = 0.21$ (Figure 1A), 0.11 (Figure 1B), 0.07 (Figure 1C), 0.02 (Figure 1D)). The only exception was observed for the attribute bready. The B1/P2 and B1/P3 pair showed significant differences (Tukey–Kramer HSD test, $p = 0.02$). For B2, the same trends were observed but generally on a weaker level (one-way ANOVA at $\alpha = 0.05$; $p = 0.79$ (Figure 1A), 0.59 (Figure 1B), 0.60 (Figure 1C), 0.72 (Figure 1D).

These analyses revealed no significant differences but only trends between proteolytic malt modification when all sampling dates were considered together. Still, this does not imply that the aging potential of individual beer samples does not differ between proteolytic malt modification.

3.2.2. Triangle Tests

Second, the goal was to determine whether the beers showed differences in a discriminative test (triangle test according to MEBAK Sensorik 3.1.3) after a long-term natural aging period (M9), as suggested by the aforementioned sensory results. M9 was chosen because the oldest sample in this study could unfold its aging potential to the highest degree. Table 2 shows the results of the triangle test. *p*-Values in bold indicate statistically significant results ($\alpha = 0.05$).

Table 2. Triangle tests to determine sensory differences in beer after 9 months of natural aging ($n = 16$) (B1: barley variety 1; B2: barley variety 2; P1: low proteolytic malt modification; P2: medium proteolytic malt modification; P3: high proteolytic malt modification).

Tested Pair	Correct Answers	Wrong Answers	*p*-Value
B1/P1–B1/P2	8	8	>0.05
B1/P1–B1/P3	12	4	<0.001
B1/P2–B1/P3	13	3	<0.001
B2/P1–B2/P2	10	6	<0.01
B2/P1–B2/P3	14	2	<0.001
B2/P2–B2/P3	14	2	<0.001

After M9, the triangle test did not reveal significant differences for B1/P1 and B1/P2, because these samples showed the smallest difference in soluble N (28 mg/100 g malt d.m.) (Table 1). However, for all other pairs, significant differences were observed. Especially, all pairs with P3, samples with the highest amounts of soluble N, showed clear results. This was the case even for B2/P3, although this sample showed the highest amount of bound SO_2 in fresh beers (Table 1). The values ranged from 1.9 to 4.4 mg/L in duplicate brews, indicating that little to medium amounts of SO_2 have no positive effect on flavor instability after M9, independent of the amount of soluble N.

Therefore, sensory analysis of the produced beers revealed differences in the aging behavior that depended on the amount of soluble N. With increasing proteolytic malt modification, we observed decreased sensory beer quality and acceptance. After 9 months of natural aging, almost all pairs showed significant differences in the triangle test, indicating a varied aging potential in fresh beer.

3.3. Behavior of Free Aldehydes

The clear sensory differences, especially for the attribute bready, in the produced samples during aging indicated that the amount of soluble N affects the number of aroma-active compounds. Amino acids, as part of the soluble N in beer, are reactants in various aldehyde-yielding reactions and direct precursors of Strecker aldehydes. These aldehydes are used as aging indicators because of the fact that they increase during aging but show high aroma activity [4]. Next, we discuss the behavior of free aldehydes: 2MP, 2MB, 3MB, Meth, PA, Benz, Fur, Hex, and T2N.

Figure 2 shows the concentrations of chosen aldehydes that showed statistically significant differences. Each boxplot contains the data of all aging points (FR, FO, M1–M9). The behavior of all other investigated aldehydes can be found in Table S3.

In the fresh condition, we observed none to only minor differences in the analyzed aldehydes between different malt modification levels (Table S3 and part 2). Generally, all aldehydes increased during aging to different extents, depending on the amount of soluble N in the samples. This indicated either N-dependent *de novo* formation or a release from the bound state. Especially, the concentration of Strecker aldehydes (2MP, 2MB, 3MB, and PA) in beer significantly increased at higher malt modification levels. Again, higher proteolytic modification (P3) showed a significant increase in the content of these aldehydes. Meth was mostly present only below its LOQ, and thus the results showed no dependency on the malt modification level despite Meth being a Strecker aldehyde. Other aldehydes such as Fur, Benz, Hex, and T2N were not significantly influenced by the

amount of soluble N. Fur is mostly influenced by the heat load during beer production [8]. Benz, even though being considered a Strecker aldehyde, appears to be more dependent on oxygen [6]. The formation of Hex and T2N is influenced by the concentration of lipids (varying by environment and cultivar) and the enzymes involved in their degradation during malting and brewing. Thus, their concentration is independent of the N species [20].

The observed increases in Strecker aldehydes explain the sensory impressions of aged beers, since these compounds are important contributors to aged aroma and especially to bready impressions [32]. A higher proteolytic malt modification lead to a higher aging potential in fresh beer and thus to increased formation of aldehydes during aging. An increased number of reactants during beer production results in a higher aging potential in the wort, which is likely to be transferred to the final beer [13].

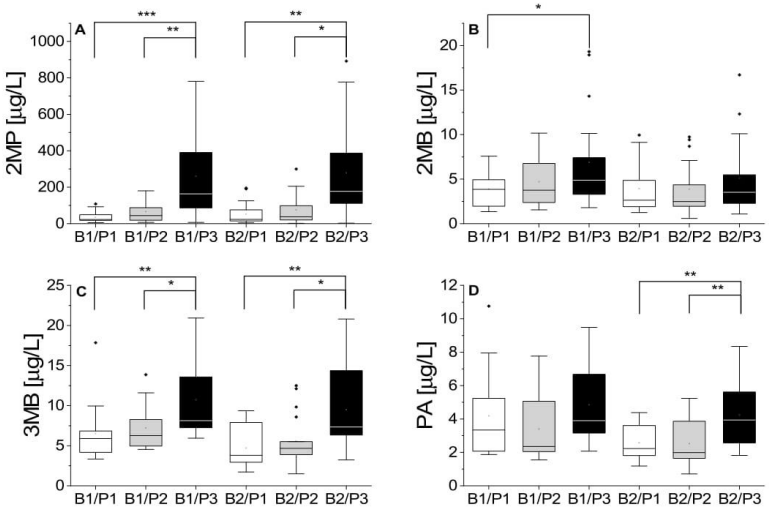

Figure 2. Boxplots of all aging points of chosen aldehydes ((**A**) 2MP; (**B**) 2MB; (**C**) 3MB; (**D**) PA) by malt modification level (B1: barley variety 1; B2: barley variety 2; P1: low proteolytic malt modification; P2: medium proteolytic malt modification; P3: high proteolytic malt modification). Linked boxplots showed a significant difference (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

3.4. Release of Bound State Aldehydes

The aging potential comes from reactive precursors, free aldehydes, and bound state aldehydes. These bound state aldehydes are relevant sources, next to *de novo* formation, of free aldehydes [3]. Thus, the behavior of bound state aldehydes during beer aging was assessed by two different methods.

First, bound state aldehydes can be released by the addition of 4VP prior to analysis. The released free aldehydes can be determined by HS-SPME. 4VP is a cysteine-trapping reagent and shows high reactivity toward thiols; thus, it acts as a binding reagent toward nucleophiles. Furthermore, the addition of 4VP to beer samples leads to a pH shift into the weak alkaline state. Baert et al. found high recoveries in model systems [11]. They ignored the observed release of bound state aldehydes from *de novo* formation of free aldehydes, observed the release of bound state aldehydes, and found a remarkable variation in releaseable aldehydes between different beer samples [17].

Second, bound state aldehydes can be released by excessive addition of ACA, which acts as a competitive agent toward other aldehydes that occur in a bound state. Upon addition, the chemical equilibrium changes and ACA being the most electrophile aldehyde

subsequently pushes out other aldehydes into their free form. Thus, these compounds can be determined as free aldehydes by HS-SPME [18].

3.4.1. Release by 4VP

Figure 3 shows the degree of bound state aldehydes after release by 4VP. The degree is a relative variable calculated as described before. A value of 0 indicates no release of bound state aldehydes, while values > 0 imply release.

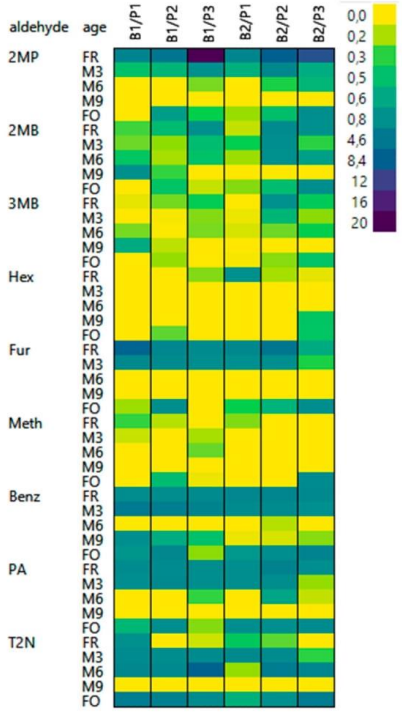

Figure 3. 4VP-releasable aldehydes (n = 6): the heatmap shows the relative concentration of bound state aldehydes in comparison to the free form (B1: barley variety 1; B2: barley variety 2; P1: low proteolytic malt modification; P2: medium proteolytic malt modification; P3: high proteolytic malt modification).

The degree of bound state aldehydes in the analyzed beers varied by the status of aging, malt modification level, and type of aldehyde. In most fresh samples, considerable amounts of aldehydes could be released by 4VP. The degree of releasable aldehydes decreased during aging. After M9, no more aldehydes could be released in most cases. This over-time-decreasing amount of bound state aldehydes was statistically significant for 2MP (one-way ANOVA: $p = 0.0002$), Fur (one-way ANOVA: $p = 0.0004$), and PA (one-way ANOVA: $p < 0.0001$). These compounds appeared to be the most promising aldehydes for use as early-stage indicators of flavor instability in fresh beer. Furthermore, Benz (one-way ANOVA: $p < 0.0001$) and T2N (one-way ANOVA: $p = 0.0024$) showed significant differences during aging. However, for these compounds, most aldehydes could be released after M3 versus M6 and forced aging. Apparently, bound state aldehydes are also released during forced aging, although to varying extents. In the case of T2N, more aldehydes were releasable after forced aging.

3.4.2. Release by ACA

Figure 4 shows the degree of bound state aldehydes after release by ACA.

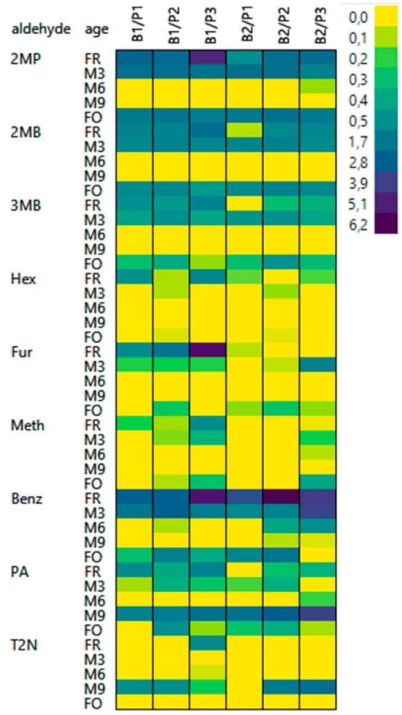

Figure 4. ACA-releasable aldehydes (n = 6): heatmap shows relative concentration of bound state aldehydes in comparison to the free form (B1: barley variety 1; B2: barley variety 2; P1: low proteolytic malt modification; P2: medium proteolytic malt modification; P3: high proteolytic malt modification).

As observed before, the degree of bound state aldehydes released by ACA varied by the status of aging, malt modification level, and type of aldehyde. For most samples, the number of ACA-releasable aldehydes showed a decrease during aging. More aldehydes could be released from fresh samples compared to natural and forced-aged samples. This was especially true for 2MP (one-way ANOVA: $p < 0.0001$), 2MB (one-way ANOVA: $p < 0.0001$), 3MB (one-way ANOVA: $p < 0.0001$), Hex (one-way ANOVA: $p = 00073$), and Benz (one-way ANOVA: $p \leq 0.0001$). Fur (one-way ANOVA: $p = 0.12$) and Meth (one-way ANOVA: $p = 0.24$) showed the same behavior but not significantly. PA and T2N also showed a similar behavior, with the exception that the highest concentrations of these compounds could be released at M9.

3.5. Influence of Aldehyde Structure on Occurrence in a Bound State

The chemical structure of aldehydes influences the degree of binding in various ways. Generally, steric effects might hinder the binding of Fur, Benz, and PA. Positive inductive effects (+I) lower electrophilicity and thus the binding affinity (e.g., for 2MB compared with 3MB). Positive mesomeric effects (+M) hinder the binding of Fur, Benz, and PA due to a higher electron density at the carbonyl group [33]. Bueno et al. investigated the equilibrium constants (K_a) of a variety of aldehydes and HSO_3^- in model wines. Higher values indicated that the equilibrium is more on the side of the bound state aldehyde.

They found that along with ACA (K_a = 485 × 10^3), Meth (K_a = 50 × 10^3) and 3MB (K_a = 29 × 10^3) showed higher affinity toward HSO_3^- compared to PA (K_a = 17 × 10^3), 2MP (K_a = 2.8 × 10^3), 2MB (K_a = 2.6 × 10^3), or Fur (K_a = 0.1 × 10^3). Higher values indicate an increased affinity toward nucleophiles [14,15].

In fresh beers, 2MP (up to 1970%), Fur (up to 760%), and PA (up to 290%) showed the highest relative release rates in the case of 4VP and, at the same time, together with 3MB, the highest absolute concentrations (Table S3). Thus, not only Ka but also process technology needs to be considered. Although 2MP is the most volatile of these compounds, it was also present in the free form in the highest quantities and might withstand processes such as evaporation during boiling at relatively high concentrations compared to other compounds. Fur and PA, in contrast, have relatively low volatilities and thus are not evaporated to the same extent but rather remain in the liquid phase. Therefore, these compounds might react more with nucleophiles in the matrix and thus occur more in a bound state. The results obtained in this study are in agreement with the literature. Baert et al. found that T2N, Fur, and 2MP could be released from fresh commercial beers by 4VP, each at an increase of more than 100% [17].

ACA revealed the presence of 2MP (up to 490%), 2MB (up to 220%), 3MB (up to 120%), Hex (up to 102%), and Benz (up to 620%) in bound states in fresh beers and their depletion during aging. Fur (except for B1/P3) and Meth did so, too, but to lesser extents. Interestingly, PA and T2N showed the same behavior, with the exception that after M9, more aldehydes were again released upon the addition of ACA. The highest absolute concentrations in the bound state were obtained for 2MP, 3MB, and PA (Table S3). The same aldehydes were among the ones that could be released by 4VP at high concentrations.

3.6. Final Discussion of Release and Bound State Aldehydes

Based on both these release methods, we finally investigated whether all the aging-related aldehydes are released during aging. Therefore, the sums of all investigated free and bound state aldehydes were calculated. Figure 5 shows the equilibrium between the sum of free aldehydes and the sum of bound state aldehydes assessed by the two applied release methods. We observed that the equilibrium shifted toward the free form during aging. After M9 versus M6, the equilibrium was fully toward the free form, indicating either hydrolysis or a different degradation of an important bound state fraction. Further possibilities could be the irreversible binding on proteins [10], proline-catalyzed aldol condensation [34], or further oxidation reactions, which will be discussed in part 2 of this study.

Furthermore, the equilibrium was influenced by the amount of soluble N in the sample. In fresh samples, the ratio of bound state and free aldehydes increased with the amount of soluble N for 4VP, and ~82% of aldehydes were free in P3, ~73% in P2, and 51% in P1 (R = 0.69). The same result was observed for ACA but to a lower extent (~57% in P3, 51% in P2, and 34% in P1; R = 0.26). A higher proteolytic malt modification level resulted in elevated concentrations of all amino acids except proline (see part 2). Therefore, amino acids are important binding agents for aldehydes. It was assumed that cysteinylated aldehydes have a major influence on beer flavor instability. In fact, they do exist in the beer matrix but only at negligible concentrations and increase during aging since they are in equilibrium with free aldehydes [12]. Thus, the N adducts that we observed in this study are more likely to be in the form of imines, either with amino acids or with larger peptides and proteins. These compounds would be hydrolyzed slowly during beer aging and thus would release free aldehydes. The degradation is favored at pH 4.0 because of the presence of a zwitterionic hemiaminal. Therefore, the targeted analysis of imines after reduction, as described in the literature, is necessary in future research [35]. Furthermore, untargeted analysis of protein-bound aldehydes seems highly promising for further elucidation of bound state species.

In summary, 4VP can release more bound state aldehydes compared with ACA. 4VP acts via a competitive (for nucleophiles) mechanism and also elevates the pH of the sample.

ACA only acts competitively. Using ACA is the softer, less invasive way of releasing bound state aldehydes. Ultimately, beer is a dynamic equilibrium system, and each method can only shift the equilibrium to a certain extent. Both presented methods, 4VP- and ACA-induced release, are promising tools for the early-stage assessment of beer flavor instability regardless of the maximum degree of release. Which method comes closest to reality is highly influenced by the samples, its way of production, and, ultimately, storage conditions.

Contrary to the literature, 4VP does not mostly release SO_2 adducts, as observed in beers with up to 10 mg/L of SO_2 [17]. 4VP and ACA can also release N-related adducts.

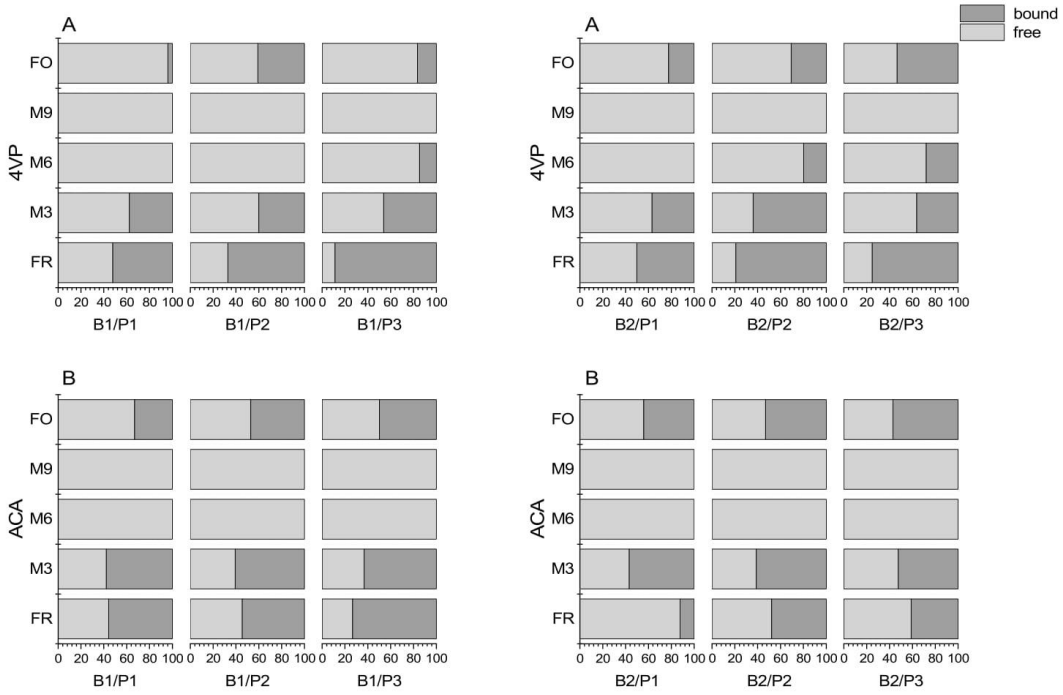

Figure 5. Ratio of sum of free and bound state aldehydes assessed by (**A**) 4VP and (**B**) ACA (B1: barley variety 1; B2: barley variety 2; P1: low proteolytic malt modification; P2: medium proteolytic malt modification; P3: high proteolytic malt modification).

4. Conclusions

Samples brewed via a standardized brewing process with a high-mashing-in procedure from two different barley varieties at different proteolytic malt modification levels showed different aging behaviors in sensory analysis, as well as free and bound state aldehydes. The soluble N in these samples ranged from 569 to 731 mg/100 g malt d.m. (ISO 65 °C mashing procedure), covering both low and high limits of the demanded brewing specifications. The sensory and analytical aging status increased with the amount of soluble N. The increase in free aldehydes, especially in the early to medium stage of aging (up to M6 of natural aging) can be explained by the release of bound state aldehydes to a strong degree. The impact of *de novo* formation will be discussed in part 2 of this study.

Aldehydes have different affinities with regard to their form of occurrence (free or bound) due to their chemical structures. The degree of binding is a combined result of abundance of the aldehyde and its electrophilicity. This equilibrium of free and bound

state aldehydes shifts toward the free form during aging. After M9 versus M6, depending on the release method, none or only a negligible impact of bound state aldehydes on the flavor instability of beer was observed. Therefore, the assessment of bound state aldehydes after their release is a promising alternative analytical method of forced aging and allows for early-stage prediction of the aging potential without thermal intake and the related problems in fresh beer.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10102432/s1, Table S1: malt and beer analysis, Table S2: sensory results, Table S3: free and bound aldehydes.

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Data Availability Statement: The data presented in this study are available within the article.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jaskula-Goiris, B.; De Causmaecker, B.; De Rouck, G.; Aerts, G.; Paternoster, A.; Braet, J.; De Cooman, L. Influence of transport and storage conditions on beer quality and flavour stability. *J. Inst. Brew.* **2018**, *125*, 60–68. [CrossRef]
2. Paternoster, A.; Jaskula-Goiris, B.; De Causmaecker, B.; Vanlanduit, S.; Springael, J.; Braet, J.; De Rouck, G.; De Cooman, L. The interaction effect between vibrations and temperature simulating truck transport on the flavor stability of beer. *J. Sci. Food Agric.* **2019**, *99*, 2165–2174. [CrossRef] [PubMed]
3. Lehnhardt, F.; Gastl, M.; Becker, T. Forced into aging: Analytical prediction of the flavor-stability of lager beer. A review. *Crit. Rev. Food Sci. Nutr.* **2019**, *59*, 2642–2653. [CrossRef] [PubMed]
4. Lehnhardt, F.; Steiner, J.; Gastl, M.; Becker, T. Prediction Power and Accuracy of Forced Ageing—Matching Sensory and Analytical Results for Lager Beer. *Brew. Sci.* **2018**, *71*, 39–48.
5. Bueno, M.; Marrufo-Curtido, A.; Carrascón, V.; Fernández-Zurbano, P.; Escudero, A.; Ferreira, V. Formation and Accumulation of Acetaldehyde and Strecker Aldehydes during Red Wine Oxidation. *Front. Chem.* **2018**, *6*, 20. [CrossRef]
6. Wietstock, P.C.; Kunz, T.; Methner, F.-J. The Relevance of Oxygen for the Formation of Strecker Aldehydes during Beer Production and Storage. *J. Agric. Food Chem.* **2016**, *64*, 8035–8044. [CrossRef]
7. Vanderhaegen, B.; Neven, H.; Verachtert, H.; Derdelinckx, G. The chemistry of beer aging—A critical review. *Food Chem.* **2006**, *95*, 357–381. [CrossRef]
8. Baert, J.J.; De Clippeleer, J.; Hughes, P.S.; De Cooman, L.; Aerts, G. On the Origin of Free and Bound Staling Aldehydes in Beer. *J. Agric. Food Chem.* **2012**, *60*, 11449–11472. [CrossRef]
9. Wietstock, P.C.; Methner, F.-J. Formation of Aldehydes by direct Oxidative Degradation of Amino Acids via Hydroxyl and Ethoxy Radical Attack in Buffered Model Solutions. *Brew. Sci.* **2013**, *66*, 104–113.
10. Lermusieau, G.; Noël, S.; Collin, S. Nonoxidative Mechanism for Development of trans-2-Nonenal in Beer. *J. Am. Soc. Brew. Chem.* **1999**, *57*, 29–33. [CrossRef]
11. Baert, J.J.; De Clippeleer, J.; Aerts, G. Exploring the Binding Behavior of Beer Staling Aldehydes in Model Systems. *J. Am. Soc. Brew. Chem.* **2015**, *73*, 100–108. [CrossRef]
12. Bustillo Trueba, P.; Jaskula-Goiris, B.; Ditrych, M.; Filipowska, W.; De Brabanter, J.; De Rouck, G. Monitoring the evolution of free and cysteinylated aldehydes from malt to fresh and forced aged beer. *Food Res. Int.* **2021**, *140*, 110049. [CrossRef]
13. Nobis, A.; Lehnhardt, F.; Gebauer, M.; Becker, T.; Gastl, M. The influence of proteolytic malt modification on the aging potential of final wort. *Foods* **2021**, *10*, 2320. [CrossRef]
14. Dugulin, A.C.; Clegg, S.C.; De Rouck, G.; Cook, D.J. Overcoming technical barriers to brewing with green (non-kilned) malt: A feasibility study. *J. Inst. Brew.* **2020**, *126*, 24–34. [CrossRef]
15. Drost, B.W.; van den Berg, R.; Freijee, F.J.M.; van der Velde, E.G.; Hollemans, M. Flavor Stability. *J. Am. Soc. Brew. Chem.* **1990**, *48*, 124–131. [CrossRef]
16. Baert, J.J.; De Clippeleer, J.; Jaskula-Goiris, B.; van Opstaele, F.; De Rouck, G.; Aerts, G. Further Elucidation of Beer Flavor Instability: The Potential Role of Cysteine-Bound Aldehydes. *J. Am. Soc. Brew. Chem.* **2015**, *73*, 243–252. [CrossRef]

17. Baert, J.J.; De Clippeleer, J.; Bustillo Trueba, P.; Jaskula-Goiris, B.; De Rouck, G.; Aerts, G. Exploring Aldehyde Release in Beer by 4-Vinylpyridine and the Effect of Cysteine Addition on the Beer's Pool of Bound Aldehydes. *J. Am. Soc. Brew. Chem.* **2018**, *76*, 257–271. [CrossRef]
18. Barker, R.L.; Gracey, D.E.F.; Irwin, A.J.; Pipasts, P.; Leiska, E. Liberation of staling aldehydes during storage of beer. *J. Inst. Brew.* **1983**, *89*, 411–415. [CrossRef]
19. Dufour, J.-P.; Leus, M.; Baxter, A.J.; Hayman, A.R. Characterization of the Reaction of Bisulfite with Unsaturated Aldehydes in a Beer Model System Using Nuclear Magnetic Resonance Spectroscopy. *J. Am. Soc. Brew. Chem.* **1999**, *57*, 138–144. [CrossRef]
20. Filipowska, W.; Jaskula-Goiris, B.; Ditrych, M.; Bustillo Trueba, P.; De Rouck, G.; Aerts, G. On the contribution of malt quality and the malting process to the formation of beer staling aldehydes: A review. *Inst. Brew. Distill.* **2021**, *127*, 107–126. [CrossRef]
21. Back, W.; Gastl, M.; Krottenhaler, M.; Narziß, L.; Zarnkow, M. *Brewing Techniques in Practice: An In-Depth Review of Beer Production with Problem Solving Strategies*; Fachverlag Hans Carl: Nürnberg, Germany, 2020.
22. Hill, A.; Stewart, G.G. Free Amino Nitrogen in Brewing. *Fermentation* **2019**, *5*, 22. [CrossRef]
23. Gastl, M.; Kupetz, M.; Becker, T. Determination of Cytolytic Malt Modification—Part I: Influence of Variety Characteristics. *J. Am. Soc. Brew. Chem.* **2021**, *79*, 53–65.
24. Stephan, A.; Kusche, M.; Stettner, G. Influence of malt quality on the generation of odour-active Strecker aldehydes during beer aging. In Proceedings of the 31st EBC Congress, Venice, Italy, 6–10 May 2007.
25. Gastl, M.; Spieleder, E.; Hermann, M.; Thiele, F.; Burberg, F.; Kogin, A. The influence of malt quality and malting technology on the flavour stability of beer. *Mon. Brauwiss.* **2006**, *72*, 163–175.
26. Malt Analysis: Industry-Wide Change in Analysis Approach for Barley Malt 5 EBC. In *BRAUWELT International 2020*; B. Deutscher Brauer-Bund e.V.: Berlin, Germany; Deutscher Mälzerbund e.V.: Frankfort on the Main, Germany; Braugersten-Gemeinschaft e.V.: Munich, Germany, 2020.
27. Lund, M.N.; Petersen, M.A.; Andersen, M.L.; Lunde, C. Effect of Protease Treatment during Mashing on Protein-Derived Thiol Content and Flavor Stability of Beer during Storage. *Am. Soc. Brew. Chem.* **2015**, *73*, 287–295. [CrossRef]
28. Jacob, F. (Ed.) *MEBAK Brautechnische Analysemethoden—Würze, Bier, Biermischgetränke: Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission*; MEBAK: Freising-Weihenstephan, Germany, 2012.
29. Jacob, F. (Ed.) *MEBAK Brautechnische Analysenmethoden—Sensorik: Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission*; MEBAK: Freising-Weihenstephan, Germany, 2013.
30. Saison, D.; De Schutter, D.P.; Delvaux, F.; Delvaux, F.R. Optimisation of a complete method for the analysis of volatiles involved in the flavour stability of beer by solid-phase microextraction in combination with gas chromatography and mass spectrometry. *J. Chromatogr. A* **2008**, *1190*, 342–349. [CrossRef]
31. Lehnhardt, F.; Becker, T.; Gastl, M. Flavor stability assessment of lager beer: What we can learn by comparing established methods. *Eur. Food Res. Technol.* **2020**, *246*, 1105–1118. [CrossRef]
32. Saison, D.; De Schutter, D.P.; Uyttenhove, B.; Delvaux, F.; Delvaux, F.R. Contribution of staling compounds to the aged flavour of lager beer by studying their flavour thresholds. *Food Chem.* **2009**, *114*, 1206–1215. [CrossRef]
33. LoPachin, R.M.; Gavin, T. Molecular Mechanisms of Aldehyde Toxicity: A Chemical Perspective. *Chem. Res. Toxicol.* **2014**, *27*, 1081–1091. [CrossRef]
34. Schmid, M.B.; Zeitler, K.; Gschwind, R.M. The Elusive Enamine Intermediate in Proline-Catalyzed Aldol Reactions: NMR Detection, Formation Pathway, and Stabilization Trends. *Angew. Chem. Int. Ed.* **2010**, *49*, 4997–5003. [CrossRef] [PubMed]
35. Zameo, S.; Vauzeilles, B.; Beau, J.-M. Direct Composition Analysis of a Dynamic Library of Imines in an Aqueous Medium. *Eur. J. Org. Chem.* **2006**, *24*, 5441–5444. [CrossRef]

4 Discussion

4.1 Deficiencies of established methods for the assessment of flavor instability

As it was introduced above, the currently established methods for the assessment of flavor instability do not allow a precise prediction of flavor instability but rather an endpoint estimation. Furthermore, they do not take into account the concept of bound state aldehydes. Therefore, there is a need to revise the established methods. In the first and second part of this study, the drawbacks of forced aging and steam distillation are discussed. The third part provides a literature review on bound state aldehydes and gives a holistic view on flavor instability. In the fourth and fifth part of this study, the behavior of free and bound state aldehydes is monitored during the wort boiling processes and beer aging and the potential of bound state aldehydes for the prediction of flavor instability is discussed.

Forced aging is an established method to accelerate aging processes and is said to resemble natural aging of 3–6 months [79]. The results of natural and forced aging showed a high similarity when assessed with more global or general approaches. The DLG scores for smell, taste, and bitterness after the applied forcing regimen were found between 3 and 5 months of natural aging. The same was observed for a hedonic acceptancy test.

When the sum of aging indicators analyzed by GC-MS was considered, again a high similarity of these samples was observed. This makes forced aging, a valid but very limited prediction method due to the fact that a large part of the sum of aging indicators is made up from the single compound FUR which is a well-known heat indicator [11]. Yet, more specific approaches resulted in a lesser consensus. The aroma profiles of the individual samples deviated in the studied aging descriptors. Figure 2 shows a principal component analysis (biplot) of 8 aging-relevant sensory attributes and 13 differently aged samples. It was found that forced aged samples were best described with descriptors such as *cardboard* and *dull*, while natural aged samples scored higher in *fruity-sweetish* descriptors.

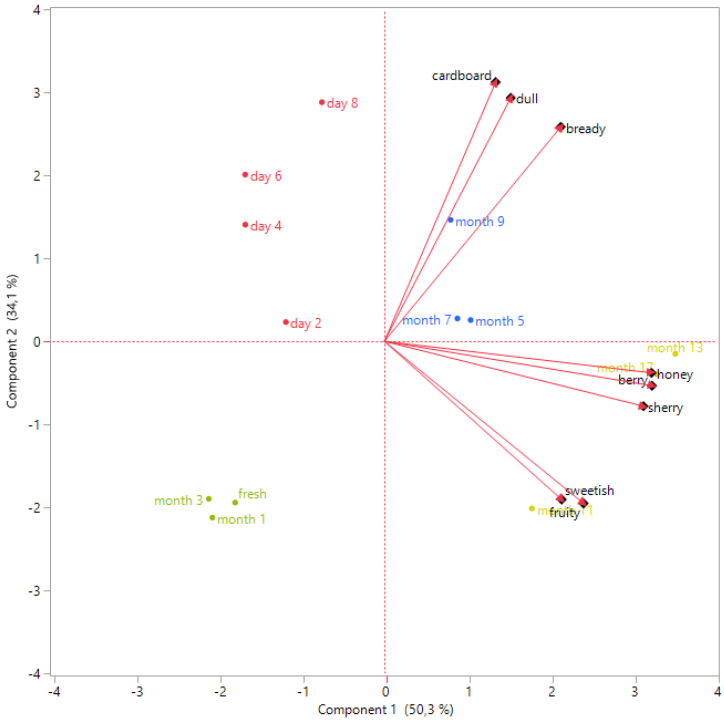


Figure 2: Principal component analysis (biplot) of 8 chosen sensory attributes and 13 different naturally and forced aged samples

Consequently, individual aroma active aging compounds showed high deviations in the different aging methods. T2N increased strongly in forced aging and after 2 days reached odor activity values (OAV) above 1, meaning that it is present above its odor threshold. During natural aging, this compound remained at stable low levels. In this specific sample, methional reached OAVs above 1 after 7 months.

Interesting behavior was observed for the linearity of the increase in individual aging compounds. From the sole view point of *de novo* formation of aging compounds, a linear behavior is expected. Due to the fact, that precursors are present in excess and ambient temperatures are constant, those reactions follow a zero-order reaction. This implies that the reaction speed is independent from the concentration of the precursors and thus, their degradation is constant. For natural aging, only 3 esters, 1 ether, and 1 aldehyde (FUR) increased linearly. For forced aging, 4 esters, 1 ether, 1 ketone, and 6 aldehydes did so. The aldehydes were comprised of 2MP, 2MB, PA, FUR, METH, and T2N. It seems that especially aldehydes from Strecker degradation and lipid degradation are affected and it points to a more complex formation behavior of these compounds during natural aging in comparison to other compounds such as afore-mentioned esters or FUR. The increase of these compounds appears to be influenced by a variety of parameters, whose impact is suppressed by the thermal intake of heat

during forced aging. While forced aging is a predominantly temperature-driven way, natural aging is more complex and possibly runs at several distinct stages.

Recently, the differences between natural and forced aging were highlighted in literature for the behavior of 3-DG. A different partition of possible degradation mechanisms of 3-DG in natural (12 months, 20 °C) and forced aging of lager beers that were spiked with 3-DG was reported. In natural aging, 3-DG predominantly interconverted to 3DGal (50 %), whereas other reactions only occurred to minor degrees (Maillard reaction (2 %), HMF formation (16 %), Strecker degradation (14 %) and others (17 %)). In forced aging, 3-DG mainly reacted to HMF (34 %), followed by interconversion to 3-DGal (17 %), Maillard reaction (8 %), and Strecker degradation (3 %). The residual 39 % took part in other reactions such as melanoidin formation and modification of proteins. This shows that forced aging results in an increase of thermally catalyzed reactions [43]. Especially the differences in participation in the Strecker degradation were confirmed in the results of this part of the thesis.

Furthermore, the currently applied extraction techniques of volatile aging compounds show substantial differences. Therefore, in order to identify the most suitable extraction for free aldehydes resp. the indirect determination of bound state aldehydes, a variety of established extraction methods in combination with GC-MS have been compared as a second part of this study. The following methods were used:

- **Headspace solid-phase microextraction (HS-SPME):** non-invasive technique; volatiles are extracted from the headspace above a sample by a sorptive fiber
- **Solvent-assisted flavor evaporation (SAFE):** invasive technique; sample is extracted with organic solvent (diethyl ether), distilled relatively gentle under vacuum at 40 °C, organic acids can be removed, the distillate can be further concentrated by distillation
- **Steam distillation (SD):** invasive technique; sample is distilled at 100 °C, organic acids are removed, ethanolic distillate is extracted with other organic solvents (dichloromethane)

At first, these methods were compared qualitatively by GC-O. A detection in this method points out that a compound is potentially aroma active in the sample matrix. Interestingly, all observed aldehydes varied among the extraction techniques and

therefore showed temperature-dependent behavior. 3MB was not observed in SPME and SAFE. In SD in contrast it showed odor activity. Methional appeared only in SAFE, not in SPME or SD. Model systems revealed its degradation and polymerization during distillation. PA showed the highest olfactometric activity in SD. Furthermore, two acetals (2,4,5-trimethyl-1,3-dioxolane and diethoxyethane) were found in SD but not in any other extraction technique. The occurrence of these products of aldehydes and alcohols suggests rather temperature-dependent formation than depletion of these bound states. The same behavior can be expected during the brewing process, especially during wort boiling.

In another experiment, SAFE and SD were compared more closely in a quantitative way. The results showed differences in certain aging indicators even though both approaches were internally calibrated and each fully validated. Among these, especially 3MB, PA, and FUR appeared to differ quite strongly. In the case of 3MB and FUR the concentrations were lower in SD, while PA was higher in SD. It was concluded that bound state precursors and possibly other intermediates are affected during sample preparation and thus affect the matrix-assisted calibration. In comparison to 3MB and FUR, PA shows a relatively high affinity towards nucleophiles (see section 4.2) being a plausible cause for the observed discrepancies. This behavior was finally confirmed in model systems. When solutions of cysteinylated FUR or 3MB were distilled according to the SD method, recoveries of about 80 – 90 % of the respective aldehyde were observed. The observed decreased recovery rates can occur on the one side due to a loss of analyte during the work-up procedure but also on the fact that the aging potential influences the matrix-assisted calibration. Since not only the added amount of analyte is detected but additionally the amount that is formed and released during the work-up the recovery rates are expected to be lower.

Thus, the type of extraction and analysis technique of volatiles is critical when assessing flavor instability. Therefore, SPME was chosen for the further analyses of free and bound state aldehydes after release in this thesis.

In practice, of course some valuable information can be drawn from the currently established assessment of flavor instability with forced aging but one should be aware of the underlying differences to natural aging. It is necessary to gain a holistic insight into the possible aging mechanisms and how they might differ between natural and forced aging. Only in this way, technological improvements can be achieved on a non-

empirical basis. Therefore, a literature review was conducted as a third part of this work. The main findings are discussed in the following lines.

4.2 Structure-dependent interactions of aldehydes in complex matrices

In complex matrices such as malt, wort and beer, as described above, all compounds possibly interact with one another and form a thermodynamical equilibrium. Since the functional carbonyl group is relatively electrophile and thus reactive, it stands to reason that, these compounds interact with the respective matrix at each production step – from malting to brewing. This takes place in the sense of nucleophilic additions and possible further reactions (such as elimination of water). Thus, free aldehydes can be masked to human perception and other processes such as evaporation during wort boiling or reduction by yeast during fermentation [12]. This binding can be either reversible or irreversible [53]. In experiments with ^{13}C-labelled amino acids it was shown that 85 % of Strecker aldehydes in the final aged beer are produced up-stream and then bound. The authors especially highlighted the importance of the wort boiling step. In that study, only 15 % are formed were found to be formed *de novo* [54].

From literature, a variety of possible bound state aldehydes and thus potential indicators of beer flavor instability could be identified. These comprise bisulfite adducts, imines, cysteinylated aldehydes, acetals, and glycosides. The respective binding agents are bisulfite (HSO_3^-), amino groups (from amino acids, peptides and proteins), cysteine, alcohols, and carbohydrates.

Since all these precursors are formed in nucleophile additions. In theory, all heteroatoms (especially nitrogen (N), oxygen (O), and sulfur (S)) with a higher electronegativity compared to carbon can act as nucleophiles in this reaction. Furthermore, the nucleophilicity of the respective reaction partners can be used to judge on the importance of adduct formation in beer.

Among the present nucleophiles in malt, wort and beer, especially cysteine (N = 23.4), proline (N = 18.1), and bisulfite (N = 16.8) show high nucleophilicity parameters [90, 91]. They are sufficiently available in wort and beer, and reaction parameters (pH, temperature) favor their formation during wort boiling (N adducts) and fermentation (SO_2 adducts) [12]. In contrast, the nucleophilicity of ethanol is expected to be relatively low as its surrogate, methanol (N = 6.0) suggests. Therefore, acetals appear of lesser

importance. Thus, imines, cysteinylated aldehydes, and bisulfite adducts can be regarded as the most important bound state precursors. It can be hypothesized that due to their distinct separation during the process, there is a hot-side (malting and brewhouse) and a cold-side (fermentation) bound pool. These two make up the bound state pool of final beer. All the aroma active compounds and their possible precursors including bound state aldehydes are defined as the **aging potential** of beer. This potential is hypothesized to be responsible for the occurrence of aged aromas.

Thus, the described bound state aldehydes need to be analyzed in order to get insights on the flavor instability of a sample. Only in this way, relevant structures, their stabilities, their behavior during aging and potential bound state indicators can be reliably identified. Furthermore, in the past, partial approaches of observation have all failed to explain the actual phenomenon and neither, solely oxidation, nor *de novo* formation nor the release from bound states will do so in future [34]. Thus, in this thesis the afore-mentioned candidates were analyzed directly and indirectly with different methods.

The direct analysis of bound state aldehydes can be challenging due to various reasons but should ultimately be strived for. In aqueous media for example, imines and corresponding amines are present in an equilibrium and the former can be rapidly hydrolyzed for example during extraction. To avoid this, reduction of the imine was described as one way to fixate these compounds [70]. Therefore, when directly investigating bound state aldehydes, strategies that preserve the original state of equilibrium need to be followed.

The indirect approach also appears promising. By addition of an agent that might release reversibly bound aldehydes the resulting free aldehydes can be determined. The amount of bound state aldehydes is then calculated based on the difference between free aldehydes after release and free aldehydes without addition of release agents. In the case of indirectly investigating them, the least invasive method for free aldehydes needs to be chosen. In combination with results after bound state aldehydes have been released, an evaluation of flavor instability is possible. A critical aspect when releasing aldehydes is the mechanism through which it is achieved. From literature, two different release agents could be identified.

It is known that 4VP competes for nucleophiles (“cysteine-trapping agent”) and elevates the pH value. It was demonstrated that the addition of this compound does

not catalyze *de novo* formation. In model systems with aldehydes and cysteine, near to complete recovery rates could be achieved [57]. Furthermore, this approach was also applied in commercial beer samples in which the highest release was observed for 2MP and FUR [92]. Additionally, acetaldehyde is also able to release other aldehydes through shifting of the chemical equilibrium due to its higher affinity towards nucleophiles. In mixtures with bisulfite it was demonstrated that the addition of acetaldehyde increases the concentrations of other aldehydes, especially unsaturated aliphatic aldehydes such as T2N [52].

In a holistic view, beer aging can be regarded as a multi-stage phenomenon in which the actually perceivable aldehydes occur due to an interplay of ROS-induced oxidation, release from N- and SO_2-adducts and *de novo* formation. Presumably, a big part of free aldehydes and their precursors including bound state aldehydes are formed during the brewing process.

Figure 3 gives an overview of the possible reactions during beer aging. N-adducts (mostly imines) are mainly formed on the hot side during malting and brewing (wort boiling) due to the favorable pH (~ 5.0–5.2). They are slowly hydrolyzed at beer pH (4.2–4.5) during aging and release free aldehydes. The formation and degradation of imines should be a 2^{nd} order reaction [93]. Therefore, the impact of release from imines is most impactful at the beginning of aging and less with increasing time.

SO_2-adducts are formed during fermentation in the green beer (cold side) and will be depleted through equilibrium-driven release since oxidation of sulfite to sulfate occurs. The same effect might occur through an ROS-mediated increase in acetaldehyde.

Finally, via *de novo* formation aldehydes can arise during aging in oxygen-independent and dependent mechanisms as described previously.

Thus, it can be hypothesized that the main drivers of the discussed aging processes are the pH-value, ΔT, t and ROS. Presumably, a big part of free aldehydes arises due to shifts in the chemical equilibrium as a result of competitive reactions among all aldehydes, which will be discussed in the following chapter. It can be argued that a beer is never fully in a chemical equilibrium due to the fact that it does not represent a closed system and the abundance of reactive compounds. Especially, the influence of temperature will be discussed in the following lines.

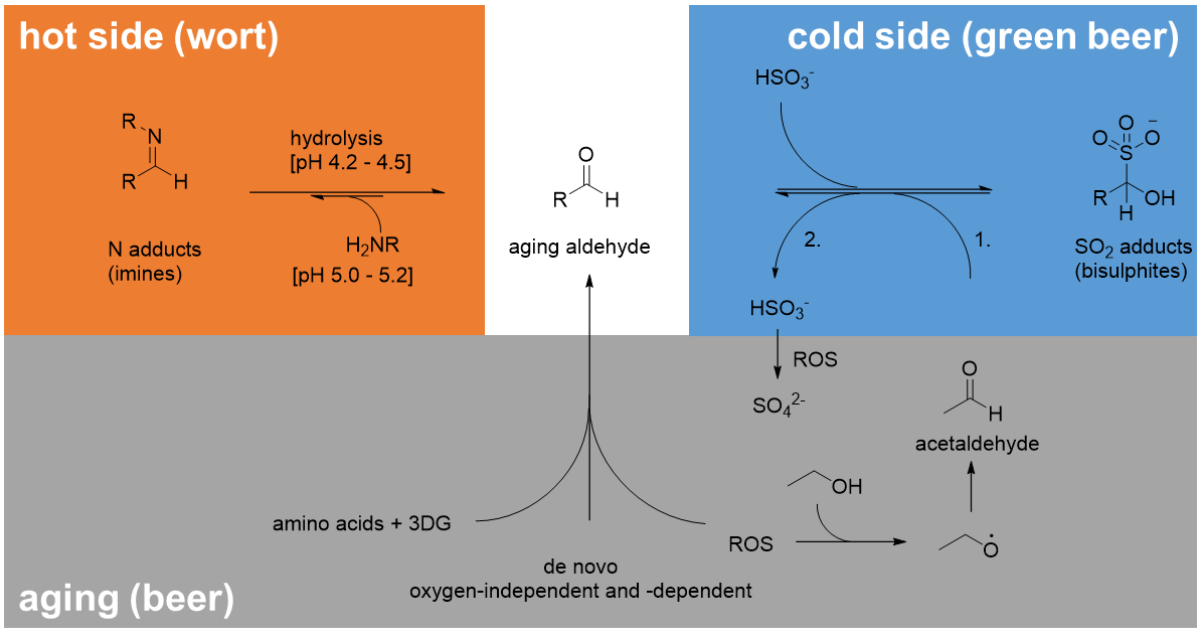
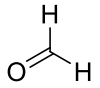
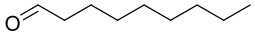
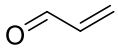
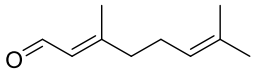
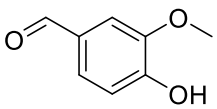


Figure 3: Schematic course of aldehyde formation during beer aging with various sources for aging aldehydes: hydrolysis of N-adducts, release from SO_2-adducts (mediated through competition with ACA (1) and oxidative depletion of sulfite (2)), and de novo *formation*

As it was shown that temperature during the analysis of free aldehydes is critical, it stands to reason that temperature plays a major role in the formation and degradation of aldehydes during the process. Especially, the wort boiling process is hypothesized to be a critical step for the formation of amino group-related adducts due to its favorable reaction conditions and abundance of reactants.

Among other parameters, such as nucleophilicity of the binding agents, also the electrophilicity of the aldehyde influences the affinity towards a bound state. Doubtless, the structure of the aldehyde influences its affinity towards being free or bound (De Azevedo 2007). Since aldehydes will be attacked nucleophilicly in the described reactions, the electrophilicity (ω) of the respective aldehyde group is a measure for its affinity towards being attacked. This is in turn influenced by the molecular structure and resulting inductive or mesomeric effects but also steric effects. Generally, short-chain aldehydes are more electrophilic than longer-chain; α,β-unsaturated aldehydes show higher electrophilicity as well as aromatic ones but sterical hindrance reduces it [94].

Table 3: Influence of aldehyde properties on electrophilicity according to LoPachin et al. [94]

Exemplary compound	Structure	Electrophilicity ω [eV]	Properties
Formaldehyde		2.62	Aliphatic Short chain No steric hindrance
Nonanal		2.16	Aliphatic Long chain No steric hindrance
Acrolein		3.82	Aliphatic α,β-unsaturated No steric hindrance
Citral		3.44	Aliphatic α,β-unsaturated Steric hindrance
Vanillin		3.32	Aromatic Steric hindrance

Based on the theoretical values of electrophilicity of the surrogates shown in table 3, T2N, BENZ, PA and FUR should be present at fairly high concentrations compared to 2MP, 2MB, 3MB, METH and HEX. Another study found that the experimental equilibrium constants of bisulfite addition in wine model systems vary greatly with the aldehyde. The highest reactivities are found for ACA (K_a = 485 × 10^3), followed by methional (K_a = 50 × 10^3), 3MB (K_a = 29 × 10^3), PA (K_a = 17 × 10^3), 2MP (K_a = 2.8 ×10^3), 2MB (K_a = 2.6 × 10^3), and lastly FUR (K_a = < 0.1 × 10^3). In these studies, HEX and T2N were not investigated but it was reported on decanal (K_a = > 100 × 10^3). As described above, the values for these aldehydes should be comparable or at least in the same magnitude [95, 96]. This highlights the complexity of the chemical equilibrium since not only the electrophilicity but also steric effects, as well as the concentrations at each production step and the following steps have to be considered. Therefore, it can be concluded that especially aliphatic aldehydes from Strecker degradation and lipid oxidation such as 2MP, 2MB, 3MB and HEX can be bound and also released relatively easy in the matrix wort and beer.

4.3 Development and degradation of amino group-related adducts

Therefore, the behavior of free and bound state aldehydes on the hot side of the brewing process was further investigated. Amino group-related adducts such as imines appear to have the highest impact on the hot side. Thus, beers were produced with a systematic variation of reactants by variation of the proteolytic malt modification.

In the fourth part of this work, the existence of bound state (4VP-releaseable) aldehydes was discovered throughout the wort boiling process. These bound state aldehydes decreased during wort boiling but less than free aldehydes and thus were present at higher levels at the end of boiling. The ratio of free and bound state aldehydes was equal for all the observed proteolytic malt modifications. A correlation of bound state aldehydes and soluble nitrogen in the sample was discovered especially for Strecker aldehydes and FUR by barley variety. 2MB (R = 0.91), FUR (R = 0.85) and METH (R = 0.82) showed the highest correlation coefficients. Therefore, it could be concluded that a considerable amount of bound state aldehydes is dependent on soluble nitrogen and therefore free amino groups.

To unravel the identity of these 4VP-releasable aldehydes, targeted analysis was applied. Previously, cysteinylated aldehydes were suggested as the most promising bound state aldehydes. In this work, at the end of boiling, only 3MB, PA and METH were present in a cysteinylated form. In average, for 3MB the cysteinylated form could explain only 1 % of 4VP-releasable aldehydes. For METH 10 % and for PA 7.5 % could be explained this way. Furthermore, no consistent trend in regard to the proteolytic malt modification was observed. All other aldehydes were not detected. This coincides with data from the literature. Other authors could detect some cysteinylated aldehydes during the process. Their concentrations are relatively high in malt but they degrade throughout the process and most quickly during fermentation [72]. Yet, during beer aging, the concentrations of cysteinylated aldehydes increase again slightly through the chemical equilibrium [74]. Overall, cysteinylated aldehydes do exist in the beer matrix but can be regarded as mostly inconsequential for beer flavor instability. Thus, other target compounds need to be chosen.

Fermentation did not affect the 4VP-releasable aldehydes as drastically as cysteinylated aldehydes, since in fresh beers (without SO_2) 4VP still showed release as the fifth part of this work indicated. This might be due to the fact that the hydrolysis rate of imines is lower than the one of 2-substituted-1,3-thiazolidine-4-carboxylic acids

and that the thiol group of cysteine shows high affinity towards other compounds. In the case of 4VP, the highest relative concentrations compared to free aldehydes in fresh beer were found for 2MP (up to 1970 %), Fur (up to 760 %), PA (up to 290 %). In the case of ACA, 2MP (up to 490 %), 2MB (up to 220 %), 3MB (up to 120 %), Hex (up to 102 %) and Benz (up to 620 %) could be released most effectively.

During aging, less aldehydes could be released, except for a few exceptions, thus supporting the hypothesis of this work. For 4VP, this over-time-decreasing behavior was statistically reliable for 2MP, Fur and PA. The behavior of BENZ and T2N deviated from the others. They showed the highest release after 3 resp. 6 months of natural aging. Thus, these aldehydes follow a more complex role in the changing equilibrium. T2N for instance is known to form diadducts with nucleophiles [53]. The ACA-releasable aldehydes decreased also during aging. This was observed especially for 2MP, 2MB, 3MB, HEX, BENZ, FUR and METH. Contrary to that, PA and T2N were present in highest concentrations after 9 months. Again, this finding highlighted the different behavior of unsaturated and possibly aromatic aldehydes compared to saturated aliphatic aldehydes.

Figure 4 shows the course of the ratio between bound (assessed by release with 4VP) and free aldehydes – exemplary for 2MP – from pre-boil wort over cast-out wort until fresh and aged beer (M6). The figure combines data from part 4 and 5 of this thesis. Values of 0 indicate that no aldehydes were releasable by 4VP.

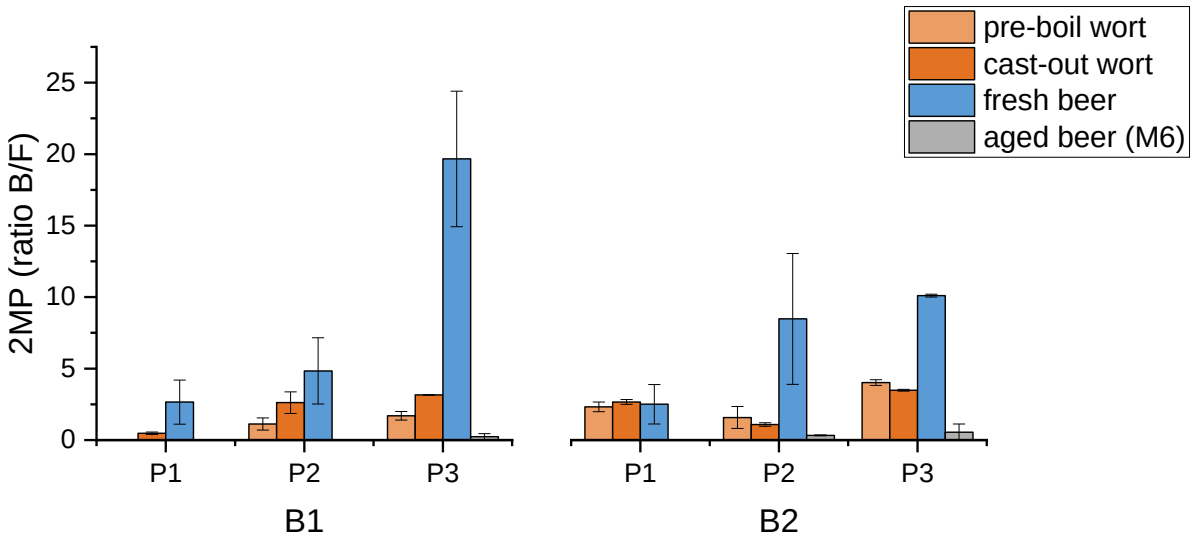


Figure 4: Course of ratio of bound (B; released with 4VP) and free (F) aldehydes from pre-boil wort, cast-out wort and fresh beer to aged beer (6 months naturally) from different barley varieties (B1 = Avalon, B2 = Marthe) and proteolytic malt modification levels (P1 = low, P2 = medium, P3 = high)

For all modifications except B1/P1, a bound state of 2MP was observed. This indicates that already during malting and mashing amino group-related adducts can be developed. In the cast-out wort, in the case of B1, the ratio of bound and free aldehydes increased, indicating a development of bound aldehydes as well as the evaporation of free aldehydes. In the case of B2, a stagnating behavior could be observed. The impact of reduction by yeast can clearly be observed in the data of fresh beer. Here, the free aldehydes are mainly reduced as indicated by the average amounts of 2MP over all proteolytic malt modifications (151.0 µg/L in cast-out wort vs. 6.2 µg/L in fresh beer). In contrast, the amount bound aldehydes decreased to a lesser extent (311.0 µg/L in cast-out-wort vs. 57.8 µg/L in fresh beer). Finally, during aging, the ratio of bound and free aldehydes is shifted towards the free form. This is discussed more in detail in Figure 5.

4.4 The use of bound state aldehydes for the prediction of flavor instability

Therefore, there is a need for the unravelling of the nature of 4VP-releasable and ACA-releasable aldehydes. As two studies of this work support the hypothesis that a higher amount of soluble N in wort and beer lead to more free and bound state aldehydes, the focus of future research should be on amino group-related bound state aldehydes such as imines. Chosen markers of bound state aldehydes should then be analyzed

by targeted methods. Especially, as discussed previously certain imines appear to be promising. For the targeted analysis of imines, the chemical equilibrium of imines can be “frozen” by the reduction of imines. Thus, they are not hydrolysable and will not be affected due to the equilibrium. The resulting secondary amine can be analyzed by HPLC [70]. Furthermore, the secondary amine, proline should be regarded as an interesting amino acid due to its high nucleophilicity (second highest among amino acids in beer [90]) and abundance in wort and beer. In the project beers, proline was present at concentrations from 325–498 mg/L.

After all, the assessment of the aging potential by release of bound state aldehydes and thus, the prediction of the flavor instability was demonstrated to reveal promising and valuable insights into the aging behavior of pale lager beers. The equilibrium of free and bound state aldehydes is pushed towards the free form during aging (Figure 5).

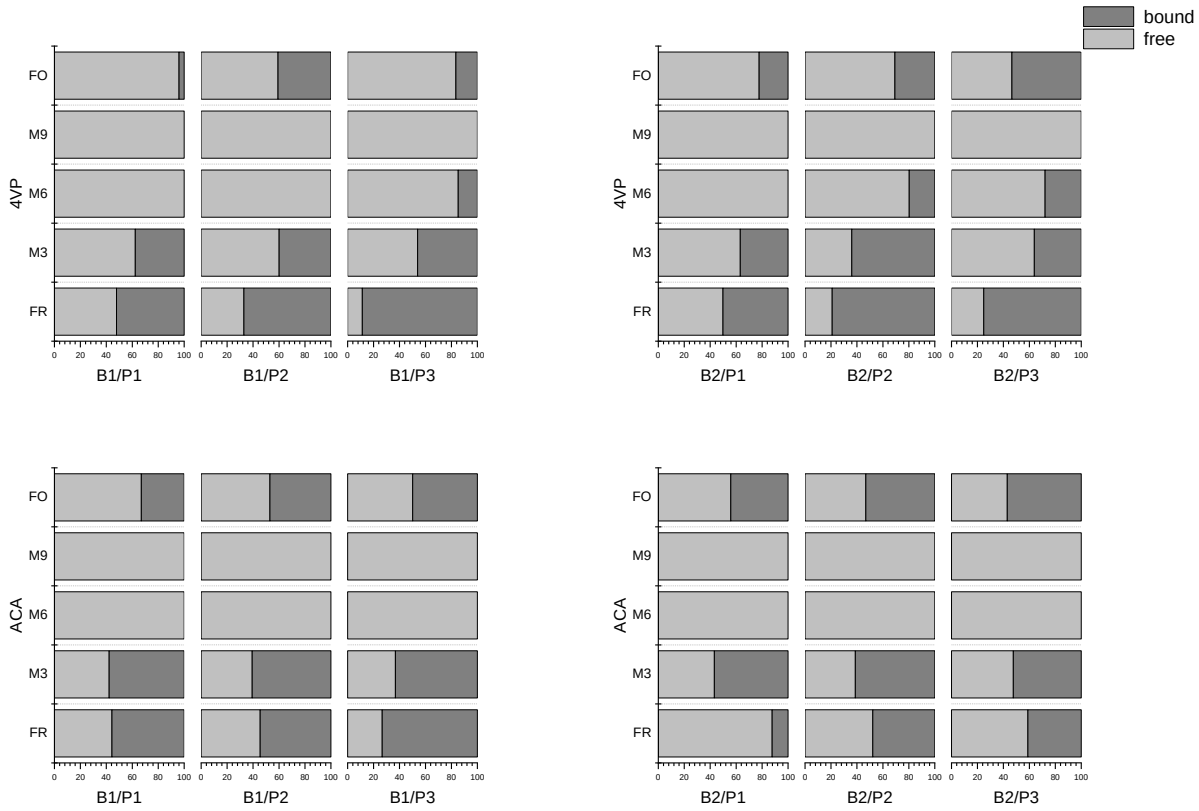


Figure 5: Ratio of free and bound state aldehydes during the course of aging assessed by 2 different release agents (4VP and ACA; B1 = barley variety Avalon; B2 = barley variety Marthe; P1 = low, P2 = medium, P3 = high proteolytic malt modification; n = 6)

Furthermore, it was also shown that the early-stage of natural aging of up to 6 months is influenced by the release of bound state aldehydes. Due to the fact that the

degradation of imines follows a 2nd order reaction [93], most aldehydes are released during this time. After that, *de novo* formation gains more importance.

Thus, the release of these compounds provides a realistic aging prediction. This approach can be regarded as an alternative to forced aging, whose drawbacks were discussed previously. In addition, results are obtained faster in this way allowing for better decisions concerning distribution and export.

5 Conclusions and Outlook

The findings in this thesis give new insights into the chemistry of flavor instability of pale lager beers and its prediction.

The currently established estimation method via forced aging and determination of volatile aging indicators using steam distillation were shown to inherit major drawbacks. It was demonstrated that forced aging results in a different quantitative profile of aroma active aging indicators and therefore a changed sensory profile compared to natural aging. Furthermore, the thermal stress during the extraction of volatiles by steam distillation resulted in substantial formation of aldehydes – through *de novo* formation as well as release from bound states. The latter group of molecules was identified to have significant impact on flavor instability especially in the early- and medium-phase of aging – the time span that is of most interest for the industry.

New insights into the formation and depletion of bound state aldehydes – indirectly assessed through the release by 4VP resp. ACA – were obtained and allow for a better understanding of the underlying mechanisms. It was shown in standardized brewing trials with variations in the proteolytic malt modification that during wort boiling, an amino group-related pool of bound state aldehydes is built up. The nature of these adducts – most likely imines – is to be determined in future. The previously discussed group of cysteinylated aldehydes was found to be inconsequential for flavor instability.

Finally, it was shown that the equilibrium of free and bound state aldehydes is shifted to the free state during aging and thus causing the observed quality loss. The mechanisms of release are a complex interplay of various chemical reactions but have to be investigated further.

In future, the initial amount of bound state aldehydes in fresh beer should be used as an important indicator to assess the aging potential and the resulting flavor instability on a day-to-day basis. As described, the indirect way (release of bound state aldehydes) provides valuable data. Yet, it should be backed up by the direct analysis of bound state aldehydes by more sophisticated LC-, MS- and NMR-based techniques. In this way, bound state indicators should be established. Furthermore, the application of (untargeted) proteomics should provide valuable insights into the interaction of aldehydes, and peptides and proteins.

Additionally, a special focus should be on the impact of yeast metabolism on bound state aldehydes. The dualism of reduction of aldehydes on the one hand and formation of bisulfite adducts on the other hand should be investigated more closely. The direct analysis of bisulfite adducts by LC-MS/MS after enrichment with SPE (ion exchange) was promising in unpublished results.

The holistic assessment of beer flavor instability – *de novo* formation, oxidation, release of bound state aldehydes – allows for better understanding of this phenomenon in all its aspects. More importantly it opens new ways towards technological approaches to reduce the loss of quality during beer aging and ultimately to more flavor stable beers.

6 References

1. Jaskula-Goiris, B.; Causmaecker, B. de; Rouck, G. de; Aerts, G.; Paternoster, A.; Braet, J. et al.: Influence of transport and storage conditions on beer quality and flavour stability, Journal of the Institute of Brewing, **125** (2018), no. 1, pp. 60-68.
2. Malfliet, S.; Opstaele, F.; Clippeleer, J.; Syryn, E.; Goiris, K.; Cooman, L. et al.: Flavour Instability of Pale Lager Beers: Determination of Analytical Markers in Relation to Sensory Ageing, Journal of the Institute of Brewing, **114** (2008), no. 2, pp. 180-192.
3. Cao, L.; Zhou, G.; Guo, P. and Li, Y.: Influence of Pasteurising Intensity on Beer Flavour Stability, Journal of the Institute of Brewing, **117** (2011), no. 4, pp. 587-592.
4. Vanderhaegen, B.; Delvaux, F.; Daenen, L.; Verachtert, H. and Delvaux, F. R.: Aging characteristics of different beer types, Food Chemistry, **103** (2007), no. 2, pp. 404-412.
5. Blanco, C. A.; Nimubona, D. and Caballero, I.: Prediction of the ageing of commercial lager beer during storage based on the degradation of iso-alpha-acids, Journal of the science of food and agriculture, **94** (2014), no. 10, pp. 1988-1993.
6. Cejka, P.; Culik, J.; Horak, T.; Jurkova, M. and Olsovska, J.: Use of chemical indicators of beer aging for ex-post checking of storage conditions and prediction of the sensory stability of beer, Journal of Agricultural and Food Chemistry, **61** (2013), no. 51, pp. 12670-12675.
7. Guido, L. F.; Boivin, P.; Benismail, N.; Goncalves, C. and Barros, A. A.: An early development of the nonenal potential in the malting process, Proceedings of the 30th EBC Congress (2005).
8. Li, H.; Liu, F.; He, X.; Cui, Y. and Hao, J.: A study on kinetics of beer ageing and development of methods for predicting the time to detection of flavour changes in beer, Journal of the Institute of Brewing, **121** (2015), no. 1, pp. 38-43.
9. Guedes de Pinho, P. and Silva Ferreira, A. C.: Role of strecker aldehydes on beer flavour stability, in Bredie, W. L. P. and Petersen, M. A. (Eds.): Flavour science: Recent advances and trends, 1st ed., Elsevier, Amsterdam, Boston, 2006, pp. 529-532.

10. Saison, D.; Schutter, D. P. de; Delvaux, F. and Delvaux, F. R.: Determination of carbonyl compounds in beer by derivatisation and headspace solid-phase microextraction in combination with GC-MS, Journal of Chromatography A (2009), no. 1216, pp. 5061-5068.
11. Lustig, S.: Das Verhalten flüchtiger Aromastoffe bei der Lagerung von Flaschenbier, 1995.
12. Baert, J. J.; Clippeleer, J. de; Hughes, P. S.; Cooman, L. de and Aerts, G.: On the Origin of Free and Bound Staling Aldehydes in Beer, Journal of Agricultural and Food Chemistry, **2012** (2012), no. 60, pp. 11449-11472.
13. https://www.tridge.com/intelligences/beer/export; last accessed on 05.05.2021.
14. Pankoke, I.: Securing beer and beverage quality during long distance distribution process - Using a special sensor system, 35th EBC Congress, Porto, 2015.
15. Paternoster, A.; Jaskula-Goiris, B.; Buyse, J.; Perkisas, T.; Springael, J.; Braet, J. et al.: The relationship between flavour instability, preference and drinkability of fresh and aged beer, J. Inst. Brew. (2020), no. 126, pp. 59-66.
16. Paternoster, A.; Jaskula-Goiris, B.; Causmaecker, B. de; Vanlanduit, S.; Springael, J.; Braet, J. et al.: The interaction effect between vibrations and temperature simulating truck transport on the flavor stability of beer, J. Sci. Food Agric. (2019), no. 99, pp. 2165-2174.
17. Martins, C.; Brandão, T.; Almeida, A. and Rocha, S. M.: Enlarging Knowledge on Lager Beer Volatile Metabolites Using Multidimensional Gas Chromatography, Foods (2020), 9, 1276.
18. Pieczonka, S. A.; Lucio, M.; Rychlik, M. and Schmitt-Kopplin, P.: Decomposing the molecular complexity of brewing, Science of Food (2020), 4:11.
19. Dunkel, A.; Steinhaus, M.; Kotthoff, M.; Nowak, B.; Krautwurst, D.; Schieberle, P. et al.: Nature's chemical signatures in human olfaction: a foodborne perspective for future biotechnology, Angewandte Chemie (International ed. in English), **53** (2014), no. 28, pp. 7124-7143.
20. Meilgaard, M. C.: Flavor chemistry of beer, part II: flavor and thresholds of 239 aroma volatiles, Tech. Q. Master Brew. Assoc. Am. (1975), no. 12, pp. 151-168.
21. Fritsch, H. T. and Schieberle, P.: Identification based on quantitative measurements and aroma recombination of the character impact odorants in a

Bavarian Pilsner-type beer, Journal of Agricultural and Food Chemistry, **53** (2005), no. 19, pp. 7544-7551.

22. Castro, L. F. and Ross, C. F.: Correlation Between Sensory Descriptive Analysis and Volatile Composition of Beer Using Multivariate Analysis: The Effect of the Nonvolatile Matrix on the Sensory Perception and Volatile Fraction Behavior, Journal of the American Society of Brewing Chemists (2018), 76:2, pp. 86-95.

23. Saison, D.; Schutter, D. P. de; Uyttenhove, B.; Delvaux, F. and Delvaux, F. R.: Contribution of staling compounds to the aged flavour of lager beer by studying their flavour thresholds, Food Chemistry, **114** (2009), no. 4, pp. 1206-1215.

24. Bamforth, C. W.: 125th Anniversary Review: The Non-Biological Instability of Beer, J. Inst. Brew. (2011), 117 (4), pp. 488-497.

25. Zufall, C.; Racioppi, G.; Gasparri, M. and Franquiz, J.: Flavour stability and ageing characteristics of light-stable beers, Proceedings of the 30 th EBC Congress (2005), pp. 617-624.

26. Dalgliesh, C. E.: Flavour stability. Proceedings of the European Brewery Convention Congress, Amsterdam, DSW, Dordrecht: The Netherlands (1977), pp. 623-659.

27. Saison, D.; Vanbeneden, N.; Schutter, D. P. de; Daenen, L.; Mertens, T.; Delvaux, F. et al.: Characterisation of the Flavour and the Chemical Composition of Lager Beer after Ageing in Varying Conditions, BrewingScience (2010), Vol. 63, pp. 41-53.

28. Rettberg, N.; Schubert, C.; Dennenlöhr, J.; Thörner, S.; Knoke, L. and Maxminer, J.: Instability of Hop-Derived 2-Methylbutyl Isobutyrate during Aging of Commercial Pasteurized and Unpasteurized Ales, Journal of the American Society of Brewing Chemists (2020).

29. Dennenlöhr, J.; Thörner, S.; Maxminer, J. and Rettberg, N.: Analysis of Selected Staling Aldehydes in Wort and Beer by GC-EI-MS/MS Using HS-SPME with On-Fiber Derivatization, Journal of the American Society of Brewing Chemists.

30. Vanderhaegen, B.; Neven, H.; Verachtert, H. and Derdelinckx, G.: The chemistry of beer aging - a critical review, Food Chemistry (2006), no. 95, pp. 357-381.

31. Wannenmacher, J.; Cotterchio, C.; Schlumberger, M.; Reuber, V.; Gastl, M. and Becker, T.: Technological influence on sensory stability and antioxidant activity

of beers measured by ORAC and FRAP, Journal of the science of food and agriculture (2019).

32. Schutter, D. P. de: The influence of thermal load during wort boiling on the flavour stability of beer, Dissertation University of Leuven, Leuven, Belgium, 2008.

33. Andersen, M. L.; Gundermann, M.; Danielsen, B. P. and Lund, M. N.: Kinetic Models for the Role of Protein Thiols during Oxidation in Beer, Journal of Agricultural and Food Chemistry (2017), no. 65, pp. 10820-10828.

34. Wietstock, P. C.; Kunz, T. and Methner, F.-J.: The Relevance of Oxygen for the Formation of Strecker Aldehydes during Beer Production and Storage, Journal of Agricultural and Food Chemistry (2016).

35. Wietstock, P. C. and Methner, F.-J.: Formation of Aldehydes by direct Oxidative Degradation of Amino Acids via Hydroxyl and Ethoxy Radical Attack in Buffered Model Solutions, BrewingScience (2013), July / August (Vol. 66), pp. 104-113.

36. Bueno, M.; Marrufo-Curtido, A.; Carrascón, V.; Fernández-Zurbano, P.; Escudero, A. and Ferreira, V.: Formation and Accumulation of Acetaldehyde and Strecker Aldehydes during Red Wine Oxidation, Frontiers in Chemistry (2018), Volume 6, Article 20.

37. Noël, S.; Liégeois, C.; Lermusieau, G.; Bodart, E.; Badot, C. and Collin, S.: Release of Deuterated Nonenal during Beer Aging from Labeled Precursors Synthesized in the Boiling Kettle, Journal of Agricultural and Food Chemistry, **47** (1999), no. 10, pp. 4323-4326.

38. Noël, S.; Metais, N.; Bonte, S.; Bodart, E.; Peladan, F.; Dupire, S. et al.: The use of Oxygen 18 in appraising the impact of oxidation process during beer storage, Journal of the Institute of Brewing, **105** (1999), no. 5, pp. 269-274.

39. Hofmann, T. and Schieberle, P.: Formation of Aroma-Active Strecker-Aldehydes by a Direct Oxidative Degradation of Amadori Compounds, Journal of Agricultural and Food Chemistry (2000), no. 48, pp. 4301-4305.

40. Pieczonka, S. A.; Hemmler, D.; Moritz, F.; Lucio, M.; Zarnkow, M.; Jacob, F. et al.: Hidden in its color: A molecular-level analysis of the beer´s Maillard reaction network, Food Chemistry (2021), no. 361, p. 130112.

41. Gibson, B.; Aumala, V.; Heiniö, R.-L.; Mikkelson, A. and Honkapää, K.: Differential evolution of Strecker and non-Strecker aldehydes during aging of pale and dark beers, Journal of Cereal Science (2018), Volume 83, pp. 130-138.

42. Hellwig, M.; Witte, S. and Henle, T.: Free and Protein-Bound Maillard Reaction Products in Beer: Method Development and a Survey of Different Beer Types, Journal of Agricultural and Food Chemistry (2016), no. 64, pp. 7234-7243.
43. Nobis, A.; Kunz, O. S.; Gastl, M.; Hellwig, M.; Henle, T. and Becker, T.: Influence of 3-DG as a Key Precursor Compound on Aging of Lager Beers, Journal of Agricultural and Food Chemistry (2021).
44. Belitz, H.-D.; Grosch, W. and Schieberle, P.: Lehrbuch der Lebensmittelchemie, 6. Auflage, Springer, Heidelberg.
45. Rizzi, G. P.: Formation of strecker aldehydes from polyphenol-derived quinones and alpha-amino acids in a nonenzymic model system, Journal of Agricultural and Food Chemistry, **54** (2006), no. 5, pp. 1893-1897.
46. Gastl, M.; Spieleder, E.; Hermann, M.; Thiele, F.; Burberg, F.; Kogin, A. et al.: The influence of malt quality and malting technology on the flavour stability of beer, Monatszeitschrift für Brauwissenschaft (2006), September / October, pp. 163-175.
47. Gijs, L.; Chevance, F.; Jerkovic, V. and Collin, S.: How Low pH Can Intensify β-Damascenone and Dimethyl Trisulfide Production through Beer Aging, Journal of Agricultural and Food Chemistry, **50** (2002), no. 20, pp. 5612-5616.
48. Yaylayan, V. A.: Recent Advances in the Chemistry of Strecker Degradation and Amadori Rearrangement: Implications to Aroma and Color Formation, Food Sci. Technol. Res. (2003), 9 (1), pp. 1-6.
49. Lermusieau, G.; Noël, S. and Collin, S.: Nonoxidative Mechanism for Development of trans-2-Nonenal in Beer, Journal of the American Society of Brewing Chemists (1999), 57(1), pp. 29-33.
50. Hofmann, T.; Münch, P. and Schieberle, P.: Quantitative Model Studies on the Formation of Aroma-Active Aldehydes and Acids by Strecker-Type Reactions, Journal of Agricultural and Food Chemistry (2000), no. 48, pp. 434-440.
51. Evans, D. J., Schmedding, D. J. M.; Bruijnje, A.; Heideman, T.; King, B. M. and Groesbeek, N. M.: Flavour Impact of Aged Beers, Journal of the Institute of Brewing (1999), Volume 105 No. 5, pp. 301-307.
52. Barker, R. L.; Gracey, D. E. F.; Irwin, A. J.; Pipasts, P. and Leiska, E.: Liberation of staling aldehydes during storage of beer, J. Inst. Brew. (1983), no. 89, pp. 411-415.
53. Dufour, J.-P.; Leus, M.; Baxter, A. J. and Hayman, A. R.: Characterization of the Reaction of Bisulfite with Unsaturated Aldehydes in a Beer Model System Using

Nuclear Magnetic Resonance Spectroscopy, Journal of the American Society of Brewing Chemists (1999), 57(4), pp. 138-144.

54. Suda, T.; Yasuda, Y.; Imai, T. and Ogawa, Y.: Mechanisms for the development of Strecker aldehydes during beer aging, Proceedings of the 31 st EBC Congress (2007).

55. Guido, L. F.: Sulfites in beer: Reviewing regulation, analysis and role, Scientia Agricola, **73** (2016), no. 2, pp. 189-197.

56. Schutter, D. P. de; Saison, D.; Delvaux, F.; Derdelinckx, G.; Rock, J.-M.; Neven, H. et al.: Release and evaporation of volatiles during boiling of unhopped wort, Journal of Agricultural and Food Chemistry, **56** (2008), no. 13, pp. 5172-5180.

57. Baert, J. J.; Clippeleer, J. de; Jaskula-Goiris, B.; van Opstaele, F.; Rouck, G. de; Aerts, G. et al.: Further Elucidation of Beer Flavor Instability: The Potential Role of Cysteine-Bound Aldehydes, Journal of the American Society of Brewing Chemists (2015).

58. Baert, J. J.; Clippeleer, J. de and Aerts, G.: Exploring the Binding Behavior of Beer Staling Aldehydes in Model Systems, Journal of the American Society of Brewing Chemists (2015), 73(1), 100-108.

59. Liu, C.; Li, Q.; Niu, C.; Zheng, F. and Zhao, Y.: Simultaneous determination of diethylacetal and acetaldehyde during beer fermentation and storage process, Journal of the science of food and agriculture (2018).

60. Kaneda, H.; Kimura, T.; Kano, Y.; Koshino, S.; Osawa, T. and Kawakishi, S.: Role of Fermentation Conditions on Flavor Stability of Beer, Journal of Fermentation and Bioengineering (1991), Vol. 72, No. 1, pp. 26-30.

61. Kaneda, H.; Takashio, M.; Osawa, T.; Kawakishi, S.; Koshino, S. and Tamaki, T.: Analysis of Aldehyde-bisulfites in Beer by HPLC-Fluorescence Detection with Post-column Derivatization, Journal of Food Science, **61** (1996), no. 1, pp. 105-108.

62. Keller, C.; Wedzicha, B. L.; Leong, L. P. and Berger, J.: Effect of glyceraldehyde on the kinetics of Maillard browning and inhibition by sulphite species, Food Chemistry (1999), no. 66, pp. 495-501.

63. Andersen, M. L.; Outtrup, H. and Skibsted, L. H.: Potential Antioxidants in Beer Assessed by ESR Spin Trapping, Journal of Agricultural and Food Chemistry (2000), no. 48, pp. 3106-3111.

64. Bueno, M.; Carrascón, V. and Ferreira, V.: Release and Formation of Oxidation-Related Aldehydes during Wine Oxidation, Journal of Agricultural and Food Chemistry, **64** (2016), no. 3, pp. 608-617.
65. Chapon, L.; Chapon, S. and Djeuga, N.: Free Sulfite in Beers - Kinetic Studies, American Society of Brewing Chemists, Inc. (1982), Vol. 40: No. 1.
66. Arapitsas, P.; Guella, G. and Mattivi, F.: The impact of SO2 on wine flavanols and indoles in relation to wine style and age, Scientific Reports (2018), 8:858.
67. Ma, L.; Watrelot, A. A.; Addison, B. and Waterhouse, A. L.: Condensed Tannin Reacts with SO2 during Wine Aging, Yielding Flavan-3-ol Sulfonates, Journal of Agricultural and Food Chemistry (2018), no. 66, pp. 9259-9268.
68. Hill, A. and Stewart, G. G.: Free Amino Nitrogen in Brewing, Fermentation (2019), 5, 22, pp. 1-11.
69. Filipowska, W.; Jaskula-Goiris, B.; Ditrych, M.; Bustillo Trueba, P.; Rouck, G. de; Aerts, G. et al.: On the contribution of malt quality and the malting process to the formation of beer staling aldehydes: a review, Institute of Brewing & Distilling (2021).
70. Zameo, S.; Vauzeilles, B. and Beau, J.-M.: Direct Composition Analysis of a Dynamic Library of Imines in an Aqueous Medium, Eur. J. Org. Chem. (2006), pp. 5441-5444.
71. Nikolov, P. Y. and Yaylayan, V. A.: Reversible and covalent binding of 5-(hydroxymethyl)-2-furaldehyde (HMF) with lysine and selected amino acids, Journal of Agricultural and Food Chemistry, **59** (2011), no. 11, pp. 6099-6107.
72. Bustillo Trueba, P.; Clippeleer, J. de; van der Eycken, E.; Guevara Romero, J. S.; Rouck, G. de; Aerts, G. et al.: Influence of pH on the Stability of 2-Substituted 1,3-Thiazolidine-4-Carboxylic Acids in Model Solutions, Journal of the American Society of Brewing Chemists (2018), 76(4), pp. 272-280.
73. Bustillo Trueba, P.; Jaskula-Goiris, B.; Clippeleer, J. de; Goiris, K.; Praet, T.; Sharma, U. K. et al.: Validation of an ultra-high performance liquid chromatography-mass spectrometry method for the quantification of cysteinylated aldehydes and application to malt and beer samples, Journal of Chromatography A (2019), 1064 (2019), pp. 460-467.
74. Bustillo Trueba, P.; Jaskula-Goiris, B.; Ditrych, M.; Filipowska, W.; Brabanter, J. de; Rouck, G. de et al.: Monitoring the evolution of free and cysteinylated aldehydes from malt to fresh and forced aged beer, Food Research International (2021), no. 140.

75. Narziss, L.: Centenary review: technological factors of flavour stability, J. Inst. Brew. (1986), Vol. 92, pp. 346-353.
76. Drost, B. W.; van den Berg, R.; Freijee, F. J. M.; van der Velde, E. G. and Hollemans, M.: Flavor Stability, Journal of American Society of Brewing Chemists (1990), no. 48, pp. 124-131.
77. Dugulin, A. C.; Clegg, S. C.; Rouck, G. de and Cook, D. J.: Overcoming technical barriers to brewing with green (non-kilned) malt: a feasibility study, Journal of the Institute of Brewing (2020).
78. Staško, A.; Brezová, V.; Biskupi, S.; Šmogroviová, D. and Selecký, R.: Stability comparison of lager with dark and nonalcoholic beers using epr spin trapping technique, Monatszeitschrift für Brauwissenschaft (2005), May/June 2005, pp. 35-44.
79. Eichhorn, P.: Untersuchungen zur Geschmacksstabilität des Bieres, 1991.
80. Soares da Costa, M.; Goncalves, C.; Ferreira, A.; Ibsen, C.; Guedes de Pinho, P. and Silva Ferreira, A. C.: Further insights into the role of methional and phenylacetaldehyde in lager beer flavor stability, Journal of Agricultural and Food Chemistry, **52** (2004), no. 26, pp. 7911-7917.
81. Baert, J.: Unravelling the Role of Free and Bound-state Aldehydes in Beer Flavour Instablity, Dissertation KU Leuven, 2015.
82. Eger, C.; Habich, N.; Akdokan, N.; Goßling, U. and Bellmer, H.-G.: Profiling of beers during ageing at different temperatures, Proceedings of the 30th EBC Congress (2005).
83. Da Costa, N. C. and Eri, S.: Chemistry and Technology of Flavors and Fragrances, Blackwell Publishing Ltd, 2005.
84. Richter, T. M.; Eyres, G. T.; Silcock, P. and Bremer, P. J.: Comparison of four extraction methods for analysis of volatile hop-derived aroma compounds in beer, Journal of separation science, **40** (2017), no. 22, pp. 4366-4376.
85. Horák, T.; Čulík, J.; Kellner, V.; Jurková, M.; Čejka, P.; Hašková, D. et al.: Analysis of Selected Esters in Beer: Comparison of SolidPhase Microextraction and Stir Bar Sorptive Extraction, Journal of the Institute of Brewing, **116** (2010), no. 1, pp. 81-85.
86. Horák, T.; Čulík, J.; Kellner, V.; Čejka, P.; Hašková, D.; Jurková, M. et al.: Determination of Selected Beer Flavours: Comparison of a Stir Bar Sorptive

Extraction and a Steam Distillation Procedure, Journal of the Institute of Brewing (2011), 117(4), pp. 617-621.

87. Thompson-Witrick, K. A.; Rouseff, R. L.; Cadawallader, K. R.; Duncan, S. E.; Eigel, W. N.; Tanko, J. M. et al.: Comparison of two extraction techniques, solid-phase microextraction versus continuous liquid-liquid extraction/solvent-assisted flavor evaporation, for the analysis of flavor compounds in gueuze lambic beer, Journal of food science, **80** (2015), no. 3, C571-6.

88. Saison, D.; Schutter, D. P. de; Delvaux, F. and Delvaux, F. R.: Optimisation of a complete method for the analysis of volatiles involved in the flavour stability of beer by solid-phase microextraction in combination with gas chromatography and mass spectrometry, Journal of chromatography. A, **1190** (2008), 1-2, pp. 342-349.

89. Nobis, A.; Roehrig, A.; Hellwig, M.; Henle, T.; Becker, T. and Gastl, M.: Formation of 3-deoxyglucosone in the malting process, Food Chemistry (2019), no. 290, pp. 187-195.

90. Brotzel, F. and Mayr, H.: Nucleophilicities of amino acids and peptides, Org. Biomol. Chem. (2007), no. 5, pp. 3814-3820.

91. Minegishi, S. and Mayr, H.: How Constant Are Ritchie's "Constant Selectivity Relationships"? A General Reactivity Scale for n-, ð-, and ó-Nucleophiles, J. AM. CHEM. SOC. (2003), no. 125, pp. 286-295.

92. Baert, J. J.; Clippeleer, J. de; Bustillo Trueba, P.; Jaskula-Goiris, B.; Rouck, G. de; Aerts, G. et al.: Exploring Aldehyde Release in Beer by 4-Vinylpyridine and the Effect of Cysteine Addition on the Beer's Pool of Bound Aldehydes, Journal of the American Society of Brewing Chemists (2018), 76:4, pp. 257-271.

93. French, T. C. and Bruice, T. C.: Rates and Equilibrium Constants of Imine Formation with Pyridine-4-aldehyde and various amino acids, Biochemical and Biophysical Research Communications (1964), Vol. 15, No. 5, pp. 403-408.

94. LoPachin, R. M. and Gavin, T.: Molecular Mechanisms of Aldehyde Toxicity: A Chemical Perspective, Chemical Research in Toxicology (2014), no. 27, pp. 1081-1091.

95. Azevedo, L. C. de; Reis, M. M.; Motta, L. F.; da Rocha, G. O.; Silva, L. A. and Andrade, J. B. de: Evaluation of the formation and stability of hydroxyalkylsulfonic acids in wines, Journal of Agricultural and Food Chemistry, **55** (2007), no. 21, pp. 8670-8680.

96. Bueno, M.; Zapata, J. and Ferreira, V.: Simultaneous determination of free and bonded forms of odor-active carbonyls in wine using a headspace solid phase microextraction strategy, Journal of chromatography. A, **1369** (2014), pp. 33-42.

7 Appendix

7.1 Non-peer reviewed publications

- Lehnhardt, F., Gastl, M., and Becker, T.: Aussagekraft der forcierten Alterung. Brauwelt 159 (21/22), (2019), 607-610.
- Lehnhardt, F., Gastl, M., and Becker, T.: Vokabular der Bieralterung. Brauindustrie 8 (2020): 10-13.
- Nobis, A. and Lehnhardt, F.: Es braut sich was zusammen. Nachrichten aus der Chemie 68(9): 80-83

7.2 Conference Contributions

7.2.1 Oral

- Lehnhardt, F., M. Gastl, and Becker, T.: Mechanismen der Bieralterung - das verborgene Potential. 51. Technologisches Seminar, Freising, Germany, 2018-02-21.
- Lehnhardt, F., Gastl, M., and Becker, T.: Beurteilung der Alterungsstabilität des Bieres - Inwieweit ist die Wahl der Analytik entscheidend? 52. Technologisches Seminar Weihenstephan, Freising, Germany, 2019-02-28.
- Lehnhardt, F., Gastl, M., and Becker, T.: Assessing sensory stability of beer-Impact of analytic methods. 37th EBC Congress, Antwerp, Belgium, 2019-06-05.

7.2.2 Poster

- Lehnhardt, F., Gastl, M., Becker, T.: What we can learn about beer aging by comparing analytical methods. 2019 ASBC Meeting, New Orleans, USA, Louisiana, 2019-06-24.