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Influence of the precursor potential of 3-deoxyglucosone from the Maillard reaction on the flavor instability of pale lager beers

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Preface

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The following six peer-reviewed publications are part of the cumulative thesis:

I. Nobis, A., Röhrig, A., Hellwig, M., Henle, T., Becker, T., and Gastl, M.: Formation of 3-deoxyglucosone in the malting process. Food Chemistry. 290. (2019): 187-195

II. Nobis, A., Wendl, S., Becker, T., and Gastl, M.: Formation and degradation of 3-deoxyglucosone as a key intermediate for ageing indicators during the wort boiling process. Journal of the Institute of Brewing. 127. (2021): 358-366

III. Nobis, A.[1], Lehnhardt, F.[1], Gebauer, M., Becker, T., and Gastl, M.: Influence of proteolytic malt modification on the formation of aging potential during wort boiling. Foods. 10. (2021): 2320

IV. 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 Agriculture and Food Chemistry. 69. (2021): 3732-3740

V. Lehnhardt, F. [1], Nobis, A. [1], Skornia, A., Becker, T., and Gastl, M.: Influence of proteolytic malt modification on the aging behavior of pale lager beers: a comprehensive evaluation of de novo formation and release of bound aldehydes. Foods. 10. (2021): 2432

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[1] Both authors contributed equally.

Abbreviations

2-MB	2-methylbutanal
2-MP	2-methylpropanal
3,4-DGE	3,4-dideoxyglucosone-3-ene
3-DG	3-deoxyglucosone
3-DGal	3-deoxygalactosone
3-MB	3-methylbutanal
AGEs	advanced glycosylation end products
CEL	N^{ε}-carboxyethyllysine
CML	N^{ε}-carboxymethyllysine
DLG	„Deutsche Landwirtschafts-Gesellschaft“
GC	gas chromatography
G	germination
Glc	glucose
GO	glyoxal
HMF	5-hydroxymethylfurfural
HPLC	high performance liqiud chromatography
FL	N-ε-fructosyllysine
Fur	furfural
K	kilning
Meth	methional
MGO	methylglyoxal
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
o-PD	ortho-phenylene diamine
PA	phenyl acetaldehyde
S	steeping
SPME	solid phase microextraction
t2N	(E)-2-nonenal

Contents

Summary

During storage, the composition of flavor-active volatiles of pale lager beer undergoes a dynamic change leading to sensory deterioration and decreased product quality. This so called flavor instability is mainly influenced by the rise of flavor-active aldehydes. They originate from various chemical reactions such as Strecker degradation, Maillard reaction, or lipid oxidation. Flavor instability is currently mainly evaluated by the increase of these aging aldehydes (aging indicators) after forced aging. However, the precursor potential of the important reactions is often neglected. Especially the Maillard reaction provides many precursors. Important and reactive substances are the dicarbonyl compounds. Within this class, 3-deoxyglucosone (3-DG) is the major compound occurring in beer in rather high concentrations up to 42 mg/L. The dicarbonyl can be degraded during beer aging and, e.g., form Strecker aldehydes which cause sensory deterioration. Therefore, the hypothesis in this thesis was stated as follows: The concentration of 3-DG in fresh pale lager beer is an analytical indicator for their flavor instability.

High contents of the dicarbonyl 3-DG (0.8–19.4 mg/100 g d. m.) are mainly formed during kilning within the malting process. The formation already leads to calculable concentrations of 1–23 mg/L in beer. An increased proteolytic malt modification level elevates the formation of low molecular weight carbohydrates and amino acids (reactants) during germination which further react to 3-DG during kilning. Therefore, the malting process plays already an important role in the 3-DG and 3-DG precursor formation. Beside the malting step, wort boiling was found as an additional key process step for 3-DG formation. There, rather 50% of the 3-DG content in final wort is formed. It could be shown that an increased malt modification level enhances the concentration of amino acids. This result in a more intensive formation of 3-DG during wort boiling. Higher contents of Strecker aldehydes are formed simultaneously, finally leading to an increased aging potential for beer from final wort after boiling by a higher malt modification level. Spiking-experiments of 3-DG in fresh beer revealed that an increased 3-DG concentration enhances Strecker degradation after prolonged storage (> 6 months of natural aging) and thus, influences the flavor instability. This finding could be confirmed in technologically varied 3-DG contents in fresh beer due to the malt modification level. An increased malt modification level leads to elevated 3-DG

concentrations in fresh beer. Further, the varied 3-DG levels in fresh beer revealed an analytically increased formation of Strecker aldehydes and simultaneously increased sensory deterioration due to higher 3-DG initial contents. It could be quantitatively shown, that a *de novo* formation of Strecker aldehydes becomes relevant after 1–4 months of natural aging depending on the malt modification level. Comparing the critical precursors of Strecker degradation, the amino acids concentration is strongly elevated compared to the dicarbonyl concentration. This finding indicates that the 3-DG concentration in fresh beer is the critical precursor for Strecker degradation. In conclusion, the hypothesis could be confirmed that a technologically increased 3-DG content in fresh beer negatively influences the flavor stability, observable in an analytical (chromatographic Strecker aldehydes determination) and in a sensory (sensory quality determination) way. Therefore, the dicarbonyl compound is suitable as an early analytical precursor indicator for pale lager beer.

Zusammenfassung

Während der Lagerung heller Vollbiere unterliegt deren Aroma dynamischen Veränderungen, die die Produktqualität aufgrund negativer sensorischer Veränderungen stark verringern. Die beschriebene Alterungsstabilität wird hauptsächlich durch die Bildung aromaaktiver Aldehyde beeinflusst. Diese entstehen aus vielfältigen chemischen Reaktionen wie dem Strecker-Abbau, der Maillard-Reaktion oder der Lipidoxidation. Bewertet wird die Alterung aktuell mit dem Anstieg wichtiger Aldehyde (Alterungsindikatoren) nach forcierter Alterung. Bisher weitgehend unbeachtet blieb das Precursorpotential. Insbesondere die Maillard-Reaktion liefert ein breites Spektrum an Precursoren. Eine wichtige und reaktive Substanzklasse sind hier die Dicarbonylverbindungen, die zum Beispiel im Strecker-Abbau eine Schlüsselrolle einnehmen. Hierbei ist 3-Desoxyglucoson (3-DG) die dominierende Verbindung, die in Bier in vergleichsweise hohen Konzentrationen bis zu 42 mg/l vorkommt und in seinem Abbau während der Lagerung Strecker-Aldehyde bilden kann. Die Hypothese der Dissertation lautet somit: Der Gehalt an 3-DG in frischem Bier ist ein analytischer Indikator für die Alterungsstabilität heller Vollbiere.

Bereits der Mälzungsprozess spielt eine entscheidende Rolle in der Bildung von 3-DG. Im Darrprozess werden hohe Mengen an 3-DG (0,8–19,4 mg/100 gTrs.) gebildet. Dies führt zu einer rechnerischen Konzentration in Bier von 1–23 mg/l 3-DG. Eine erhöhte proteolytische Malzlösung verstärkt die Bildung von niedermolekularen Zuckern (Glucose) und Aminosäuren, die während des Darrens dann verstärkt zu 3-DG reagieren. Neben der Mälzung wurde die Würzekochung als entscheidender 3-DG-Bildungsschritt in der Bierherstellung identifiziert. Hier bilden sich ca. 50 % des gesamt vorliegenden Gehaltes an 3-DG. Auch hier konnte gezeigt werden, dass eine erhöhte proteolytische Malzlösung den Gehalt an Aminosäuren zu Beginn der Kochung erhöht und sich folgend auch verstärkt 3-DG während des Würzekochens bildet. Gleichzeitig werden höhere Gehalte an freien Streckeraldehyden gebildet, was bereits nach der Kochung zu einem erhöhten Alterungspotential in der Ausschlagwürze führt. In Spiking-Experimenten konnte bestätigend nachgewiesen werden, dass ein erhöhter 3-DG-Gehalt in seinem Abbau nach 6 Monaten Lagerung verstärkt Streckeraldehyde bildet und somit die Alterungsstabilität stark beeinflusst. Dieses Verhalten konnte auch durch eine technologisch induzierte proteolytische Malzmodifikation, die zu

variierenden 3-DG-Gehalt führte, in frischen Bier bestätigt werden. Eine erhöhte Malzlösung führt zu erhöhten Gehalten an 3-DG in frischen Bier, die in der Alterung mit einer erhöhten Bildung an Streckeraldehyden und verstärkter sensorischer Alterung einhergehen. Quantitativ konnte belegt werden, dass eine Neubildung der Streckeraldehyde ab Monat 1–4 gegenüber einer Freisetzung selbiger Aldehyde bevorteilt ist. Hierbei sind die Aminosäuren im Precursorvergleich stark im Überschuss vorhanden, weshalb die Dicarbonylverbindungen (3-DG) im Strecker-Abbau heller Vollbiere die entscheidende Rolle spielen. Somit kann die Hypothese bestätigt werden, dass ein erhöhter Gehalt von 3-DG aufgrund von technologischer Variation, gemessen sowohl analytisch (chromatographische Bestimmung von Streckeraldehyden) als auch sensorisch (sensorische Qualitätstests), einen negativen Einfluss auf die Alterungstabilität von Bier hat. Die Verbindung kann somit als analytischer Precursorindikator für helle Vollbiere verwendet werden.

1. Introduction

In 2017 a study revealed beer as the most favoured alcoholic beverage in the European Union (1). This fact is taken to be due to the brewers commitment to maintaining highest product quality. For consumer's satisfaction, the key task is to provide a constant flavor during transport and storage of beer until consumers consumption (2). Unfortunately, especially during these two steps the volatilome of beer changes due to occurring vibrations, increased storage temperatures, or exposure to light (3). Recently, this problem raised because of increasing distribution distances in a globalized world. Therefore, the key challenge brewers are facing today is to minimize the changes in the volatilome of beer with time – the so called flavor instability.

This thesis investigates the problem of flavor instability of pale lager beer, which is the most favoured beer type worldwide. Facing the problem, the thesis focusses on the precursor potential of the beer aging relevant Maillard reaction (4). Minimizing the precursor potential for chemical reactions is likely to be a good tool to decrease flavor instability. Within the Maillard reaction, dicarbonyl compounds such as 3-DG are promising aging-relevant compounds due to their precursor potential to form sensory active beer aging indicators such as Strecker aldehydes (5). The next paragraphs introduce in more detail the flavor instability, its evaluation in the brewing industry, the potential of 3-DG towards flavor instability of beer, the chemistry of the dicarbonyl compounds, and the potential from the malt and beer production for Maillard precursor compounds.

1.1 Flavor instability of beer

The flavor of beer is a complex mixture of various flavor-active compounds – the beer volatilome (4). This complex mixture undergoes a dynamic change starting from fresh beer directly after bottling. This change leads to flavor instability and causes beer aging (6). The term "flavour instability" was established in the previous literature and can be used concurrently to the term "flavour stability" (7-10). People mainly favoured fresh beer regarding consumers acceptancy studies (2). This phenomenon is evident, because the flavor changes during beer aging decrease the sensory beer quality, the so called sensory deterioration (10). Zufall et al. (2005) qualitatively investigated the

sensory changes of a pale lager beer during forced aging (11). They found that sensory changes comprise of decreasing and increasing aroma impressions. The decreasing sensory attributes are the *bitterness* and *sulfury* impressions. The most important increasing effects are *bready*, *sweet*, *caramel* and *cardboard* attributes. At an advanced aging stage, the impression *sherry* rises and finally dominates the aged beer (11).

The period of aging stages and the intensity of sensory impressions depend on raw materials (12), technological variations in beer production (12), oxygen uptake (13), exposure to light (14), and storage conditions. These influences cause variations in forming aging-relevant volatiles in beer, the so-called “aging indicators”. Table 1 summarizes important sensory-active aging indicators, their aroma impressions and the formation reaction (10, 15) according to the main sensory impression occurring during beer aging (11).

Table 1 Aging indicators for the main sensory categories occurring during beer aging (10, 15)

Sensory category	Substance	Flavor description	Origin
Bready/malty	2-Methylpropanal (2MP)	grainy, varnish, fruity	Strecker degradation
	2-Methylbutanal (2MB)	almond, apple-like, malty	Strecker degradation
	3-Methylbutanal (3MB)	malty, chocolate, cherry, almond	Strecker degradation
Sweet/fruity	Acetaldehyde	green apple, fruity	Oxidation of ethanol
	Phenyl acetaldehyde (PA)	hyacinth, flowery, roses	Strecker degradation
	2-Acetylpyrazine	sweet, caramel	Pyrazine formation
Caramel	Furfural (Fur)	caramel, bready	Maillard reaction, caramelization
	5-Hydroxy-methylfurfural (HMF)	bready, caramel	Maillard reaction, caramelization
	Acetylfuran	nutty, almond, burnt	Maillard reaction, caramelization
Cardboard	(E)-2-Nonenal (t2N)	cardboard, papery, cucumber	Lipid oxidation
	(Z,Z)-2,4-Decadienal	deep-fried, papery	Lipid oxidation

The aging indicators are formed by several aging-relevant reactions. According to Table 1, the important reactions are Strecker degradation, the Maillard reaction, caramelization, and the lipid oxidation. All reaction types take place simultaneously during aging and depend on level of reactants, pH-value, storage conditions and oxygen level in beer (6).

1.2 The evaluation of flavor instability

The occurring aging indicators through the different aging-relevant reactions are used to determine beer aging. Currently, the flavor instability is evaluated by using forcing methods and comparing of the fresh and the forced aged sample. According to the forced method of *Eichhorn and Lustig*, the beer should be agitated for 24 h and incubated for 4 d at 40 °C. This treatment should simulate natural beer aging from 3–5 months. The fresh and the forced aged samples are analyzed by sensory tests as well as gas chromatographic (GC) methods analysing the aging indicators (16). Regarding the sensory tests, mainly the DLG 5-point-Scheme and the Eichhorn-Test are used. The DLG-Scheme evaluates general beer quality issues where aging as criterion is included. Here, the purity of *smell*, the purity of *taste*, *palate fullness*, *freshness*, and *bitterness* are evaluated ranging from 0 (inadequate) to 5 (quality expectations totally reached). The Eichhorn-Test focusses on beer aging. Here, *smell*, *taste* and *bitterness* are rated at a scale from 1 (none-aged) to 4 (critical aged). Furthermore, the hedonic acceptancy is evaluated on a scale from 0% (no acceptancy) to 100% (full acceptancy). Regarding the quantification of aging indicators, various GC methods were applied with various sample preparation techniques such as steam distillation (SD), solid phase extraction (SPE), or derivatization reactions (17). Generally, it can be summarized that currently the flavor instability is evaluated by an increase of sensory attributes and aging indicators by using forcing methods (4).

The forced aging methods are considered as critical. They are time-consuming, expensive, and the incubation at 40 °C may cause differences in the reactivity of precursors. These facts represent the lack of knowledge. However, there is no aging prediction method available based on fresh beer so far, which simplifies the critical problem of flavor instability evaluation. The precursor compounds of the aging-relevant reaction can be seen as promising indicators, because they are already present in

fresh beer and result up-stream from the beer and malt production. In case of being representative, they can replace the forcing methods.

1.3 3-DG as a key aging precursor compound from the Maillard reaction

The Maillard reaction provides a high number of precursor compounds (10, 18). In its advanced stage reactive dicarbonyl compounds are formed (19). These compounds are particularly promising as early precursor-based flavor instability evaluation, because they are able to further react in the aging-relevant reactions Strecker degradation and Maillard reaction to aging indicators (5). Within this substance class, 3-DG is the predominant compound in beer and its intermediate stages (20-22). Furthermore, 3-DG was found in fresh pale lager beers in concentrations up to 42 mg/L (22, 23). This concentration range can be regarded as high and provides a broad reaction potential, if 3-DG is significantly degraded during beer aging. Considering these aspects, the question arises whether 3-DG is usable to influence or predict the beer flavor instability.

3-DG has a qualitative and quantitative precursor potential during beer aging. Qualitatively, the compound can react to Strecker aldehydes (5) or HMF (24), which are common aging indicators (4). Regarding its quantitative potential, Bravo et al. (2008) monitored several dicarbonyls during beer production and beer aging at 28 °C (20). The authors observed a degradation of 3-DG of about 8.1 mg/L during an aging period of 105 d (~ 3.5 months). There, a degradation of dicarbonyl compounds was firstly observed during beer aging (20, 25). Based on the absolute degradation rate (20), Table 2 shows the quantitative potential of 3-DG during beer aging at Strecker degradation.

Table 2 Calculated max. concentration increase of Strecker aldehydes and their relative conversion rates to reach their aroma thresholds by 3-DG degradation based on study of Bravo et al. (2008) (20)

Strecker aldehyde	Calculated max. increase (20)	Aroma thresholds (15)/(26)	3-DG conversion rate to reach thresholds
	mg/L	mg/L	%
2-Methylpropanal	3.6	0.086/ 0.065	1.8–2.4
2-Methylbutanal	4.3	0.045/ 0.035	0.8–1.0
3-Methylbutanal	4.3	0.056/ 0.046	1.1–1.7
Phenyl acetaldehyde	6.0	0.105/ 0.100	1.7–1.8

The maximum concentration increase of each listed Strecker aldehydes was calculated by a 100% conversion rate of 3-DG within Strecker degradation and the corresponding amino acid according to the absolute degradation of 3-DG of 8.1 mg/L at the study of Bravo et al. (2008) (20). Therefore, e. g. 2-MP could be formed up to 3.6 mg/L by 3-DG degradation. Comparing the calculated maximum increase of the Strecker aldehydes and their aroma thresholds in beer (15, 26), it could be stated that only minor percentage conversion rates are mandatory for the aldehyde to surpass their thresholds. Surpassing the thresholds means a decreased sensory beer quality by beer aging. This calculation indicates the high importance of 3-DG as a precursor from the Maillard reaction on flavor instability of beer.

1.4 Chemical survey of 3-DG: formation and degradation

In the previous section, 3-DG was described as an important precursor for the influence of the Strecker degradation on the beer flavor instability. Further, the chemistry of 3-DG needs to be considered. Figure 1 summarizes formation and degradation pathways of 3-DG in beer.

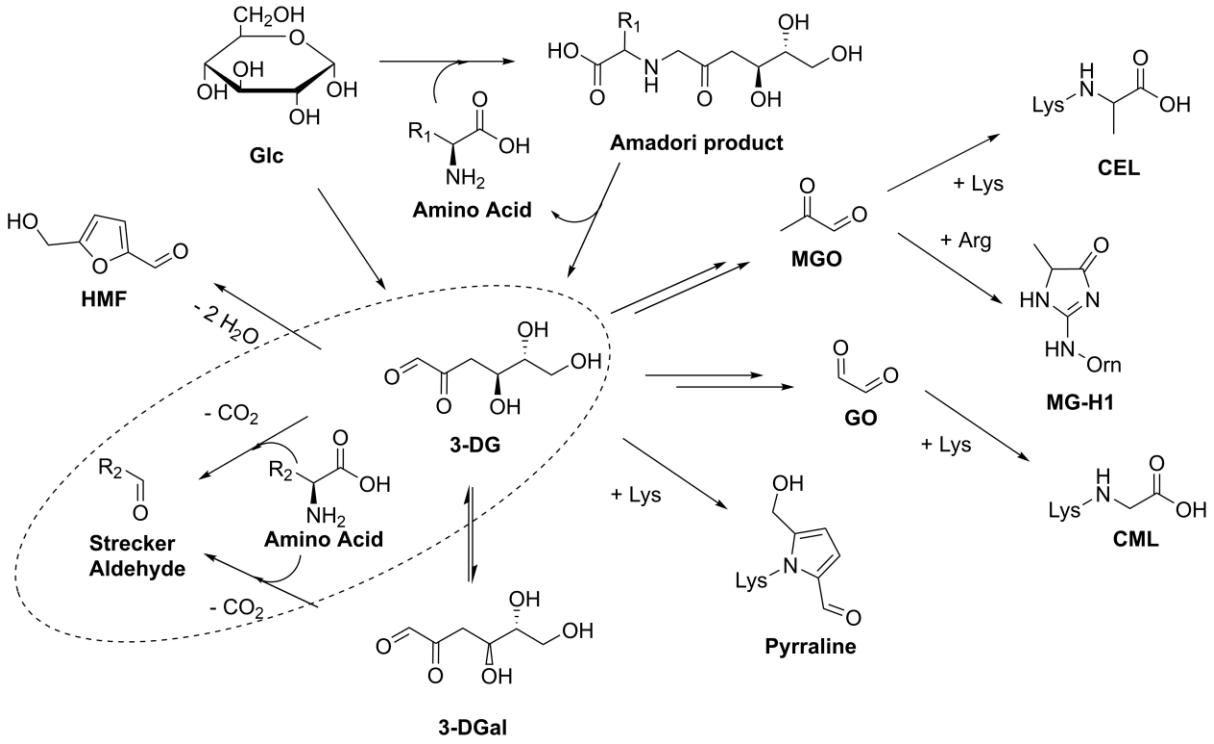

Figure 1 Overview about beer-related formation and degradation pathways of 3-DG; dashed ellipse marks formation of the aging indicators Strecker aldehydes

Regarding the formation of 3-DG, the caramelization and the Maillard reaction are the most important reactions (27-30). Considering caramelization, low molecular weight carbohydrates react in dehydration and enolization reactions and form dicarbonyls such as 3-DG (18). Reactants can be glucose (Glc) or fructose as well as oligosaccharides (31, 32). Exemplarily, Hollnagel et al. (2000) postulated a “peeling-off”- mechanism of oligosaccharides resulting in dicarbonyl compounds, where low molecular carbohydrates showed a higher reactivity onto 3-DG formation (31).

The Maillard reaction is another formation pathway for 3-DG. In the early stage the carbonyl group of carbohydrates reacts with the amino group of amino acids and forms the Amadori product by the Amadori rearrangement reaction (18). Exemplarily, glucose forms with the ε-amino group of lysine the Amadori product ε-fructosyllysine (FL) (33). The Amadori products are stable intermediates and their formation characterizes the early stage of the Maillard reaction (19). Nevertheless, the Amadori products can be further degraded by release of the amino acid and form α-dicarbonyl compounds such as 3-DG (34). This reduction of the Amadori products is characterized as the advanced stage of the Maillard reaction (18, 19, 35). Several studies confirmed the formation of 3-DG from Amadori products in watery and dry model systems in single substance

incubations (36-38). The 3-DG formation is catalyzed by thermal treatment and acidic pH-values (32).

3-DG is degraded in various pathways. Thus, the dicarbonyl can undergo dehydration or condensation reactions as well as further react with amino acids. An important reaction for flavor formation in food is the Strecker degradation (39). Here, the dicarbonyls react with amino acids forming an α-imino carbonyl which undergoes decarboxylation and results in the Strecker aldehyde and an 2-amino ketone (40). As already introduced, Strecker aldehydes are sensory active compounds, which arise during beer storage and cause flavor instability (10, 41). Beer aging-relevant amino acids and their corresponding Strecker aldehydes are valine and 2MP, isoleucine and 2MB, leucine and 3MB, phenylalanine and PA as well as methionine and Meth (4, 42). Besides the Strecker aldehydes, the dicarbonyl backbone results in a 2-amino ketone. These substances can further condense forming a pyrazine sub-structure (6). Pyrazines also contribute to the aroma of beer. For example, Giis et al (2002) considered 2-methoxypyrazine (roasted) and 2-acetylpyrazine (caramel, sweet) as increasing compounds during beer aging (43). Respectively, 3-DG was found in the literature as reactive dicarbonyl for pyrazine formation (44, 45).

Another possible reaction of 3-DG is the final stage of the Maillard reaction (19). Here, dicarbonyls react further with nucleophilic amino acids forming advanced glycosylation end-products (AGEs) (46). The AGEs are considered stable products in the final stage of the Maillard reaction (18). Especially the ε-amino group of lysine or the guanidino group of arginine are likely nucleophilic reaction partners for the electrophilic dicarbonyl structure. For example, 3-DG can react with lysine forming pyrraline (47). Furthermore, lysine or arginine can react with fragmentation products of 3-DG. The dicarbonyl can undergo cleavage reactions and form smaller vicinal diketones such as methylglyoxal (MGO), glyoxal (GO), or diacetyl (48, 49). The resulting short chain dicarbonyls are more reactive and also able to form AGEs. Lysine derives N^{ε}-carboxyethyllysine (CEL) by reaction with MGO (50) and N^{ε}-carboxymethyllysine (CML) by reaction with GO (51). Additionally, arginine can be modified with MGO forming a methylglyoxal-derived hydroimidazolone 1 (MG-H1) (52). Other occuring AGEs are formyline and maltosine. These are derived from 3-deoxypentosone (formyline) (53) and from 1-deoxyglucosone (maltosine) (54). However, the AGEs as final products of the Maillard

reaction are only described in fresh beer focussing on different beer types (55). Thus, their behaviour during beer aging is still to be investigated.

3-DG can also be degraded by dehydration reactions. After elimination of one water molecule 3,4-dideoxyglucoson-3-ene (3,4-DGE) is formed (22). Based on 3,4-DGE, two main reaction pathways are possible. First, the dicarbonyl can be further dehydrated leading to 5-hydroxymethylfurfural (HMF) (24). HMF is a well-known aging indicator in beer and characterizes intensive thermal treatment (4). Second, 3,4-DGE can again undergo addition of water and form 3-deoxygalactosone (3-DGal). This so called interconversion reaction is reversible (56). Both dicarbonyls are C4-epimers and can equally undergo all described reactions. Another follow up reaction of 3-DG is the melanoidin formation. Melanoidins are heterocyclic brown coloured structures with a high molecular weight (up to 100 kDa) (57). 3-DG can undergo polymerisation reactions like aldol condensation leading to melanoidin structures (58). Several studies confirmed an involvement of 3-DG in coloured melanoidin formation (58-60). The reaction of 3-DG to melanoidins is important because these are considered as causing color increase during beer aging (4, 61).

Up to here, the reaction pathways were introduced qualitatively. The presented reactions will now be considered quantitatively. Table 3 summarizes occurring concentrations of selected metabolites in malt, final wort, and fresh beer focussed on pale lager beers found in the literature.

Table 3 Summary of occurring concentrations of 3-DG related metabolites in malt, final wort and fresh beer (20-22, 55, 62-72)

Substance class	Metabolite	Malt [mg/kg]	Final wort [mg/L]	Fresh beer [mg/L]
Amino acids	Phenylalanine	720.0–1390.0 (62, 63)	63.0–226.0 (62, 63)	10.0–80 (62, 63)
	Valine	610.0–1120.0 (62, 63)	78.0–214.0 (62, 63)	50.0–100.0 (62, 63)
	Isoleucine	320.0–870.0 (62, 63)	48.0–155.0 (62, 63)	10.0–50.0 (62, 63)
	Leucine	700.0–1890.0 (62, 63)	100.0–316.0 (62, 63)	10.0–100.0 (62, 63)
	Methionine	n.d.[a]–270.0 (62, 63)	20.0–67.0 (62, 63)	n.d.[a]–10.0 (62, 63)

Substance class	Metabolite	Malt [mg/kg]	Final wort [mg/L]	Fresh beer [mg/L]
	Lysine	510.0–860.0 (62, 63)	65-0–177.0 (62, 63)	10.0–50.0 (62, 63)
Amadori products	N-ε-fructosyllysine	37.6–105.7 (21)	-	6.8–13.6 (55)
	N-ε-maltulosyllysine	8.9–30.0 (21)	-	3.7–27.3 (55)
Dicarbonyls	3-DG	45.2–154.2 (21)	29.2 (20)	8.1–42.5 (22, 64)
	3-DGal	1.5–6.0 (21)	-	3.2–17.4 (22, 64)
Strecker aldehydes	2MP	612.0–3480.0 µg/kg (65-67)	24.2–1531.0 µg/L (68, 69)	1.9-7.9 µg/L (15, 70)
	2MB	54.4–2411 µg/kg (65-67, 71)	9.2–1245.0 µg/L (13, 68, 69)	0.6–2.7 µg/L (13, 15, 70)
	3MB	63.0–4271.0 µg/kg (65-67, 71, 72)	39.5–1654.0 µg/L (13, 68, 69)	0.6–36.0 µg/L (13, 15, 70)
	Meth	224–1014 µg/kg (65-67)	26.0–359.6 µg/L (13, 68, 69)	n.d.[a]–2.8 µg/L (13, 15, 70)
	PA	198–1014 µg/kg (65-67)	34.7–295.6 µg/L (13, 68, 69)	1.3–22.0 µg/L (13, 15, 70)
Dehydration products	HMF	n.d.[a]–8.8 (21)	1.1–2.7 (62)	0.3–2.0 (22, 64)
	Furfural	n.d.[a]–0.7 (21, 65, 72)	70.0–2646.0 µg/L (62, 69)	9.0–38.4 µg/L (15, 64, 70)
AGEs	Pyrraline	0.4–1.7 (21)	-	0.2–0.3 (55)
	MGH-1	0.2–0.3 (21)	-	0.3–1.5 (55)

[a] n.d. – none detectable

The substance classes of amino acids, Strecker aldehydes and dehydration products are broadly investigated due to technological influences and sample variation (15, 63, 65). Regarding the Amadori products, the dicarbonyls, and the AGEs, only few studies focussed on commercial samples were previously published (21, 22, 55). However, less is known about the formation of 3-DG as representative of the dicarbonyls during the malt and beer production due to technological modification such as a varied steeping degree. This knowledge would be of significant importance to adjust

dicarbonyl formation during the production processes and, furthermore to use the dicarbonyl content as an early prediction possibility of flavor instability evaluation.

1.5 The influence of the beer and malt production on the dicarbonyl chemistry

Although the beer and malt production comprise suitable production steps such as kilning or wort boiling for caramelization or the Maillard reaction, less is known about the formation of dicarbonyls such as 3-DG during the processes. In the following, the potential provided by the consecutive steps for 3-DG formation and degradation are introduced in more detail.

Malting

The malting process is mandatory to increase the functionality of cereal grains in the brewing process. Here, amylolytic, cytolytic, and proteolytic degradation pathways play an important role for changing of the metabolic profile during the process.

The germination step has two critical effects which influence the dicarbonyl chemistry: (i) the formation of amylolytic enzymes and (ii) the formation of amino acids and low molecular sugars as reactants able to form 3-DG (73, 74). Regarding the enzyme formation, they can be distinguished into amylolytic and proteolytic active enzymes. Critical amylolytic enzymes are α- and β-amylase. β-amylase already exists in barley but is also formed like α-amylase during germination (75, 76). Important proteolytic enzymes formed during germination are endoproteases, carboxyproteases, and metalloproteases (77-79). These enzymes cause formation of amino acids from proteins (proteolytic enzymes) (79, 80) and low molecular weight carbohydrates from polysaccharides (amylolytic enzymes) (81) during malting and mashing. Therefore, their activity provides a formation potential for dicarbonyls such as 3-DG (18). A higher malt modification level with elevated steeping degree, germination temperature, or germination time increases the enzyme activity (82) and, therefore, is prone to be a suitable adjustment for the 3-DG formation potential of the malting process. Previous studies already showed that a higher malt modification level increased the concentration of free amino nitrogen (includes free amino acids) (83) or low molecular sugars (81) in malt, which covers the second critical effect on germination (formation of reactants). Therefore, this thesis speculated that the varied precursor potential by a varied malt modification level influences the 3-DG formation during malting.

The kilning step is also critical for 3-DG formation. The thermal load provides reaction potential for the Maillard reaction and caramelization (18). Previous studies showed that the HMF content raises with higher kilning temperatures (21, 84). It can be assumed that 3-DG as intermediate in HMF formation is subject to similar effects. Especially the low water activity during kilning provides very good conditions for dehydration reaction within 3-DG formation (28, 34). Beside formation of the dicarbonyl, the thermal load also could cause 3-DG degradation. The appearance of follow up products such as HMF or pyrraline indicates a 3-DG degradation during kilning (21). Therefore, the thesis assumes the kilning step as critical for observed 3-DG contents in malt.

Mashing

Mashing is a key process for substrate formation in beer production. Enzymes formed during malting hydrolyse polymers such as proteins or starch into their oligomers, dimers, or monomers. As already discussed, proteolytic and amylolytic degradation provides precursors for 3-DG formation.

Regarding the amylolytic degradation, Vriesekoop et al. (2010) showed an increase of glucose, fructose, maltose, and maltotriose during mashing where higher molecular weight dextrins were decreased (85). Although 3-DG could be also formed by dextrin degradation, carbohydrates with a smaller chain length yield a higher amount of 3-DG (31). Therefore, the mashing process enhances the qualitative and quantitative formation potential of 3-DG. Furthermore, previous studies showed that the resulting carbohydrate profile after mashing depends on the barley variety, mashing procedure, and the enzymatic activity formed during the germination process (85, 86). Regarding the proteolytic degradation, free amino acids were formed representing precursors for Amadori products which can be further degraded to dicarbonyls (77). Several studies showed the solubilization of proteins due to different enzyme activity based on germination conditions, pH-values, and mashing temperatures, where all studies confirmed the formation of free amino acids from barley proteins (78, 79, 87, 88). In particular, a rest at 45 °C was found to increase the amino acid content after the mashing procedure (77).

Therefore, this thesis speculated that a varied mashing procedure determines the reactant concentrations and, thus, influences the 3-DG formation in further beer production steps such as wort boiling. However, the influence of a varied enzyme

activity by the malt modification level on 3-DG precursor formation may turn out to be more important than varying different mashing temperatures. Therefore, the thesis assumes the effect of the mashing procedure being less important for 3-DG formation and focussed on the variation of an additional proteolytic rest.

Wort Boiling

Wort boiling is a critical step providing most intense thermal load during beer production. Besides the expected functionalities such as bitterness formation or wort sterilization, a high reaction potential is induced and initialises flavor formation. Besides chemical formation, the metabolites undergo a concentration increase because of the occurring evaporation effect (8–10%).

Regarding precursor compounds, amino acids (except glutamine) and low molecular sugars remain constant or show a slight increase during wort boiling (89-92). However, compared to published 3-DG contents in wort (Table 3), e.g. glucose concentration (5.0–15.0 g/L (92)) is highly elevated. This means, that a slight decrease of glucose can critically affect the dicarbonyl concentration in final wort. Witmann and Eichner (1989) showed a decrease of several Amadori products during boiling indicating dicarbonyl formation (89). Bravo et al. (2008) showed a strong increase of 3-DG (from 13.0 to 29.2 mg/L) after 2 h of boiling. However, the result indicates the high reaction potential of wort boiling for 3-DG formation, but the influence of technological variation such as varied malt modification level or varied mashing procedure onto dicarbonyl formation is still unknown. Based on the high thermal load, the thesis assumes the wort boiling step as the most important step for 3-DG formation.

Besides 3-DG formation, wort boiling very likely induces degradation reactions of 3-DG and, therefore, forms follow up products of the dicarbonyl. 3-DG degradation is indicated by HMF (93) or Strecker aldehyde formation (68) during wort boiling. However, less is known about 3-DG degradation during wort boiling yet, but it can be expected that 3-DG formation and degradation take place simultaneously during wort boiling.

Fermentation

The fermentation step mainly metabolizes the formed substrate potential (low molecular sugars and amino acids) during mashing and wort boiling to ethanol and beer flavor compounds (94). In the following, possible effects of this process step on

3-DG reactivity are discussed. Generally, fermentation of bottom fermented beer types is conducted at temperatures of about 8 °C (95). Therefore, the temperature cannot be taken as critical for dicarbonyl chemistry. Another aspect is the pH drop during the fermentation. The pH-value decreases down to 4.2–4.6 (62). The more acidic conditions can catalyze 3-DG degradation reactions such as HMF formation (18), but the lower temperature strongly reduces the reactivity for such reactions.

Furthermore, yeast provides many enzymes for the biotransformation of wort. Using model systems, recent studies reported a metabolism of MGO to D-lactate via glyoxalase system occurring in yeast. This system is known to reduce dicarbonyl stress in humans (96, 97). Hellwig et al. (2021) reported a conversion of 17.8% of 3-DG to 3-deoxyfructose in single substance focussed model studies by *Saccharomyces cerevisiae*, but the fermentations were conducted at 30°C and sucrose (10 g/L) was used as substrate (98). Both conditions are significantly different to brewing fermentations conditions (95).

A few yeast enzymes are able to metabolize 2-oxoaldehydes. Sanchez et al. (2003) showed a purified NADPH-dependent oxidoreductase enzyme which reduces the dicarbonyl content during wort fermentation (99). The used enzyme showed a 96% similarity to the Old Yellow Enzyme (OYE; EC 1.6.99.1) which is an oxidoreductase and reduces the carbonyl groups to their corresponding alcohols (100, 101). Bravo et al. (1999) suggested the additional dosage of the described enzyme during fermentation of malt beverages to stabilize the flavor (102). The European patent can also be applied on beer, but an enzyme dosage is not permitted in Germany because of the German purity law. Furthermore, Liang et al. (2000) also reported a purified NADPH-dependent enzyme from brewer's yeast which was able to degrade 3-DG in higher activity levels (103). However, all the references show reactivity for purified enzymes and a pH-optimum at 7.0 (99, 103). At pH-values below 6.0 the enzyme was considered unstable (103). Further, it has to be considered that 3-DG is partly present in ring from, which can sterically inhibit enzymatic degradations. Therefore, the thesis speculated the effect of the fermentation step on the 3-DG content as negligible and it was decided to use standardized fermentations for the brewing trials.

1.6 Thesis Outline and Motivation

In the previous sections, the chemistry of 3-DG, the relevance of 3-DG for flavor stability, and its formation potential has been described in detail. Hence, the initial situation and motivation of this thesis can be summarized:

- 3-DG is an important precursor compound for Strecker degradation and HMF formation which are common aging indicators in fresh beer. Therefore, the dicarbonyl provides a qualitative potential for flavor instability. Observed concentrations of 3-DG in fresh beer were degraded during beer aging. If only about as much as 1% of the degraded 3-DG is converted to Strecker aldehydes, they cause sensory deterioration. This fact indicates the quantitative potential of 3-DG onto flavor instability.

- 3-DG is formed during the Maillard reaction and caramelization. Educts for the formation are carbohydrates, amino acids, and Amadori products. 3-DG is a reactive compound and can form Strecker aldehydes, AGEs, HMF, 3-DGal, and melanoidins. The degradation products can be used to evaluate the reactivity of 3-DG during beer aging.

- The malt and beer production show a crucial potential for 3-DG formation. Especially the kilning, mashing, and wort boiling steps provide a high thermal load and produce high concentrations of precursor compounds. It can be assumed that technological variation such as the malt modification level by an adjusted steeping degree vary the 3-DG formation during the malt and beer production.

Based on this initial situation the main hypothesis is as follows: **The concentration of 3-DG in fresh beer is an analytical indicator for flavor instability of pale lager beers.** In order to achieve the objective, two main questions are formulated in this thesis.

(i) At which stages of the malt and beer production is 3-DG mainly formed and can the formation be influenced technologically?

1. Investigation of 3-DG formation during malting by variation of reactants low molecular sugars and amino acids (variation of malt modification level) and final kilning temperature.
2. Investigation of 3-DG reactivity (formation and degradation) during wort boiling by variation of malt modification level and mashing procedure in lab-scale to understand the reaction pathways.
3. Investigation of 3-DG formation during wort boiling by variation of malt modification level on a pilot scale to verify the observed reaction pathways.

(ii) Does the formed 3-DG during malt and beer production influence the flavor instability of beer?

1. Investigation of the influence of artificially spiked 3-DG during beer aging to investigate the distribution of aging-relevant follow-up reactions.
2. Investigation of a technologically varied (different malt modification levels) initial 3-DG concentration on the flavor instability (analytical and sensory evaluation) of lager beer. Evaluation of a possible use of 3-DG as an early prediction possibility for flavor instability.

2. Methods

2.1 Sample production

The malt samples in part I and II were produced in a pilot malting system at a 1 kg scale at the Chair of Brewing and Beverage Technology.

The wort and beer samples of part III, IV, and V were produced on a pilot scale brewing system (80 L scale) at the Chair of Brewing and Beverage Technology. Fermentation was performed in cylindroconical vessels and bottling in a semiautomatic filling unit (Alfred Gruber GmbH, Eugendorf, Austria).

2.2 Sample treatment

Solid samples (malt samples) were first milled and then extracted with a tempered (25 °C) water-methanol (1:1 v/v%) solution for 30 min. Next, the samples were centrifuged and the liquid phase was used for chromatographic analysis.

Liquid samples (wort and beer samples) were directly used without any further treatment.

2.3 High pressure liquid chromatography (HPLC) analysis

HPLC methods were applied at all parts of the thesis. Prior to HPLC analysis, samples with high carbon dioxide content were degassed and filtered through a 0.45 µm membrane filter.

Non-volatiles were determined according to several HPLC techniques in all parts. Amino acids were quantified by liquid chromatography separation and tandem mass spectrometry detection (LC-MS/MS) (104). Low molecular sugars were analyzed by anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (105). Dicarbonyls (3-DG and 3-DGal) were first derivatized with ortho-phenylene diamine (o-PD) to obtain stable quinoxalines. Quinoxalines and HMF were determined by HPLC-UV (23, 106). AGEs were quantified by LC-MS/MS (52).

2.4 Gas chromatographic (GC) analysis

Volatiles were analysed at a GC system coupled to a mass spectrometer (MS) in parts II, III, IV, V and VI. Quantification of aging carbonyls was conducted by an optimized head space solid phase microextraction (HS-SPME)-GC-MS method based on Saison et al. (2008) (107). The aldehydes were derivatized with o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) to obtain stable oximes.

2.5 Sensory analysis

Sensory Analysis was done in part V and part VI. The beers were tasted according to the DLG 5-point scheme, the Eichhorn aging scheme (108), and a descriptive scheme using a check-all-that-apply (CATA) approach.

2.6 Data analysis

The Statistical analysis was made use of with the software JMP Pro 14 (SAS Institute GmBH; Heidelberg, Germany). Results are shown as average ± standard deviation. Differences between averages were checked by ANOVA (Tukey test) for a significance level of 0.05.

3. Results

3.1 Summary thesis publication

Part I: Formation of 3-deoxyglucosone in the malting process

Pages 23-31

The aim of the study was to investigate the formation of 3-DG due to different malt modification levels and final kilning temperatures. To explore the reaction pathways of 3-DG formation, the composition of reactants (sugars, amino acids) was varied by using different malting modification levels (germination time 5–7 d; steeping degree 42–45%; germination temperature 12–14 °C) and a final kilning temperature (60–100 °C).

3-DG concentration was found to be between 0.8–19.5 mg/100 g d. m. in all malt samples. The study shows that 3-DG formation is enhanced by a higher malt modification level and increased final kilning temperatures. The resulting higher concentrations of glucose at the beginning of withering acts as the major precursor compound and promotes the formation of 3-DG at higher temperatures during kilning. The malt modification level has no significant influence on the relative formation rate of 3-DG, but it has a strong effect on the absolute final content of 3-DG.

The contents of 3-DG and FL (3-DG precursor) were remarkably higher than the HMF (degradation product) concentration at all variations. This indicates that the Maillard reaction stays in an early stage in the malting process. In further investigations of the formation of 3-DG, a kilning model system was used to show that caramelization leads to higher absolute amounts of 3-DG than the Maillard reaction at malt kilning conditions. Based on Glc being the precursor compound, 3-DG was formed up to 16.1 mg/100 g d.m. under Maillard reaction conditions and up to 19.9 mg/100 g d.m. under caramelization conditions. In conclusion, the abundant formation of 3-DG in the malting process is already comparable to the occurred brewing process concentration. (105)

Authors´ contribution:

The PhD candidate worked out the hypothesis and experimentals. He established the applied methodology and conducted the main malting trials and model experiments. Furthermore, he evaluated the resulting data and wrote the original draft. All authors reviewed and approved the final manuscript.

Part II: Formation and degradation of 3-deoxyglucosone as a key intermediate for ageing indicators during the wort boiling process

Pages 32-40

The aim of the study was to investigate the formation of 3-DG due to a varied malt modification level of the used malt and a varied mashing procedure (additional proteolytic rest to increase the free amino acid concentration). Furthermore, selected degradation reaction pathways (3-DGal, HMF, and Strecker aldehyde formation) of 3-DG were observed during the wort boiling process in order to describe the influence of 3-DG reactivity during boiling on the final sensory quality of beer. Experiments were performed in a closed system at lab scale.

The study shows, that a higher content of amino acids (reactant) caused by an additional proteolytic rest in the mashing procedure has no significant effect on the 3-DG formation during wort boiling. A higher malt modification level enhances the 3-DG formation. The study highlights that rather close to 50% of the final 3-DG concentration in wort is formed during wort boiling, which is consequentially the most important process of 3-DG formation in beer production. Besides dicarbonyl increase, the Amadori compound FL is mainly decreased, indicating that the Maillard reaction is going further to the advanced stage. Regarding degradation reactions, HMF is formed during boiling, but the compound has a low conversion rate from 3-DG of 0.7%. The interconversion of 3-DGal is formed stronger, but only 4.0% of 3-DG were interconverted. Investigating this phenomenon, model reactions showed that the dicarbonyl is mainly stable under wort boiling conditions. Only 16% of the absolute 3-DG content is degraded, whereby 25% of the degraded 3-DG is interconverted to 3-DGal. Generally, the study shows that wort boiling generates the main dicarbonyl potential by forming 3-DG as a key intermediate for aging indicators. Therefore, the

results indicate that wort boiling strongly influences the flavor instability of beer by 3-DG formation (109).

Authors´ contribution:

The PhD candidate worked out the hypothesis and experimentals in discussion with the other authors. He established the applied methodology and conducted the main boiling trials and model experiments. Furthermore, he evaluated the resulting data and wrote the original draft. All authors reviewed and approved the final manuscript.

Part III: The influence of the proteolytic malt modification on the aging potential of final wort

Pages 41-58

The aim of the study was to investigate the influence of the proteolytic malt modification level (technologically varied 3-DG and amino acid concentration at the beginning of wort boiling) assessed by the liquid nitrogen content on the formation of the aging potential during wort boiling. Here, the formed 3-DG concentration as an important aging precursor contributes directly to the aging potential of wort.

The dynamic changes of beer flavor are determined by its aging potential which comprises free and bound aldehydes as well as their precursors (e. g. 3-DG). These compounds are mainly formed upstream in the brewing process through the Maillard reaction, Strecker degradation or lipid oxidation. Especially wort boiling is a critical production step for the important reactions due to its high temperature and favorable pH-value (catalyzed aging reactions).

Six worts (malt of two malting barley varieties at three different proteolytic malt modification levels) were produced. Regarding precursors, especially the concentrations of Strecker relevant amino acids and dicarbonyls (3-DG and 3-DGal) increase significantly by an enhanced malt modification level. Concentrations of free and bound aldehydes are highest at the beginning of boiling and decrease towards the end of boiling. A dependency of the malt modification level to the degree of free and bound aldehydes was observed for 2MP, 2MB, and 3MB. This significant increase of Strecker aldehydes in final wort by a higher malt modification level can be explained by the elevated concentration of amino acids and dicarbonyls (3-DG and 3-DGal) at

the beginning of wort boiling. Generally, a higher proteolytic malt modification level tends to increase the content of free and bound aldehydes at the end of wort boiling. In conclusion, the aging potential formation during boiling is increased by an intensified malt modification level (110).

Authors´ contribution:

The PhD candidate worked out the hypothesis and experimentals. He established the applied methodology and conducted the main malting and brewing trials and model experiments. Furthermore, he evaluated the resulting data and as a shared principal author wrote the original draft. All authors reviewed and approved the final manuscript.

Part IV: Influence of 3-DG as a Key Precursor Compound on Aging of Lager Beers

Pages 59-67

The aim of the study was to investigate the influence of 3-DG degradation during storage of pale lager beer. Therefore, different concentrations of 3-DG were added to the freshly brewed beer at the beginning of storage (immediately after filling).

Analysis of degradation products of 3-DG such as 3-DGal, 5-HMF, Strecker aldehydes, and AGEs during beer aging revealed that a higher initial 3-DG concentration increases the formation of the products after prolonged storage (>6 months). Hence, the study shows for the first time that artificially increased content of 3-DG leads to accelerated beer aging, in particular enhanced Strecker degradation causing aged flavor. Furthermore, the relative distribution of the described degradation pathways was quantitatively observed. The study shows that the forced aging method could not predict 3-DG reactivity during natural aging up to 12 months. In particular, the interconversion to 3-DGal comprises 50% of 3-DG degradation during natural aging, but covers only 17% after forced aging. However, besides the investigated degradation pathways, the increased incubation temperature at forced aging promotes other reactions of 3-DG (39% of degraded 3-DG). In conclusion, the significant importance of 3-DG as a key precursor compound in beer aging has been shown, in particular by the increase of Strecker aldehydes (111).

Authors´ contribution:

The PhD candidate worked out the hypothesis and experimentals. He established the applied methodology and conducted the main brewing and aging. Furthermore, he evaluated the resulting data and wrote the original draft. All authors reviewed and approved the final manuscript.

Part V: A comprehensive evaluation of flavor instability of beer – part 1: Influence of release of bound-state aldehydes

Pages 68-83

The aim of the study was to investigate the influence of the varied aging potential in wort due to different malt modification levels on the flavor instability of pale lager beers. There, the brewed beers of part 3 were naturally (9 months) and forced aged. Thus, 3-DG content in fresh beer was technologically varied by the malt modification level. The flavor instability was evaluated by sensory analysis as well as by investigating of the behavior of precursors (amino acids and dicarbonyls), free, and bound aldehydes during natural aging. The study comprised two parts. Part 1 (part V in the cumulative thesis) focuses on the release of bound aldehydes while part 2 (part VI of the cumulative thesis) investigates on the *de novo* formation of aging aldehydes.

For beers based on increased initial content of 3-DG by a higher malt modification level (increased reactant contents during beer production) of the used malt, an enhanced formation of aging aldehydes was observed. The aldehydes increase in general during aging and in particular the Strecker aldehydes 2-methylpropanal, 3-methylbutanal, and phenyl acetaldehyde increase significantly with the proteolytic malt modification level. From beers with a higher malt modification, more aldehydes are present in a bound form. Furthermore, the equilibrium between free and bound aldehydes shifts towards the free state during aging. For beers using malts with a higher proteolytic modification Sensory analysis show an increased flavor instability. These beers show decreased DLG overall score and acceptancy as well as an increase of the attribute *bready* (CATA-analysis). Triangle tests further reveal sensory significant differences between beers at different malt modification levels after 9 months of natural aging.

These results indicate the existence of a nitrogen-dependent bound-pool of aldehydes which is depleted during aging. The bound aldehydes are responsible for an aged aroma especially during the early and medium stage of aging. The amount of free and

bound aldehydes, and thus, the aging potential is influenced by the proteolytic malt modification level (increased initial content of 3-DG) (112).

Authors´ contribution:

The PhD candidate worked out the hypothesis and experimentals. He established the applied methodology and conducted the brewing trials and aging experiments. Furthermore, he evaluated the resulting data and as a shared principal author wrote the original draft. All authors reviewed and approved the final manuscript.

Part VI: A comprehensive evaluation of flavor instability of beer – part 2: Influence of *de novo* formation of aging aldehydes

Pages 84-98

This study focused on *de novo* formation pathways of Strecker aldehydes. The sample set was equal to part V.

The study shows that the amino acid and dicarbonyl concentrations as precursors are elevated by higher malt modification levels. 3-DG and 3-DGal are degraded during aging. Especially, 3-DG concentration in fresh beer is increased and more strongly degraded by a higher malt modification level. Thus, Strecker aldehyde contents increase also more strongly during beer aging at higher malt modification levels. AGEs as possible degradation products show no consistent formation during aging. Therefore, Strecker degradation for dicarbonyls is promoted during beer aging. An alternative oxidative formation of Strecker aldehydes from their corresponding alcohols could not be confirmed. In correspondence to part V (preceding article), the study shows that *de novo* formation and release of aging aldehydes occur simultaneously. *De novo* becomes crucial to the rise in aldehydes mainly after 4 months of natural aging. Significant sensory changes occurre at a range of 3–5 months of natural aging (preceding article). Therefore, it can be assumed that the release of aldehydes basically provides the potential for sensory changes. In conclusion, de novo formation of Strecker aldehydes by 3-DG degradation mainly reinforces and accelerates sensory beer aging after 3–5 months according to this study (113).

Authors´ contribution:

The PhD candidate worked out the hypothesis and experimentals. He established the applied methodology and conducted the brewing trials and aging experiments.

Furthermore, he evaluated the resulting data, as a shared principal author wrote the original draft and submitted it. All authors reviewed and approved the final manuscript.

3.2 Formation of 3-deoxyglucosone in the malting process

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Formation of 3-deoxyglucosone in the malting process

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ABSTRACT

3-Deoxyglucosone (3-DG) is a metabolite from sugar degradation obtained by the Maillard reaction. It is an important precursor compound in Strecker reactionism that directly leads to known beer aging indicators and can influence the final sensory beer quality. However, the conditions of 3-DG formation in the malting process have not yet been described.

To investigate the reaction pathways of 3-DG formation, we varied the composition of reactants (sugars, amino acids) by using different malting modification levels (germination time 5–7 d; steeping degree 42–45%; germination temperature 12–14 °C); final kilning temperature (60 °C to 100 °C).

After its derivatization with ortho-phenylenediamine, we analyzed 3-DG with HPLC-UV. 3-DG concentration was between 5 and 120 µmol/100 g dry weight. The formation of 3-DG increased for high malt modification levels and high final kilning temperature.

The abundant formation of 3-DG in the malting process is already comparable to the occurred brewing process concentration.

1. Introduction

Malting is an important process used to increase the functionality of cereal grains in the brewing process. In addition to cytolytic and proteolytic metabolomic pathways, the amylolytic degradation of starch is also important in the malting process. Glucose and maltose, which are formed during the process, are important reactants in the formation of 3-deoxyglucosone (3-DG).

For the formation of maltose, β-amylase and its activity are important and are also major quality parameters of barley malt (Bera, Sabikhi, & Singh, 2018; Delcour & Verschaeve, 1987; Gibson, Solah, Holmes, & Taylor, 1995). First, the steeping process increases the water content in the barley grains and induces the germination step which includes enzyme expression. β-amylase already exists in barley grains, but it is also expressed like α-amylase during the germination process whereby increasing rates of β-amylase activity are lower than α-amylase activity enhancement (Arends, Fox, Henry, Marschke, & Symons, 1995; Georg-Kraemer, Mundstock, & Cavalli-Molina, 2001). Although the germination temperature (12–18 °C) is below the optimum temperature of the described enzymes (62–72 °C), polysaccharide degradation is induced. During the expression of amylase enzymes, barley starch is degraded to low molecular weight carbohydrate compounds (Hollo & Laszlo, 1972; Smith & Gill, 1986; Vinje, Duke, & Henson, 2015). Besides the less reactive sucrose, glucose is the main product of low molecular weight sugar compounds in the malting process and is therefore a possible precursor in the formation of 3-DG (Allosio-Ouarnier, Quemener, Bertrand, & Boivin, 2000; MacLeod, Travis, & Wreay, 1953).

Low molecular weight reducing sugars can react in enolization and dehydration reactions in caramelization processes to obtain 3-DG. A second pathway is the formation during the Maillard reaction. Possible precursor compounds include Amadori rearrangement products such as fructosyllysine (FL). In general, degradation reactions of low molecular weight carbohydrates are catalyzed by thermal treatment, a low pH-value and a low water activity (Hellwig & Henle, 2014; Ledl & Schleicher, 1990; Liedke, 1999; Zhang et al., 2016). Consequently, heat treatment in a careful kilning process provides good conditions for the formation of 3-DG, which is also a major aroma precursor in the Strecker reaction. The vicinal dicarbonyl structure easily reacts with amino acids to form Strecker aldehydes such as 3-methylbutanal or 2-methylbutanal under decarboxylation (Baltes, 1980; Lehnhardt, Gastl, & Becker, 2018; Weenen & Tjan, 1994). Additionally, 3-DG formed in the malting process is directly transferred in the brewing process together with the malt. The transferred 3-DG has the potential to react as

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a precursor compound for aging compounds like Strecker aldehydes during the thermal load of the brewing process and consequently affect flavor stability and the final sensory quality of beer.

Recently, 3-DG was quantified in a small number of malt samples (Hellwig, Nobis, Witte, & Henle, 2016; Ruiz-Matute, Castro Vazquez, Hernandez-Hernandez, Sanz, & Martinez-Castro, 2015). The studies focused on various commercial beer types and 3-DG was identified as the major α-dicarbonyl compound in beer in addition to 3-deoxygalactosone. The 3-DG concentration increased with the thermal load during beer production like mashing or wort boiling and it decreased in the beer aging process (Bravo et al., 2008; Bravo, Sanchez, Scherer, Herrera, & Aldao, 2003; Damm & Kringstad, 1964; Degen, Hellwig, & Henle, 2012; Hellwig, Nobis et al., 2016; Hellwig, Witte, & Henle, 2016). There is nothing known about 3-DG formation in the malting process and less is known about its final content in pale barley malts, which is the most important ingredient in beer production. We hypothesize that the final concentration of 3-DG in malt may be influenced by the technological malting conditions.

The potential of 3-DG in malt could be of significant importance for aroma formation in beer production, because its role as a precursor compound in the Strecker reaction has already been proven (Baltes, 1980; Weenen & Tjan, 1994). Therefore, the aim of this study is to investigate the formation of 3-DG in the malting process by systematically varying the technological malting conditions.

2. Material and methods

2.1. Chemicals

5-Hydroxymethylfurfural (HMF), L-lysine, [13C, 15N] labelled amino acids, ammonium acetate, potassium dihydrogen phosphate, calcium chloride dihydrate, amylopectin, Avicel PH-101, methanol (LC-MS-grade), o-phenylenediamine (OPD), glucose, maltose, sucrose, hydrochloric acid and citric acid were obtained from Sigma-Aldrich (Steinheim, Germany). Disodium hydrogen phosphate dihydrate, acetic acid, magnesium sulphate, and water (LC-MS-grade) were purchased from Merck (Darmstadt, Germany). Acetonitrile used for liquid chromatography mass spectrometry (LC-MS) analysis was used from VWR (Darmstadt, Germany). Barley starch was purchased from Altia PLC (Helsinki, Finnland). 3-DG (> 95%) was obtained from Apollo Scientific Ltd. (Cheshire, UK). N^{ε}-fructosyllysine (79.6%) was provided by the Chair of Food Chemistry of TU Dresden (Dresden, Germany). Prior to use, the water for solutions, buffers, and the high-performance liquid chromatography analysis with ultraviolet detection (HPLC-UV) was treated by a micropore water purification system (Thermo Fisher Scientific Inc., Waltham, USA).

2.2. Malting experiments

Barley malt samples (500 g) were produced in a micromalting system. The malting conditions are presented in Table 1. Steeping was performed as follows: 5 h immersion, 17 h resting in air (95% humidity), 4 h immersion and 20 h resting in air (95% humidity). The sample extracted after the second immersion step, having steeped for 1 day and 4 h, is defined as S1d4h. After 48 h (2 days) of germination, another sample was taken and is defined as G2d. The other samples were similarly labeled according to their processing. The soluble nitrogen was measured according to MEBAK (2006) (Jakob, 2006) and indicated the level of malt modification as a quality parameter. Accordingly, M1 refers to very high level of malt modification, M2 indicates malting conditions are at an average level and M3 denotes a comparably low malt modification.

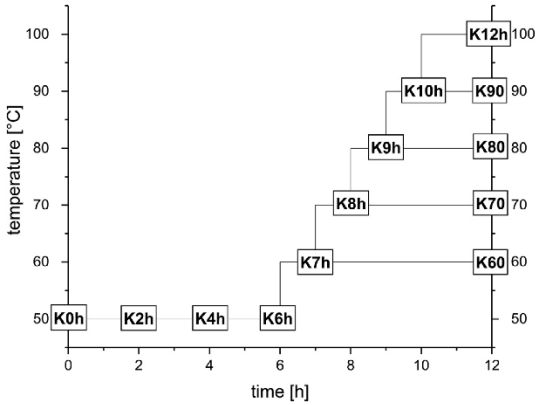

Fig. 1. Sampling points of the kilning process at various final kilning temperatures.

After the steeping and germination processes each green malt sample was dried by a special kilning program. The final kilning temperature was varied in steps of 10 °C from 60 °C to 100 °C (K60, K70, K80, K90, K12h). The sampling points are defined in Fig. 1. The samples were placed in the kiln, and the temperature was raised after staying 6 h at 50 °C to the final kilning temperature over 6 h at a rate of 10 °C increments per hour.

All samples were produced in three biological replicates.

2.3. Sample preparation

Barley grains (K4h, K6h, K7h, K8h, K9h, K10h, K12h, K90, K80, K70, K60) were homogenized and ground with a milling gap of 0.2 mm. K2h was first frozen in liquid nitrogen prior to milling. Samples S1d4h, G2d and K0h were lyophilized before grinding. Any roots and sprouts found in the sample grains were separated from the seeds before milling.

Five grams of the ground samples were extracted with a 25 °C tempered methanol–water solution (1:1) for 30 min by stirring at 300 rpm. After the extraction step, the liquid phase was centrifuged for 10 min at 13,000 rpm. For the HPLC analysis, samples were filtered (0.45 µm).

2.4. Quantitation of moisture content

The amount of moisture present in the samples was measured according to MEBAK 2006 (Jakob, 2006). The moisture content of samples S1d4h, G2d and K0h was analyzed by weighing before and after lyophilization.

Table 1
Malt modifications.

Malt modification	Modification level	Germination time [d]	Germination temperature [°C]	Steeping degree [%]	Soluble nitrogen [mg/100 g dry weight]
M1	high	7	14	45	941.3 ± 5.9
M2	medium	6	12	42	732.5 ± 6.9
M3	low	5	12	42	683.8 ± 4.1

2.5. Quantitation of 3-DG

Derivatization and HPLC-conditions were applied as previously performed by Degen et al. (2012). The HPLC system consisted of the UltiMate 3000 Autosampler, an UltiMate 3000 pump module, an UltiMate 3000 column compartment and the UltiMate 3000 Diode Array Detector. All devices were from Thermo Fisher Scientific Inc. (Waltham, USA). Data evaluation was performed by Chromeleon 6.80 software from Thermo Fisher Scientific Inc. (Waltham, USA).

2.6. Quantitation of HMF

The HMF concentration was determined according to the method reported by Rufian-Henares, Delgado-Andrade, and Morales (2006). A Kinetex 5 µm C18, 100 Å, 250 × 4.6 mm column was used. Solvent A was 5% acetonitrile in water and solvent B was methanol. The gradient mode was modified (0 min 100% A; 9 min, 100% A; 9.5 min, 20% A; 11.5 min, 20% A; 12 min, 100% A; 17 min, 100% A). The flow rate was 1.0 ml/min, the column oven temperature was set to 32 °C, the injection volume was 20 µl and detection was carried out at 280 nm. The HPLC-system was the same as that described in Section 2.5. Samples were directly measured after extraction und filtration (0.45 µm).

2.7. Quantitation of low molecular weight sugars

Fermentable sugars (glucose, maltose, sucrose) were quantified using high-performance anion exchange chromatography pulsed amperometric detection (HPAEC-PAD). The Dionex CarboPack PA10 analytical column (2 × 250 mm) and the Dionex CarboPack PA10 guard column (2 × 50 mm) were used. Eluent A (250 mM NaOH) and eluent B (water) were mixed in gradient mode (0 min 90% B; 20 min, 90% B; 21 min, 20% B; 45 min, 20% B; 46 min, 5% B; 48 min, 5% B; 49 min, 90% B; 60 min, 90% B) at a flow rate of 0.25 ml/min.

The HPAEC system consisted of an ICS AS/AP autosampler, an ICS 5000 DP pump module, an ICS 5000 DC column compartment, and an ICS 5000 PAD detector. All devices were from Thermo Fisher Scientific Inc. (Waltham, USA). Data evaluation is described in Section 2.5.

2.8. Quantitation of amino acids

The availability of 19 amino acids was determined according the method reported by Sonntag, Kunert, Dunkel, and Hofmann (2010) by high-performance liquid chromatography tandem mass spectrometry (HPLC MS/MS) in the multiple reaction monitoring (MRM) mode. Samples were separated first on an amide security guard pre-column (Waters, Eschborn) and on an XBridge amide column (3.5 µm 2 × 150 mm) at a flow rate of 0.4 ml/min. The mobile phases were (A) 5% (v/v) acetonitrile (7.5 mM ammonium acetate, pH 3) and (B) 95% (v/v) acetonitrile (7.5 mM ammonium acetate, pH 3). Gradient mode was applied (0 min, 95% B; 1 min; 95% B; 1.5 min 90% B; 3.5 min 90% B, 8 min, 70% B; 10 min, 40% B; 13 min 40%; 13.3 min, 20% B; 14.3 min, 20% B; 15 min, 95% B; 20 min, 95% B). The HPLC Agilent 1200 series system (Agilent, Waldbronn) consisted of a HiP-ALS SL autosampler, a 1200 series bin pump module, a 1200 series degasser and a 1100 series column oven was coupled to the Triple Quad 4500 MS (SCIEX, Darmstadt). The ion spray voltage was set to 5,500 V, the curtain gas pressure was set to 35 psi, the nebulizer gas pressure was 55 psi and the heater gas pressure was 65 psi. The turbogas temperature was set to 450 °C. The calibration was conducted by using isotopically labeled 13C,15N-standards. All used transitions for quantification are shown in Supplementary material (Supplementary Excel 1).

2.9. Quantitation of fructosyllysine (FL)

The determination of FL in the samples was carried out with the chromatographic system described in Section 2.8. Gradient mode was applied after modification (0 min, 95% B; 1 min, 95% B; 2 min, 80% B; 16 min, 66% B, 17 min, 66% B; 17.3 min, 20% B; 18.3 min 20%; 19 min, 95% B; 24 min, 95% B).

Available FL was also determined using mass transitions (FL quantifier: m/z 309 → 84; qualifier: m/z 309 → 225). Quantitation of the FL of each sample was done by standard addition using final concentrations of 6.72, 13.43, 26.86 and 53.72 µmol/l.

2.10. Kilning model reactions

A kilning model was developed using microcrystalline cellulose as an unreactive substrate. Based on data available in the literature regarding the mineral contents of barley grains (Krüger & Anger, 1990) a liquid buffer was prepared with the following final concentrations potassium dihydrogen phosphate (26.74 mmol/l), magnesium sulphate (21.60 mmol/l), citric acid as the major organic acid (2.99 mmol/l) and calcium dichloride dihydrate (12.60 mmol/l). The buffer was set to pH 5.6 with potassium hydroxide.

The substrates used for incubation in the model system were starch (55 g/100 g dry weight), amylose (11 g/100 g dry weight), amylopectin (44 g/100 g dry weight), glucose (1.98 g/100 g dry weight), and maltose (1.80 g/100 g dry weight). The contents of starch, amylose and amylopectin were set to the values reported for barley seeds (Krüger & Anger, 1990). Glucose and maltose concentrations were defined to the values measured at the sampling point K0h and for malt modification M2, thus providing the concentration of the sugars at the beginning of the kilning process. Each substrate was prepared with and without lysine to characterize the MR and the caramelization reaction. The lysine concentration was set to 140 µmol/100 g dry weight, thus corresponding to the measured concentration at point K0h and malt modification M1. Here the malt modification M1 was used to look for ideal Maillard reaction conditions.

The solid samples were then homogenized in the prepared buffer, frozen and lyophilized. Water activity of the sample was set to 0.99 over water in a desiccator, which is comparable to the water activity of green malt.

After adjusting the water activity, the samples were placed in a drying chamber according to the kilning program with the final kilning temperature of 100 °C (Fig. 1). The samples were cooled down and directly extracted as described in Section 2.3. The components HMF, FL, and 3-DG were studied using the methods described herein.

2.11. Statistical analysis

The statistical analysis was performed with the software JMP® Pro 12 (SAS Institute GmbH; Heidelberg, Germany).

3. Results

3.1. Formation of selected reactants in the malting process

We first investigated the formation of possible reactants, low molecular weight sugars and amino acids, in the pathway of 3-DG formation.

Preceding studies pointed out that 3-DG is the major dicarbonyl compound in beer and beer intermediate products like mash and wort (Bravo et al., 2008; Degen et al., 2012; Hellwig, Nobis et al., 2016). Additionally, the formation of 3-DG in the Maillard reaction or by caramelization is already well described in the literature (Ledl & Schleicher, 1990; Liedke, 1999). Consequently, and especially in the presence of free nitrogen compounds, low molecular weight carbohydrates like glucose or fructose are well suited precursors for 3-DG formation. Nanamori et al. (2011) showed that low molecular weight sugars and amino acids are formed as possible reactants in the germination process (Nanamori et al., 2011). Based on the literature, we speculated that the substances generated in the germination process

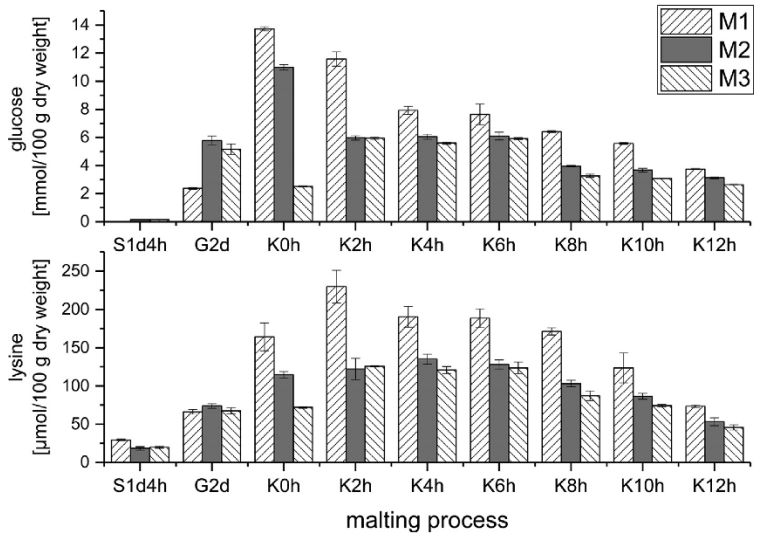

Fig. 2. The formation of glucose and lysine in the malting process; n = 3.

react in the kilning process, thus being catalyzed by thermal treatment especially in the end of kilning by the final kilning temperature.

Fig. 2 shows the formation of glucose and lysine during the malting process at different malt modification levels.

According to the literature (Nanamori et al., 2011) the contents of glucose and lysine increase during the steeping and germination processes. Here, the largest amount of glucose was detected in the green malt (K0h) for high and medium modification levels, M1 and M2. Glucose at M3 and lysine at all modification levels have the highest concentration after 2 h heat treatment in the kiln (K2h). This phenomenon can be explained by enzymatic activities. At the beginning of the kilning process, the moisture in the K2h grains is still relatively high, 24.6%, and consequently, enzymatic protein or polysaccharide degradation processes can proceed with less activity. However, these are promoted at higher temperatures, e.g. 50 °C, depending on the optimum temperature of the specific enzymes. After reaching the maximum amount glucose and lysine are degraded during the kilning process until K12h and that applies for all malt modification levels. Consequently, it can be assumed that the formation and degradation reactions of glucose and lysine take place simultaneously; the balanced state is dominated by degradation processes through increasing time and temperature in the kilning process. The final concentration levels after 12 h (K12h) of heat treatment are significantly different to each other and increase as the malt modification level is increased, i.e., following: M3 < M2 < M1 (low < medium < high). Higher malt modification levels induce higher proteolytic and amylolytic activity, especially in germination process, and result in higher amounts of amino acids or glucose in the final malts. Due to the chemical degradation of the analyzed reactants the rate of decreasing is also significantly different at various malt modification levels and follows: M1 > M2 > M3. Regarding the whole spectrum of amino acids (Supplementary Excel 2) all analyzed amino acids show the same declining trend in the kilning process. Further, considering sucrose and maltose levels (Supplementary Excel 2), sucrose shows a consistent trend whereby maltose follows more the reaction pathway of glucose. Therefore, the potential of 3-DG formation during the kilning process is maximized by high malt modification levels because of the increasing consumption rate of the reactants.

3.2. Formation of 3-DG subject to malting conditions and final kilning temperature

After considering the formation potential of 3-DG in the malting process, the dicarbonyl compound was measured in all final malt samples. The results are presented *in* Table 2 (for samples K60, K70, K80, K90, K12h).

3-DG was measured over the range of 4.91–120.44 µmol/100 g dry weight (0.8–19.5 mg/100 g dry weight). By increasing the final kilning temperature, the concentration of 3-DG increased at all modification levels. The formation of 3-DG is affected by water activity (Liedke, 1999). Therefore, the water activity at a higher final kilning temperature can reach a lower minimum, thus catalyzing the 3-DG formation and leading to higher final 3-DG amounts. The major effect of increased 3-DG formation could be caused by the enhanced temperature which is already described in the literature. The results of studies using different substrates showed thermal effects in model reaction solutions (Kanzler, Schestkowa, Haase, & Kroh, 2017; Kocadagli & Gokmen, 2016a) and in baking experiments, the latter of which is a system comparable to kilning but with less water activity (Kanzler et al., 2017; Kocadagli et al., 2016c). In all reported work, the increased concentration of 3-DG reached a maximum upon more intensive thermal treatment. Therefore, increasing thermal load by implementing higher final kilning temperatures is also a cause for maximum 3-DG concentration in the final malt samples.

Regarding the malt modification levels, the 3-DG content increases from low to high level at all final kilning temperatures, where the greatest increase is for M1 (M1 > M2 > M3). In Fig. 2 the formation and degradation of glucose in the malting process is depicted. Higher malt modification levels are characterized by a higher chemical degradation of glucose, thus resulting in higher final 3-DG concentrations. Furthermore, the amount of lysine also increases according to higher malt modification levels. Consequently, the Maillard reaction is catalysed and results in more 3-DG.

Hellwig, Nobis et al. (2016) quantified 3-DG in pilsner malt samples up to concentrations of 49 µmol/100 g which is comparable to the 3-DG content obtained at a final kilning temperature of 80 °C (Hellwig, Nobis et al., 2016). Furthermore, Degen et al. (2012) investigated the amount of 3-DG in food for evaluating daily intake. The 3-DG content in our study being in a range of 4.91–120.44 µmol/100 g dry weight is

Table 2
The formation of MRPs in the malting process when subject to malt modification and final kilning temperature; n = 3.

Malt modification[a]												
Time	M1				M2				M3			
	3-DG	FL	$\frac{3\text{-DG}}{FL}$	HMF	3-DG	FL	$\frac{3\text{-DG}}{FL}$	HMF	3-DG	FL	$\frac{3\text{-DG}}{FL}$	HMF
K0h	n.d.	1.56 ± 0.23	–	n.d.	n.d.	1.26 ± 0.10	–	n.d.	n.d.	0.75 ± 0.11	–	n.d.
K6h	n.d.	2.11 ± 0.36	–	n.d.	n.d.	1.02 ± 0.03	–	n.d.	n.d.	0.89 ± 0.07	–	n.d.
K7h	2.23 ± 0.05	3.12 ± 0.81	0.71	n.d.	1.63 ± 0.09	2.21 ± 0.18	0.74	n.d.	1.13 ± 0.03	1.53 ± 0.16	0.74	n.d.
K8h	6.14 ± 0.11	6.97 ± 0.57	0.90	n.d.	4.24 ± 0.05	4.70 ± 0.16	0.90	n.d.	3.04 ± 0.11	3.79 ± 0.11	0.80	n.d.
K9h	18.38 ± 0.17	15.87 ± 2.12	1.16	n.d.	13.73 ± 0.05	11.83 ± 1.48	1.16	n.d.	9.54 ± 0.09	8.98 ± 1.58	1.07	n.d.
K10h	50.47 ± 0.68	24.07 ± 4.29	2.10	0.40 ± 0.01	37.34 ± 0.11	22.63 ± 1.72	1.65	0.25 ± 0.02	27.11 ± 0.17	17.55 ± 1.51	1.54	0.18 ± 0.01
K12h	120.44 ± 1.13	45.41 ± 3.40	2.65	4.15 ± 0.12	90.31 ± 0.19	31.22 ± 2.58	2.89	3.02 ± 0.01	72.27 ± 0.63	26.83 ± 0.44	2.69	2.56 ± 0.01
Δ(12 h–0 h)	120.44	43.85		4.15	90.31	29.96		3.02	72.27	26.08		2.56
Final kilning temperature												
°C	3-DG	FL	$\frac{3\text{-DG}}{FL}$	HMF	3-DG	FL	$\frac{3\text{-DG}}{FL}$	HMF	3-DG	FL	$\frac{3\text{-DG}}{FL}$	HMF
K60	9.61 ± 0.08	6.42 ± 0.20	1.50	n.d.	6.64 ± 0.06	4.10 ± 0.31	1.62	n.d.	4.91 ± 0.29	4.11 ± 0.61	1.19	n.d.
K70	19.85 ± 0.51	16.49 ± 0.62	1.20	n.d.	13.61 ± 0.15	11.18 ± 0.34	1.22	n.d.	9.90 ± 0.07	9.04 ± 0.73	1.10	n.d.
K80	41.01 ± 0.17	28.06 ± 4.14	1.46	0.33 ± 0.01	24.61 ± 0.23	15.94 ± 6.35	1.54	0.15 ± 0.01	22.51 ± 0.08	14.78 ± 1.99	1.52	0.13 ± 0.01
K90	76.25 ± 0.71	36.89 ± 0.03	2.07	1.52 ± 0.03	71.32 ± 0.13	31.52 ± 2.64	2.26	1.49 ± 0.01	48.43 ± 0.24	24.49 ± 2.64	1.98	1.25 ± 0.01
K12h	120.44 ± 1.13	45.41 ± 3.70	2.65	4.15 ± 0.12	90.31 ± 0.19	31.22 ± 2.58	2.89	3.02 ± 0.01	72.27 ± 0.63	26.83 ± 0.44	2.69	2.57 ± 0.01

[a] Data are in μmol/100 g dry weight; n.d. – not detectable.

comparable to that reported in the literature for bread (8–382 µmol/100 g) and cookies (5–238 µmol/100 g) (Degen et al., 2012); those foods are also subjected to heating processes with low water activity. Considering daily intake, malt is not important because it is further processed as a raw material in the production of beer.

3-Deoxyglactosone (3-DGal), which is formed under isomerisation directly from 3-DG, is also described as the second most important dicarbonyl compound in beer in several studies (Degen et al., 2012; Hellwig, Nobis et al., 2016). However, in our study 3-DGal was not detectable, which indicates that the isomerisation reaction from 3-DG to 3-DGal is not present in the malting process.

3.3. 3-DG in the Maillard reaction pathway

In addition to 3-DG, we measured FL and HMF content during the process to evaluate 3-DG formation in the Maillard reaction pathway. Comparing the amounts of these three substances, it is possible to evaluate the stage of the Maillard reaction in the malting process. All data are presented in Table 2 (samples K0h, K6h, K7h, K8h, K9h, K10h, K12h).

Only FL was detected at the beginning of the kilning process (K0h). 3-DG was first determined after 7 h (K7h) and HMF could only be measured 2 h before the completion of the kilning process (K10h). The observed sequence follows the chronological pathway of the Maillard reaction outlined exemplarily in Fig. 3.

With glucose as a precursor compound, the Maillard reaction proceeds via Amadori rearrangement and forms FL as a first MRP. Afterwards 3-DG is formed, which can undergo dehydration reactions, resulting in a final sugar degradation product HMF. Besides the Maillard reaction 3-DG can be formed immediately from glucose by enolization and dehydration reactions catalyzed by thermal treatment. The latter reaction is called caramelisation. Both pathways take place concurrently.

In the kilning process described in Table 2, the onset of the formation of individual MRPs is delayed according to the Maillard reaction pathway (FL > 3-DG > HMF), but the products are then formed, also simultaneously, during the whole kilning process. The concentration of all MRPs increases during the process and reaches a maximum at the end of the kilning process (K12h). By increasing kilning time, the 3-DG formation rate is faster than the FL formation rate. HMF is generated later in the kilning process and compared to FL (4.10–45.41 µmol/100 g dry weight) and 3-DG (4.91–120.44 µmol/100 g dry weight), it has a significantly lower concentration, which ranges from a nondetectable to 4.15 µmol/100 g dry weight, in the final malts (K60, K70, K80, K90, K12h), though 3-DG is a precursor of HMF and is described as a reactive compound (Baltes, 1980; Weenen & Tjan, 1994). Therefore, it can be shown that the Maillard reaction stays in an early phase in the malting process. Regarding the formation of the selected MRPs it can be assumed generally that formation and degradation reactions of the MRPs take place simultaneously, adjusting a balance situation that is focused at a stage of 3-DG, thus representing an early phase of the Maillard reaction.

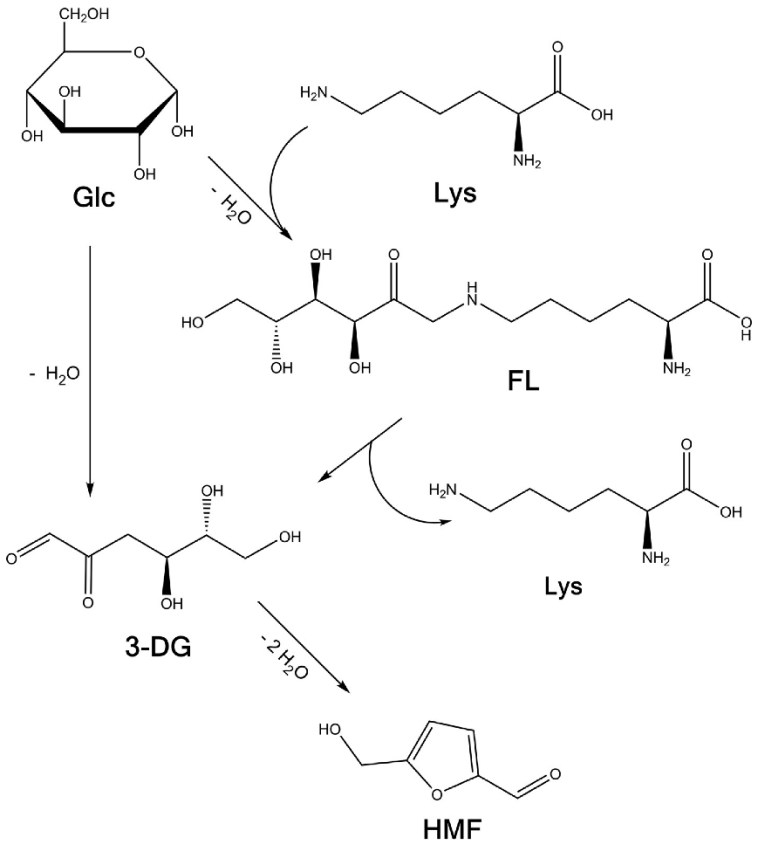

Fig. 3. The formation of 3-DG from glucose in the kilning process.

Comparing the analyzed MRPs at all sampling points, a higher malt modification level (M1 > M2 > M3) accelerated the formation of all MRPs. This is similar to the effect already discussed for the 3-DG concentration according to malt modification and final kilning temperature. Corresponding to the differences in the precursor reactants (Fig. 2) and according to malt modification levels, the potential for MRP formation increases with higher germination time, temperature and steeping degree, thus leading to higher concentrations of MRPs. All malt modification levels are characterized by increasing MRP concentration up to the end of the malting process and they additionally have 3-DG as the major compound. At all modification levels, the MRP formation starting times were very similar. The Maillard reaction stays at an early phase for all variations, which is demonstrated by lower concentration and late formation of HMF.

Furthermore, the ratio of 3-DG and FL was calculated in Table 2 to evaluate the status of the Maillard reaction at each step and to compare the amount of 3-DG and FL throughout the process. After heat treatment in the kiln for 8 h (K8h), the ratio exceeds 1, thus meaning that first the 3-DG concentration is higher than the FL concentration. Considering the entire process, the ratio increases continuously, which also shows that the 3-DG formation rate increases faster than FL formation rate, which we described earlier. Considering the different malt modification levels, the ratio is similar without a significant trend at one level. Therefore, the malt modification has no influence on the relative reaction rate of 3-DG or FL.

Comparing the influence of the final kilning temperature the concentration of all MRPs increases with higher temperatures, whereby 3-DG is always the major compound. When comparing the ratio achieved for the final kilning temperature of 70 °C, we found the ratio increases for higher final kilning temperatures. Up to 100 °C the ratio is enhanced by about 134% when compared to that obtained at 70 °C. 3-DG can be formed by the Maillard reaction as well as caramelization (Hellwig & Henle, 2014; Ledl & Schleicher, 1990; Liedke, 1999). The increasing ratio and 3-DG concentration may be caused by higher thermal treatment wherein it can be expected that the percentage of caramelization is also enhanced by higher temperature. Regarding the different malt modification levels for various temperatures, 3-DG:FL ratios are not significantly different. Therefore, malt modification has also no influence on the relative formation rate of 3-DG and FL in the kilning process for various final kilning temperatures.

3.4. 3-DG formation in Maillard reaction and caramelization

To verify the formation of 3-DG for the proposed precursor compounds, a kilning model was developed to simulate the kilning process. We speculated that 3-DG was formed from glucose and other low molecular weight carbohydrates and not from the thermal degradation of polysaccharides. Measured 3-DG concentrations are shown in Fig. 4.

Polysaccharides as substrates result in extremely low 3-DG content, through the caramelization of starch is catalyzed in the presence of minerals (Moreau, Bindzus, & Hill, 2011a, 2011b). Therefore, these high molecular weight saccharides may have been degraded to other products. Hollnagel and Kroh (2000) suggest a "peeling off" mechanism of oligosaccharides in the presence of amino compounds leading to the major dicarbonyl compound 1,4-dideoxyosone in systems with less water activity (Hollnagel & Kroh, 2000). Our system is comparable to the described study, and so it is possible that polysaccharides are degraded from the reducing end to 1,4-dideoxyosone. Furthermore, high molecular weight saccharides are less reactive in non-enzymatic pathways. To form low molecular weight reaction products, enzymatic hydrolysis by amylases is required. Rakete, Klaus, and Glomb (2014) showed that maltodextrins react mainly to glyoxal instead of C5- or C6-dicarbonyl compounds in forced beer aging model systems, although it is discussable if these model systems are comparable to our reaction model with low water activity (Rakete et al., 2014). Further, FL and HMF are non-detectable by thermal treatment of the used polysaccharides which supports the assumption that the degradation of them undergoes alternative pathways as already described in the literature (Rakete et al., 2014).

Maltose shows less conversion to 3-DG. It could be also degraded according to the "peeling off" mechanism (Hollnagel & Kroh, 2000). Recent studies showed also that maltose is degraded more to heterocyclic products like furfural or HMF and to dicarbonyl compounds based on maltose like maltosone, 3-deoxymaltosone or 1-deoxymaltosone in aqueous model systems (Kanzler et al., 2017; Rakete et al., 2014; Smuda & Glomb, 2011). Contrary to Kanzler et al. (2017) (Kanzler et al., 2017) in our study glucose is more degraded to HMF than maltose. The incubation of glucose formed 3.4 µmol/100 g dry weight HMF without lysine and 2.0 µmol/100 g dry weight HMF with lysine. The degradation of maltose only formed 0.4 µmol/100 g dry weight HMF without lysine and 0.5 µmol/100 g dry weight with lysine. The levels of HMF in the model incubation is comparable to the concentration of HMF in the malting experiments (Table 2). However, it could be expected that maltose is mainly degraded to maltosone or 3-deoxymaltosone in our dry model system. Additionally, less 3-DG was formed by maltose and consequently, it could be assumed that a low percentage of maltose could be hydrolyzed by thermal catalysis to glucose and further degraded to 3-DG.

Glucose degradation induced high 3-DG concentrations that are comparable to the values found in malting experiments with a final kilning temperature of 100 °C (72–120 µmol/100 g dry weight). Consequently, glucose was identified as the major precursor compound in the kilning model. It is formed by amylolytic degradation of polysaccharides (Fig. 2) in the steeping and germination processes. Afterwards it is degraded to 3-DG in the kilning process Table 2) in the pathways of the Maillard reaction and caramelization according to Fig. 3.

In the kilning reaction model, the 3-DG concentration is significantly higher in the absence of lysine (caramelization conditions) when compared to that in the presence of lysine (MR) by degradation of glucose. The contrast cannot be explained by the FL-content (2 µmol/100 g dry weight). Furthermore the result is different from the study by Hollnagel and Kroh (2000), who detect more 3-DG for Maillard reaction conditions. Finally, the studies are not comparable because of different thermal treatment procedures and because our study is based on a model buffer system. Especially phosphate can accelerate the browning rate and influence the degradation pathways of sugars by catalyzing the formation of diacetyl, glyoxal and pyruvaldehyde (Rizzi, 2005). It could be expected that glucose also reacted to an Amadori product at the α-amino-group of lysine. In a kinetic model presented in the literature reactions of several MRPs under low water activity conditions showed that Amadori products mainly react to 1-DG, and 3-DG is rather directly formed from glucose (Kocadagli & Gokmen, 2016b). Therefore, in the caramelization part of our study, glucose does not react to FL, which would be degraded as a precursor mainly to 1-DG. Glucose can be directly dehydrated by thermal treatment to 3-DG, thus leading to higher amounts of 3-DG as compared to the yield from the Maillard reaction conditions. Further, it is possible, that 3-DG is more strongly degraded to other intermediates. Possible pathways are the Strecker degradation of lysine, direct reaction with lysine to pyrraline (Hellwig & Henle, 2014) or the formation of imine isomers of 3-DG and lysine. Overall, our results show that under the described kilning model conditions, 3-DG is formed from both pathways, but caramelization leads to a higher absolute content of 3-DG than the Maillard reaction.

4. Conclusion

Our study investigated the formation of 3-DG in the malting process to evaluate its precursor potential for aging reactions already formed in the malting process. Therefore, we showed that 3-DG formation is enhanced by higher germination time and temperature, steeping degree and final kilning temperatures. The resulting higher concentrations of

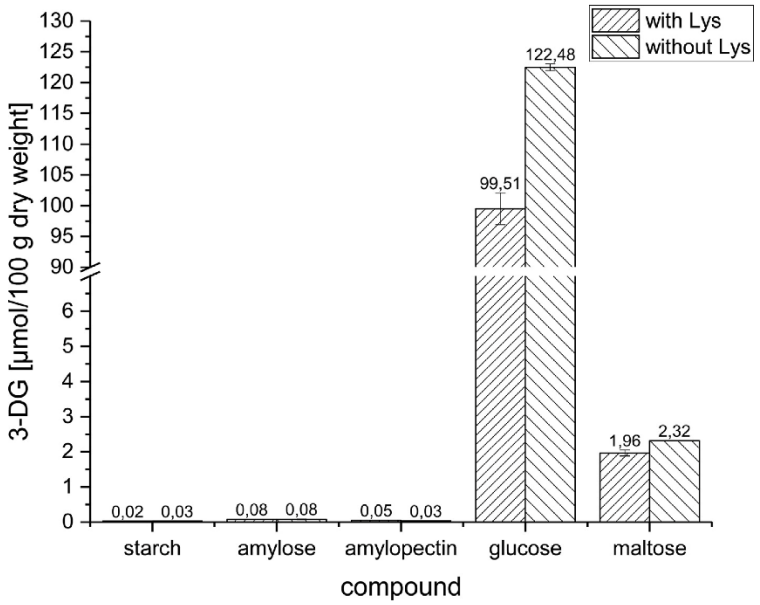

Fig. 4. The formation of 3-DG from various precursor compounds in kilning model reactions; n = 3.

glucose acted as the major precursor compound and promoted the formation of 3-DG at higher temperatures, which proves our hypothesis, stating that the final amount of 3-DG is influenced by technological malting conditions. 3-DGal was not detectable, which indicates that the isomerisation reaction doesn't occur in the malting process.

The malt modification level has no significant influence on the relative formation rate of 3-DG, but it has a strong effect on the absolute final content of 3-DG. On average, at all final kilning temperatures the high malt modification level (M1) increased the 3-DG amount by 40% when compared to that for the medium level (M2) and by 80% when compared to that for the low malt modification level (M3).

The amount of 3-DG and FL were remarkably higher than the HMF concentration at all variations. This indicates that the Maillard reaction stays in an early phase in the malting process. In further investigations of the formation of 3-DG, we used in a kilning model system to show that caramelization leads to higher absolute amounts of 3-DG than the Maillard reaction at malt kilning conditions. Based on glucose being the precursor compound 3-DG was formed up to 99.51 µmol/100 g dry weight under Maillard reaction conditions and up to 122.48 µmol/100 g dry weight under caramelization conditions. In the kilning process of barley grains, both pathways played a role, and their respective influence depended on the final kilning temperature.

Common final kilning temperatures are around 80 °C. We calculated the content of 3-DG at the beginning of the mashing process, which is the first process in beer production, arriving at the 3-DG concentration of 64 µmol/l; our calculation was based on a medium malt modification level (M2), a common ratio of malt to water of 4:1 and a complete transfer of 3-DG in the water phase. Comparing this previous work on maceration (80 µmol/l) (Bravo et al., 2008), the potential of 3-DG yielded with malt in beer production could be evaluated as strongly influential at the beginning of the brewing process. Even during the mashing process 3-DG can react as a precursor compound for aroma active compounds and, thus influence the molecular precursor potential for the constitution of known beer aging indicators. The amount of 3-DG in malt could already have a strong effect on the final sensory beer quality owing to its reaction potential. Therefore, we conclude that the malting conditions strongly influence the potential of aroma substance formation in mash and wort based on 3-DG reactivity.

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Conflict of interest statement

There is no conflict of interest among the authors.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.03.144.

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3.3 Formation and degradation of 3-deoxyglucosone as a key intermediate for ageing indicators during the wort boiling process

Research article

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Formation and degradation of 3-deoxyglucosone as a key intermediate for ageing indicators during wort boiling

Arndt Nobis, Stefan Wendl, Thomas Becker and Martina Gastl*

The metabolite 3-deoxyglucosone (3-DG) is formed by carbohydrate caramelisation or the Maillard reaction. 3-DG is a precursor in the Strecker reaction forming beer ageing compounds, such as 2-methylbutanal or 3-methylbutanal. Although 3-DG is known as intermediate, recent studies have focused on 3-DG in beer. Foremost, the thermal load during wort boiling provides the best conditions for 3-DG formation and degradation, however, the reactivity of the dicarbonyl during the boiling process has not yet been explained. As a key intermediate, 3-deoxyglucosone could be a critical indicator for beer ageing stability. The 3-DG formation and reactivity during wort production depends on its precursor reactants (amino acids and glucose). The concentration in wort of these substances was varied using two malts with different malt modification along with two different mashing programmes. 3-Deoxyglucosone reactivity was observed by analysing dehydratisation to HMF (HPLC-UV), interconversion to 3-deoxygalactosone (3-DGal, HPLC-UV) and selected Strecker aldehydes (GC-SPME-MS). This study shows that wort boiling is the most important process in 3-DG formation as it contributes 47% of the final content compared with malting (28%) and mashing (25%). With degradation reactions, 3-DG is mainly interconverted to 3-DGal and, contrary to the literature, it could not be confirmed that enhanced 3-deoxyglucosone content affects Strecker reactions. The interconversion reaction during wort boiling determines the dicarbonyl potential of beer and influences the ageing stability. © 2021 The Authors. *Journal of the Institute of Brewing* published by John Wiley & Sons Ltd on behalf of The Institute of Brewing & Distilling.

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

Keywords: 3-deoxyglucosone; wort boiling; 3-deoxygalactosone; Maillard reaction; brewing

Introduction

Wort boiling is a key process step in beer production. The thermal load provides the best conditions for forming precursor substances. Typical compounds are dicarbonyls, which form aroma active substances in several chemical reactions. Specifically, the high content of low molecular sugars (glucose or fructose as precursor reactants) induces the best conditions for the Maillard reaction *(1)*. These carbohydrates can react through the Maillard reaction or caramelisation in enolisation and dehydration reactions to α-dicarbonyl compounds *(2, 3)*, with 3-deoxyglucosone (3-DG) the major compound in beer and wort *(4–6)*. Figure 1 summarises possible formation and degradation reactions of 3-DG during the wort boiling process.

Low molecular sugars such as glucose (Glc) can react with amino acids to form Amadori rearrangement products such as fructosyllysine (FL) that are precursor compounds for 3-DG or other dicarbonyl compounds *(2)*. After formation, 3-DG becomes a reactive compound and can be degraded in several consecutive reactions. Well known pathways include dehydration through formation of 3,4-dideoxyglucosone (3,4-DGE) to 5-(hydroxymethyl) furfural (HMF) *(5)*, the formation of Strecker aldehydes *(2, 7)* and the interconversion of 3-deoxyglucosone to 3-deoxygalactosone (3-DGal) *(4, 5)*. 3-DGal can react similarly to 3-DG *(5, 8)*. Beside these reactions, 3-DG can react in several pathways within the Maillard reaction, including the formation of melanoidins *(9)* or pyrraline by reacting with lysine *(10)*.

Regarding the formation of aroma active compounds, the Strecker degradation of amino acids is a dominant pathway that forms important aroma active compounds. Typical beer ageing flavours result from degrading valine (forming 2-methylpropanal), leucine (3-methylbutanal), isoleucine (2-methylbutanal) and phenylalanine (phenyl acetaldehyde) *(11)*. The free formed aldehydes during wort production cannot be transferred directly to beer, as yeast reduces them during fermentation. However, there are several masking reactions where aldehydes are first bound and then released in the final beer during the ageing process *(12)*. Possible reactions are the formation of bisulphite adducts *(13)*, imine adducts *(14)* or 2-substituted thiazolidine-4-carboxylic acids (cysteine adducts) *(15)*. It is reported that 85% of Strecker aldehydes formed during mashing and boiling are transferred to beer through masked reactions *(16)*. Therefore, the formation of ageing indicators in wort boiling are significant for final beer quality and sensory stability.

Until now, the reactivity of 3-DG to Strecker degradation has been described in several model reactions using single amino acids and dicarbonyl compounds *(17–22)*. Regarding the Strecker r-

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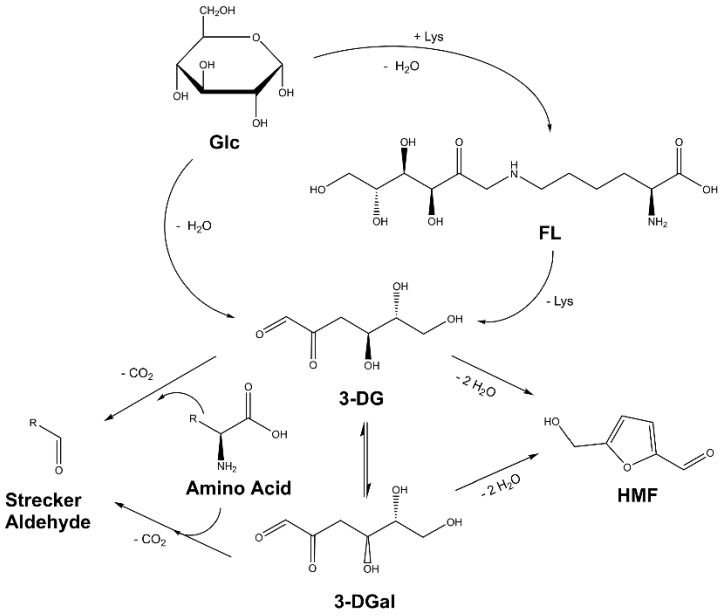

Figure 1. 3-DG metabolism during wort boiling

eactions, dicarbonyl is described as a reactive precursor compound *(22, 23)*. Within these experiments it was shown that small cleavage products of 3-DG, such as methylglyoxal or glyoxal, have an even higher reactivity towards Strecker degradation *(24, 25)*. Akagawa et al. showed the reaction to accelerate at higher pH values and at higher oxygen levels in model reactions *(18)*. Although the dicarbonyl potential for reacting in Strecker degradation exists *(6)*, until now, the 3-DG reactivity is described only in model reactions and not in complex food matrices *(22)*.

In beer production, there is limited insight into 3-deoxyglucosone formation. In the malting process a high amount of 3-DG (3.5–6.6 mg/100 g dry weight) is formed *(5, 26)*. Bravo et al. reported the concentration of 3-DG *(6)* during mashing (13 mg/L) and wort boiling (29 mg/L). During the boiling process, it was shown that the 3-DG content increased rapidly. The occurrence of the interconverted 3-DGal was first described by Bravo et al. *(6)*, but the interconversion of 3-DG and 3-DGal has not been reported in wort production. Recent studies have focused on beer with 3-deoxyglucosone a the major dicarbonyl compound, quantified in a range of 18–54 mg/L *(4–6)*. However, the formation and degradation of 3-DG as a key intermediate in wort production has not been investigated.

In this study, the hypothesis is that a varied initial 3-DG content and the potential of available reactants (amino acids, sugars) caused by different malt modification levels and intensity of mashing procedures influences the concentration of the dicarbonyl at the end of the wort boiling and subsequent reactions during the process. The reactivity of 3-DG could affect the final beer ageing stability in two ways. Firstly, the content of 3-deoxyglucosone and 3-deoxygalactosone provides the dicarbonyl potential in beer and induces ageing by improving the formation of beer ageing compounds. Secondly, the Strecker aldehydes exist in masked form in wort and beer and are released in the final beer, consequently accelerating beer ageing. Therefore, the aim of the study was to investigate the formation and selected degradation reactions of 3-DG as a key intermediate for ageing indicators during the wort boiling process by varying the initial content of 3-DG and its precursor reactants.

Material and methods

Chemicals

5-Hydroxymethylfurfural (HMF), all L-amino acids, [13C, 15N] labelled amino acids, L-lysin-6-13C-dihydro chloride, potassium dihydrogen phosphate, methanol (LC-MS-grade), o-phenylenediamine (OPD), calcium chloride dihydrate, glucose ammonium acetate, maltose, fructose, sucrose, hydrochloric acid, 4-fluorobenzaldehyde, citric acid, disodium hydrogen phosphate dihydrate, 3-methylcrotonaldehyde, acetic acid, magnesium sulphate, 2-methylbutyraldehyde (2MB), 3-methylbutyraldehyde (3MB), isobutyraldehyde (2MP), pheylacetaldehyde (PA), and water (LC-MS-grade) were obtained from Merck (Darmstadt, Germany). Acetonitrile for liquid chromatography mass spectrometry (LC-MS) analysis was purchased from VWR (Darmstadt, Germany). 3-DG (>95%) was from Apollo Scientific Ltd. (Cheshire, UK). 3-deoxygalactosone (>90%) was obtained from Carbosynth Ltd. (Berkshire, UK). The Chair of Food Chemistry of TU Dresden (Dresden, Germany) provided N^6-fructosyllysine (79.6%). Before use, water for analysis was purified using a micropore water purification system (Thermo Fisher Scientific Inc., Waltham, USA). Distilled water was used for the mashing trials.

Wort production and boiling

According to Nobis et al. *(26)*, two malts were produced by high (M1) and low (M2) proteolytic modification levels (assessed as soluble nitrogen) to vary the initial concentration of precursor

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reactants of 3-DG. The desired specification of soluble nitrogen for brewing of barley malts is 650–750 mg/100 g dry weight (according to Congress mashing procedure) *(27)*. The observed levels were 733 mg/100 g dry weight (M1) and 684 mg/100 g dry weight (M2) analysed using MEBAK (R-205.11.030 [2016-03]) *(28)*. The definition of high (M1 – upper limit of specification) and low (M2 - lower limit of specification) malt modification level was as the required specification and is not comparable to the previous study by Nobis et al. *(26)*. To influence the enzymatic hydrolysis during mashing (substrate/wort production), each malt was further mashed using two different laboratory mashing procedures ('45' at 45°C and '63' at 63°C). Laboratory procedure 45 impacts the activity of proteolytic and cytolytic enzymes resulting in higher concentration of low molecular proteolytic reactants, mainly amino acids. Procedure 63 focusses on amylolytic enzymes and is the reference procedure. Malt (50 g) was ground with a DLFU disk mill from Bühler (Braunschweig, Germany) at a disk gap of 0.2 mm. Afterwards, 200 mL of tempered water was added and the suspension was mashed using procedure 45 (20 min: 45°C; 10 min: 45°C–63°C; 30 min: 63°C; 6 min: 63°C–73°C; 30 min: 73°C; 5 min: 73°C–78°C; 2 min: 78°C) and procedure 63 (30 min: 63°C; 6 min: 63°C–73°C; 30 min: 73°C; 5 min: 73°C–78°C; 2 min: 78°C). Table 1 presents an overview of the variations produced in the study. Subsequently, all mashes were filtered through a laboratory folded paper filter (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). After filtration, the laboratory worts were adjusted to 11.5% mass with water according to the wort extract content of pale lager beer. Extract content was measured according to MEBAK guidelines method 2.9.6.2 *(29)*. In all, four laboratory worts with different (assumed) levels of reactants (45M1 > 45M2 > 63M1 > 63M2) were produced.

For the boiling trials, each variation (6 x 8 mL) was boiled in a water bath for 60 min in 15 mL sealed glass tubes with screw caps. Individual tubes were sampled every 10 min and the tubes immediately cooled down with water. For each analysis 0.5 mL (amino acids, dicarbonyls, HMF, sugars, FL) and 5 mL (Strecker aldehydes) were sampled and frozen. Transfer procedures of all wort samples were standardised. Each wort was incubated with and without spiking of 16 mg/L of 3-DG to investigate the influence of increased 3-DG content before boiling. All experiments were performed in triplicate.

Quantitation of Strecker aldehydes

Selected Strecker aldehydes (2MP, 2MB, 3MB, and PA) were determined using gas chromatography headspace solid phase microextraction mass spectrometry (GC-HS-SPME-MS) technique based on Saison et al. *(30)* as published by Lehnhardt et al. *(31)*. Methylcrotonaldehyde (2MP, 2MB and 3MB) and 4-fluorobenzaldehyde (PA) were used as internal standards. The calibration range of all Strecker aldehydes was 1–100 µg/L. Wort sample (1 mL) was mixed with 0.15 mL internal standard and 3.85 mL phosphate buffer (pH 5.5, 50 mM), placed in a 20 mL headspace vial and incubated at 40°C. The fibre was injected splitless at 270°C. The GC-system included Trace 1300 GC, TriPlus RSH autosampler and ISD QD mass spectrometer (Thermo Fisher Scientific Inc. Waltham, USA).

Quantitation of 3-deoxyglucosone (3-DG) and 3-deoxygalactosone (3-DGal)

Derivatisation and HPLC conditions were as previously performed by Degen et al. *(4)* and Nobis et al. *(26)*. A Kinetex column (2.6 µm phenyl-hexyl, 100 Å, 150 x 2,1 mm) was used. Solvent A was 0.075% acetic acid in water and solvent B was 20% solvent A in methanol. The gradient mode was as follows - 0 min, 90% A; 15 min, 55% A; 16 min, 25% A; 19 min, 25% A; 19.5 min, 90% A; 28 min, 90% A. The flow rate was 0.2 mL/min, the column oven temperature was 30°C, the injection volume was 5 µL and detection wavelength was 312 nm. Wort samples were measured directly after derivatisation and membrane filtration (0.45 µm).

Quantitation of 5-hydroxymethylfurfural

5-Hydroxymethylfurfural (HMF) was quantified by HPLC analytics according to the method published by Rufian-Henares et al. *(32)*. A Kinetex column (2.6 µm C18, 100 Å, 150 x 2,1 mm) was used. Solvent A was 2% acetonitrile in water and solvent B was methanol. The gradient mode was - 0 min, 100% A; 7 min, 100% A; 7.5 min, 20% A; 10.5 min, 20% A; 11 min, 100% A; 16 min, 100% A. The flow rate was 0.2 mL/min, the column oven temperature was 32°C, the injection volume was 15 µL and detection wavelength 280 nm. The HPLC system consisted of the UltiMate 3000 Autosampler, an UltiMate 3000 pump module, an UltiMate 3000 column compartment and the UltiMate 3000 Diode Array Detector (Thermo Fisher Scientific Inc. Waltham, USA). Data evaluation was performed by Chromeleon 6.80 Software from Thermo Fisher Scientific Inc.

Quantitation of low molecular weight sugars

Fermentable sugars (glucose, fructose, maltose, and sucrose) were determined by high-performance anion exchange chromatography pulsed amperometric detection (HPAEC-PAD). Eluent A (250 mM NaOH) and eluent B (water) were mixed in gradient mode (0 min, 90% B; 20 min, 90% B; 21 min, 20% B; 45 min, 20% B;

Table 1. Overview of laboratory mashes

Variation	Malt modification level	Mashing procedure
45M1	low malt modification level (M1: 684 mg/100 g dry weight soluble nitrogen concentration)	additional rest at 45°C to increase amino acid formation
45M2	high malt modification level (M2: 733 mg/100 g dry weight soluble nitrogen concentration)	additional rest at 45°C to increase amino acid formation
63M1	low malt modification level (M1: 684 mg/100 g dry weight soluble nitrogen concentration)	reference procedure
63M2	high malt modification level (M2: 733 mg/100 g dry weight soluble nitrogen concentration)	reference procedure

46 min, 5% B; 48 min, 5% B; 49 min, 90% B; 60 min, 90% B) at a flow rate of 0.25 mL/min. A Dionex CarboPack PA10 analytical column (2 x 250 mm) and Dionex CarboPack PA10 guard column (2 x 50 mm) were used (Thermo Fisher Scientific Inc. Waltham, USA). The HPAEC system consisted of an ICS AS/AP autosampler, an ICS 5000 DP pump module, an ICS 5000 DC column compartment, and an ICS 5000 PAD detector (Thermo Fisher Scientific Inc. Waltham, USA).

Quantitation of amino acids

Nineteen amino acids were determined according to Sonntag et al. *(33)* by high-performance liquid chromatography tandem mass spectrometry (HPLC MS/MS) in the multiple reaction monitoring (MRM) mode and as reported by Nobis et al. *(26)*.

Quantitation of fructosyllysine

Determination of fructosyllysine (FL) was performed as reported by Nobis et al. *(26)*. The HPLC Agilent 1200 series system (Agilent, Waldbronn) consisted of a HiP-ALS SL autosampler, a 1200 series bin pump module, a 1200 series degasser and a 1100 series column oven coupled to a Triple Quad 4500 MS (SCIEX, Darmstadt). The ion spray voltage was set to 5500 V, the curtain gas pressure was set to 35 psi, the nebuliser gas pressure was 55 psi and the heater gas pressure was 65 psi. The turbogas temperature was set to 450°C. The analysis was conducted using 13C-labelled lysine as an internal standard.

Model experiments with 3-deoxyglucosone

Investigations of 3-DG degradation and interconversion were performed by model incubations (2 mL) with phosphate buffer (50 mM) at different pH values (5.0, 5.2, 5.4, 5.6, 5.8 and 6.0). The first experiment was performed by varying the initial concentration of 3-DG at levels of 10, 20, 30, 40, 50 and 60 mg/L at pH value 5.6, which represents a typical wort pH. The second experiment was performed at different pH values (5.0, 5.2, 5.4, 5.6, 5.8 and 6.0) with an initial concentration of 30 mg/L 3-DG. Both variations were further conducted in the presence and absence of glycine at a concentration of 14 mg/L to evaluate the influence of the Maillard reaction. All samples were boiled for 60 min in closed systems (plastic tubes) and analysed in triplicate.

Statistical analysis

Statistical analysis was performed using the software JMP® Pro 12 (SAS Institute GmBH, Heidelberg, Germany).

Results and discussion

Sugars and amino acids in the wort boiling process

The levels of low molecular sugars and amino acids in the worts (45M1 > 45M2 > 63M1 > 63M2) were investigated. Notably as glucose is a well known precursor of 3-deoxyglucosone *(2)*, with all variations there was no significant degradation of glucose during wort boiling. Nevertheless, formation of 3-DG by glucose degradation could take place as formation and degradation of glucose occur simultaneously. The malt modification levels, and mashing procedures slightly affected the resulting glucose concentration in wort. Mashing procedure 45 showed an initial glucose concentration of 5.8 ± 0.3 g/L for both malt modification levels. Mashing procedure 63 resulted in glucose concentrations of 6.3 ± 0.7 g/L (M1) and 4.6 ± 0.2 g/L (M2). In this approach a higher malt modification resulted in a higher initial glucose concentration (precursor reactant) in wort at the beginning of wort boiling. The more intensive malting procedure supported the amylolytic enzymatic activity *(34)*, thereby inducing an accelerated starch degradation during the mashing.

The second group of potential precursors for 3-DG formation in the Maillard reaction are amino acids, which were analysed during the boiling process. Table 2 shows the difference between the start and end of the boiling process of amino acids in all experimental approaches (45M1, 45M2, 63M1 and 63M2). Differences in amino acids indicate their reactivity during wort boiling. Additionally, 3-DG was spiked (16 mg/l) with each variation to investigate the influence of an increased concentration of the dicarbonyl on amino acid reactivity. Leucine (Leu), isoleucince (Ile), valine (Val), and phenylalanine (Phe) are common precursor compounds for Strecker reactions. Lysine is also shown as it was the only amino acid that was degraded constantly with all variations and is known as a key amino acid in the Maillard reaction *(2)*. The concentration of all measured amino acids and glucose during wort boiling are reported in the Supporting Information.

Comparing the initial level of amino acids (45M1: 2372 mg/L; 45M2: 3278 mg/L; 63M1: 2102 mg/L; 63M2: 1839 mg/L), it was confirmed that the mashing procedure which included a 45°C step increased the free amino acids. The enhanced proteolytic

Table 2. Changes (60 – 0 minutes) during wort boiling in selected amino acids, 3-DGal and HMF

Mashing procedure	Malt modification	3-DG addition (mg/L)	Δ leucine (mg/L)	Δ isoleucine (mg/L)	Δ valine (mg/L)	Δ phenylalanine (mg/L)	Δ lysine (mg/L)	Δ Σ amino acids (mg/L)	Δ 3-DGal (mg/L)	Δ HMF (mg/L)
45	M1	0	63.7	58.2	39.8	42.7	-113.8	416	4.1	0.3
		16	39.8	48.7	26.2	27.8	-131.4	364	4.7	0.4
	M2	0	16.5	28.3	10.7	14.7	-203.1	-177	2.7	0.2
		16	-6.3	26.8	2.7	-1.5	-206.7	-799	3.4	0.3
63	M1	0	-14.7	10.5	7.0	7.1	-177.3	-287	3.1	0.3
		16	0.9	5.4	2.6	3.8	-182.3	-354	3.6	0.4
	M2	0	-11.2	-11.8	-7.9	-7.8	-154.8	-294	2.0	0.2
		16	-4.5	-0.4	-7.7	-7.9	-153.8	-384	2.5	0.2

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enzyme activity at 45°C results in higher amounts of the reactants *(35)*. Regarding the effect of malt modification (assessed by soluble nitrogen), there was no consistent trend in the sum of amino acids in the initial wort. The higher malt modification level (M1) has a lower initial sum of amino acids than M2 at the mashing procedure with enhanced proteolytic activity (procedure 45) and this effect is reversed at the reference mashing procedure (procedure 63). Generally, higher malt modification induces higher soluble nitrogen *(36)*, peptide *(37)* or amino acid content *(26)* in malt. Therefore, presumably 45M2 has a higher content of peptides in malt that are degraded during mashing, resulting in a higher initial content of amino acids during wort boiling. 45M1 has a higher amount of amino acids in malt *(26)* which are more greatly degraded during mashing and therefore reduced at the beginning of boiling.

Besides 45M1, all variations showed a decrease in total amino acids during wort boiling. Presumably, the increase in 45M1 is caused by higher levels of Amadori products (FL in Figure 2), which are degraded and then released as amino acids by dicarbonyl formation *(2)* during wort boiling. The 3-DG spiked samples demonstrated a trend for the inhibited formation or enhanced degradation of amino acids (Supporting Information and Table 2). This overall effect indicates enhanced Strecker degradation *(7)* or alternative Maillard reaction pathways, such as the formation of advanced glycation end products by a higher initial 3-DG concentration.

As important precursor substances and reaction partners, amino acids such as lysine were extensively degraded in all variations. This was slightly increased by 3-deoxyglucosone addition and could be caused by pyrraline formation *(2)*. Selected precursors (leucine, isoleucine, valine and phenylalanine) for known Strecker aldehydes showed no significant degradation in type 45 mashing samples. 3-DG addition partly affected the formation of the amino acids during wort boiling by decreasing formation. Only variation 63M2 showed a consistent degradation of leucine, isoleucine, valine and phenylalanine during wort boiling. Generally, there was no indication of the influence of 3-DG towards Strecker degradation of the selected amino acids. Although the amino acids (Table 2) showed a trend in formation in most experiments, Strecker reactions occur as formation and degradation proceed simultaneously.

Formation of fructosyllysine and 3-deoxyglucosone during wort boiling

Following the reaction order, fructosyllysine was investigated as a direct precursor of 3-DG. In a complex wort matrix, caramelisation or the Maillard reaction does not only occur in wort boiling as it is already initiated in malting *(26)* and during mashing. Therefore, the initial concentration of the compounds varies during wort boiling due to malt modification and mashing. The formation and degradation of FL and 3-DG during wort boiling indicate the progress of the Maillard reaction and are directly linked to the 3-deoxyglucosone concentration in final wort. Figure 2 shows the concentration of FL and 3-DG during wort boiling. FL is mainly degraded, and 3-DG is formed in all variations without spiking (45M1, 45M2, 63M1, and 63M2). Recently it was shown that both compounds are formed simultaneously and the Maillard reaction is initiated in malt production *(5)*. Therefore, as demonstrated, the Maillard reaction goes further, focusing on the formation of dicarbonyls *(38)*.

The mashing procedure with the additional step of 45°C rest induces a higher FL initial concentration. Assumably, the enhanced lysine content reacts more strongly supported by a longer mashing procedure (and more reaction time) to FL. A higher malt modification level also results in a higher initial FL concentration. A more intensive malting process leads to higher contents of the Amadori compound in malt *(26)* and causes an increase in the initial FL concentration in wort. By including the recently published concentration of FL in the same malts *(26)*, it can be calculated that 37% at M1 and 50% at M2 of initial FL is already accounted from

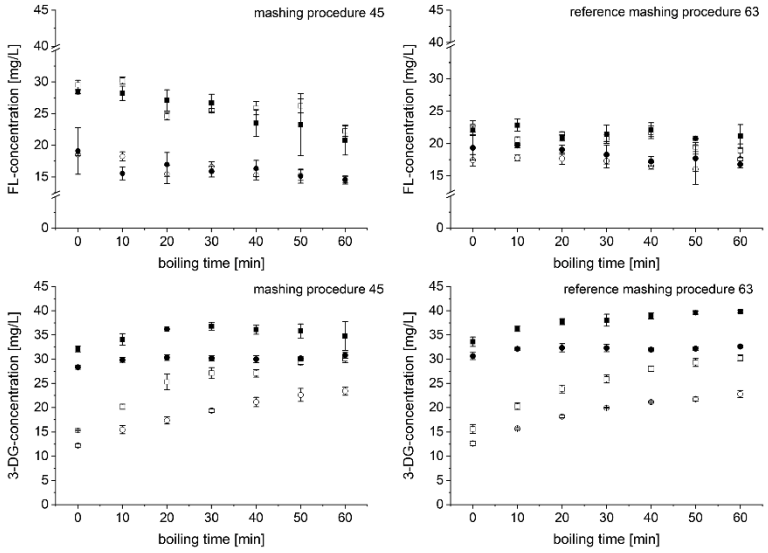

Figure 2. Concentration of 3-DG and FL during wort boiling at a high (M1, □) and low (M2, ○) malt modification level; white: without 3-DG; black: with 16 mg/L 3-DG; n = 3

the malt. Therefore, the FL formation during mashing could be low, through degradation during mashing. During boiling, FL is degraded in the following order: 45M1 > 45M2 ≥ 63M1 > 63M2. Accordingly, the increased concentration of the reactants (lysine and glucose) form higher amounts of FL during mashing, which is subsequently degraded during boiling. 3-DG spiking has no influence on FL degradation.

Fructosyllysine degradation results in the formation of 3-deoxyglucosone (Figure 2). The course of the curve of 3-DG concentration is comparable at all variations without spiking. Initially, the dicarbonyl is strongly formed for up to 20 minutes, but the rate of formation decreases during boiling. The relative formation of 3-DG is approximately 90% and independent of the variations (45M1, 45M2, 63M1 and 63M2). Unexpectedly, the results indicate that the mashing procedure does not affect the initial 3-DG concentration. In contrast, the level of malt modification shows a higher influence on the initial concentration of 3-deoxyglucosone. A greater modification level increases the initial 3-DG content, probably caused by a higher potential of the dicarbonyl due to the more intensive malting procedure *(26)*.

The 3-DG potential is the sum of the compound in malt, its precursor reactants (sugars and amino acids) and the enzyme potential for reactant formation during mashing. By evaluating the impact of the consecutive steps of wort production and comparing with the concentration of the same malt *(26)*, it could be calculated that independent of the variation, 28% of final 3-DG was formed during malting, 25% during mashing and 47% during wort boiling. Therefore, wort boiling mostly influences 3-DG formation in wort. The level of malt modification influences only the absolute content of 3-DG. Final concentrations are 30.0 ± 0.7 mg/L at 45M1 and 23.4 ± 0.8 mg/L at 45M2. These concentrations are comparable to Bravo et al. with 29.2 mg/L of 3-deoxyglucosone in the final wort *(6)*. Varying the initial concentration of 3-DG, the relative formation of the dicarbonyl decreased rapidly to 10%. The course stays more linear. Therefore, presumably the 3-DG degradation is catalysed in spiked samples, indicating that the Maillard reaction goes further to the final stage.

Degradation of 3-deoxyglucosone

The first reaction in the interconversion of 3-deoxyglucosone, where 3-deoxygalactosone is formed by dehydration and rehydration *(8)*. The concentration of 3-DGal at all sampling points is shown at Supplementary Information. The dicarbonyl compound is formed linearly in all variations. The mashing procedure with the additional rest at 45°C results in a higher initial concentration (45M1: 0.67 ± 0.02 mg/L; 45M2: 0.67 ± 0.05 mg/L) than the reference procedure (63M1: 0.50 ± 0.10 mg/L; 63M2: 0.34 ± 0.04 mg/L). Therefore, the interconversion occurs during mashing and is accelerated by a more intensive thermal load by an additional rest in the mashing procedure. At the end of wort boiling, the concentration of 3-DGal is the following order: 45M1 (4.72 ± 0.21 mg/L) > 65M1 (3.61 ± 0.31 mg/L) > 45M2 (3.39 ± 0.15 mg/L) > 65M2 (2.37 ± 0.12 mg/L). The absolute formation of 3-DGal is enhanced by the increased initial concentration of 3-DG at the beginning of wort boiling (Figure 2). Wort boiling results in approximately 85% of the 3-DGal content in the final wort. Samples spiked with 3-DG show a higher rate of formation during boiling (Table 2) and a higher final content of 3-DGal (Supplementary Information). The concentration of 3-deoxygalactosone was enhanced by 13% at the more intensive malt modification level and surprisingly by 20% in the M2 variation. Presumably, 3-DG has more reaction partners in M1 (e.g., amino acids) and other pathways are also stimulated. Generally, it could be shown that the enhanced initial concentration of 3-DG accelerates 3-DGal formation although only a minor amount of added 3-deoxyglucosone (about 4%) contributes through interconversion to 3-deoxygalactosone. The calculation of interconversion rate was based on the difference of the increased 3-DGal formation in spiked and non-spiked samples (-Supplementary Information). Presumably, spiked 3-DG is partly stable, forming the intermediate 3,4-dideoxyglucosone-3-ene (3,4-DGE) *(5)* or reacts in other pathways such as the formation of HMF.

The second degradation reaction was the dehydration of 3-DG to HMF. The concentration of HMF at each sampling point is shown in the Supplementary Information. The thermal load of wort boiling forms HMF constantly at all variations between 0.33 and 0.51 mg/L. These levels are comparable to reported values where HMF was quantified up to 1 mg/L *(39)*. The mashing procedure and malt modification show no influence on the final content of HMF. Except for 63M2, the final HMF concentration is increased by adding 3-DG (Table 2), suggesting that the dicarbonyl partly reacts to HMF during wort boiling. The relative increase varies between 27–48%, but the absolute increase is surprisingly low at 0.09 mg/L, reflecting a conversion rate of 0.7% of spiked 3-DG. Regarding the absolute formation, the interconversion of 3-DG to 3-DGal is more important than its degradation to HMF.

The third pathway to be investigated was the formation of Strecker aldehydes. The concentration of all Strecker aldehydes are reported in the Supplementary Information and Figure 3 shows the final concentration of 2MB, 2MP, 3MB and PA. Strecker aldehydes are formed during boiling. Contrary to De Schutter et al. *(40)*, the formation is non-linear, as in our study a temporary maxima appear during wort boiling. Surprisingly, the mashing procedure showed no significant influence on the formation of Strecker aldehydes, although the amino acid concentration rapidly increased with the additional 45°C mashing step. Therefore, some of the aldehydes are already bound before and released during wort boiling. Specifically, kilning provides the best conditions for aldehyde formation and masking reactions such as imine or 2-substituted thiazolidine-4-carboxylic acid formation**.** It can be assumed, that an increased malt modification level enhances the final concentration of Strecker aldehydes. Here, the initial concentrations of quantified aldehydes are already increased (-Supplementary Information). It is proposed that the level of malt modification enhances the overall potential for Strecker aldehyde formation. Although Hofmann et al. in model studies suggested 3-DG as a precursor compound in Strecker reactions *(41)*, spiked samples showed no significant increase in the final content of Strecker aldehydes in all variations. Therefore, 3-DG is either partly stable under wort boiling conditions or undergoes further consecutive reactions such as the formation of advanced glycation end products, fragmentation to methylglyoxal or protein modification *(2)*. Further Strecker aldehydes can be formed and be partly bound during wort boiling.

Interconversion of 3-deoxyglucosone in model solution

The interconversion of 3-DGal was the most critical reaction of the observed degradation reactions. Additionally, the wort boiling results indicate stability of 3-deoxyglucosone during wort boiling. Therefore, the interconversion of 3-DG in a wort model system was investigated by varying the pH value and the initial concentration of 3-DG with and without glycine. A variation in pH over the

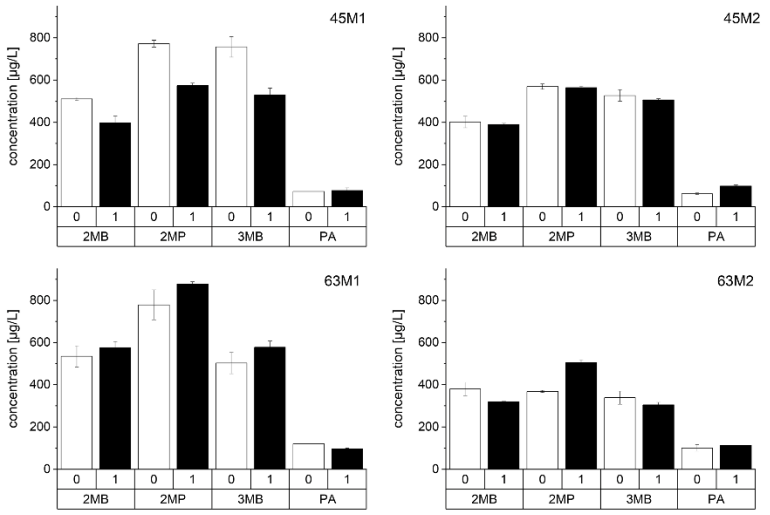

Figure 3. Final concentration of selected Strecker aldehydes during wort boiling; white bars (0): without 3-DG; black bars (1): with 16 mg/L 3-DG; n = 3

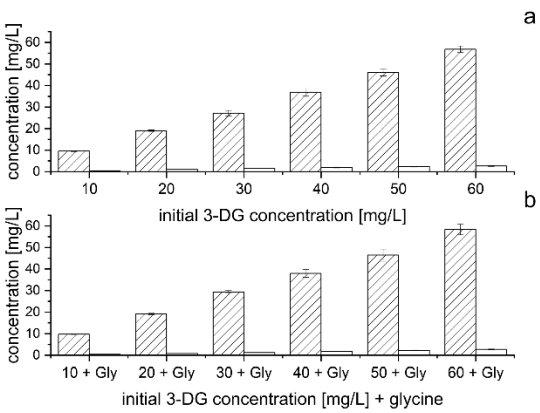

Figure 4. Dicarbonyl concentration after 3-DG incubation in wort boiling model systems at a pH 5.6; (a) varied initial concentration of 3-DG; (b) varied initial concentration of 3-DG + glycine; cross-striped bars: 3-DG; white bars; 3-DGal; n = 3

range of 5–6 does not affect the interconversion of 3-DG (data not shown). Figure 4 shows the concentration of 3-DG and 3-DGal after incubation with 3-DG degraded in all variations. The absolute amount of the degraded dicarbonyl is increased at higher initial concentrations. Therefore, the reaction potential is improved by an increased initial content of 3-DG. Regarding the relative degradation rate, there is no difference between the approaches. The reduction rate was about 17% in all variations. Therefore, most of 3-deoxyglucosone is stable under wort boiling conditions. The addition of glycine showed no significant difference in the degradation rate.

Further, the interconversion reaction was observed in the model incubations. The enhanced initial concentration of 3-DG results in a higher content of 3-DGal. Contrary to these results, the relative interconversion rate is independent of the initial 3-DG concentration with 25% of degraded 3-deoxyglucosone converted to 3-deoxygalactosone. Assumably, 3-DG undergoes further reaction such as polymerisation or dehydration to 3,4-DGE *(5)*. The addition of glycine did not affect the interconversion reaction. The 3-DGal formation in the model experiment explains the formation of 3-DGal in the wort boiling experiments and the increasing effect of 3-DGal formation by spiking experiments.

Conclusions

This study investigated the formation and selected degradation reactions of 3-deoxyglucosone during wort boiling. It was shown that a higher content of amino acids (precursor reactant) caused by an additional proteolytic rest in the mashing procedure had no significant effect on 3-DG formation during wort boiling. However, higher levels of malt modification enhance 3-DG formation. The study highlighted that close to 50% of the final 3-DG concentration in wort is formed during wort boiling and is the most important process of 3-DG formation in beer production. Besides the increase in dicarbonyl, the Amadori compound fructosyllysine decreased, indicating the Maillard reaction goes further to the advanced phase. In addition, the study investigated the degradation reaction to 5-hydroxymethylfurfural, the interconversion to 3-deoxygalactosone and the formation of selected Strecker aldehydes to evaluate the influence of 3-DG reactivity on the sensory quality of beer. HMF was formed during boiling, but the compound had a low conversion rate of 0.7% to 3-DG. Although the interconversion to 3-DGal was formed more strongly, only 4% of 3-DG was interconverted. Investigations of this phenomenon with model reactions showed that the dicarbonyl is stable under wort boiling conditions. Only 16% of 3-DG was degraded, whereby 25% of the degraded 3-DG was interconverted to 3-DGal. Comparing all the investigated degradation reactions, interconversion is the most important. Although 3-DG is known as a Strecker precursor compound *(22)*, an enhanced initial concentration of the dicarbonyl did not increase the final concentration of Strecker aldehydes. It can be assumed that additionally formed ageing compounds are partly bound or that 3-DG undergoes further reactions

such as AGE formation, cleavage reactions or protein modification. Generally, the study showed that wort boiling generates the main dicarbonyl potential by forming 3-deoxyglucosone as a key intermediate for ageing indicators. Therefore, the results indicate that wort boiling strongly influences the ageing stability in beer through the reactivity of 3-DG.

Author contributions

Arndt Nobis: conceptualisation, visualisation, investigation, writing (original draft).
Stefan Wendl: formal analysis, investigation.
Martina Gastl: conceptualisation, writing (review and editing), supervision, funding acquisition.
Thomas Becker: writing (review and editing), supervision

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Conflict of Interest

The authors declare there are no conflicts of interest.

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Supporting information

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

3.4 The influence of the 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

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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.

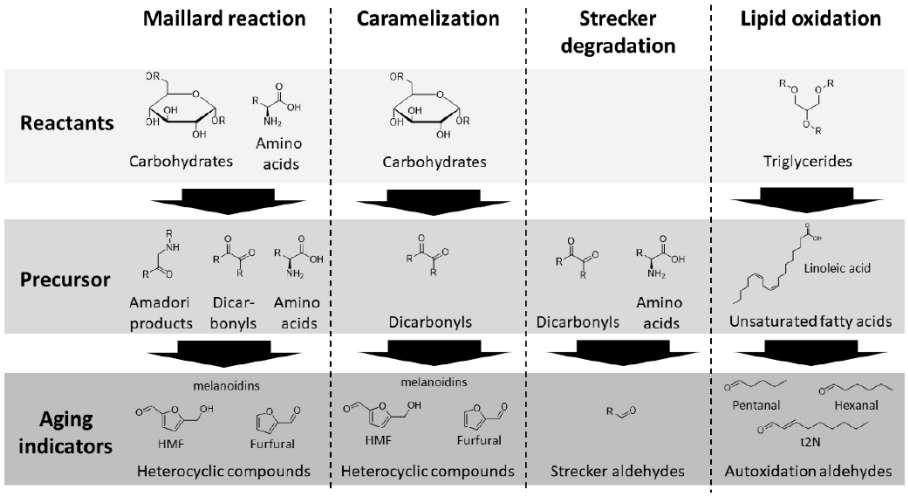

Figure 1. Overview of selected aging-relevant reactions and their key reactants, precursors, and aging indicators.

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.

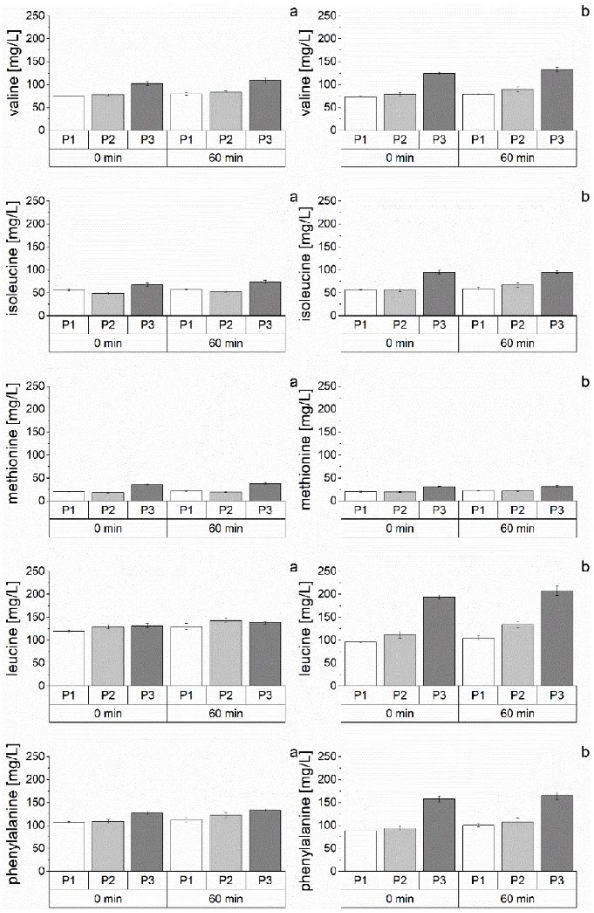

Figure 2. Concentration of selected amino acids at the start (0 min) and end (60 min) of boiling at different malt modification levels (P1 < P2 < P3; n = 3); (**a**) = B1; (**b**) = B2.

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.

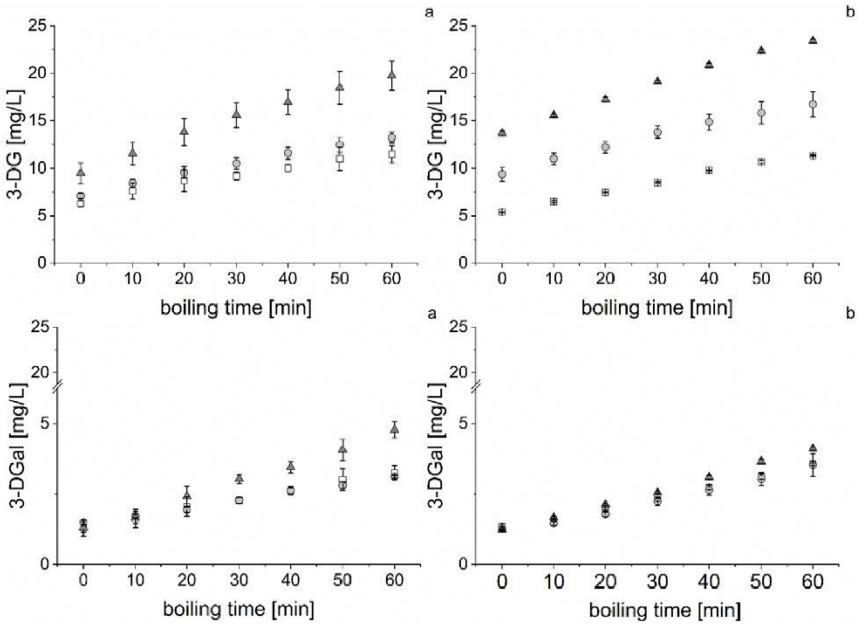

Figure 3. Formation of 3-DG and 3-DGal ((**a**) = B1, (**b**) = B2)) during wort boiling; squares: P1, points: P2, triangles: P3 (n = 3).

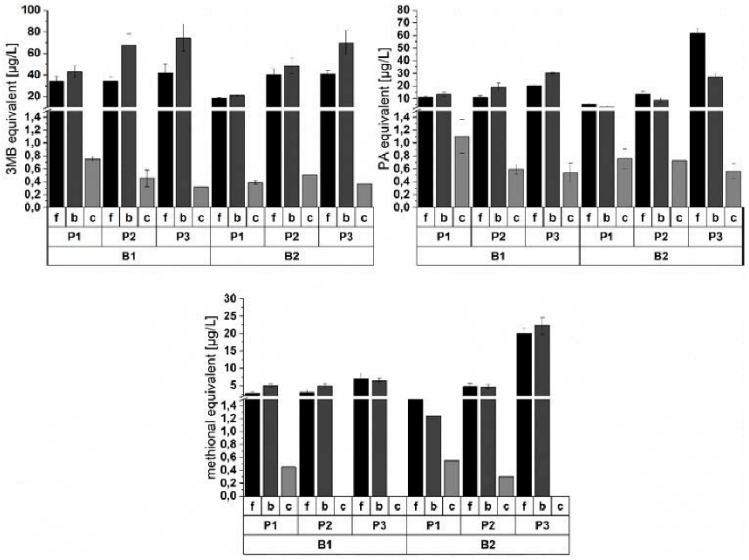

Figure 4. Concentration equivalent of free (f), 4-VP-releasable (b), and cysteinylated (c) forms of 3MB, PA, and methional at the end of boiling (n = 3).

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.

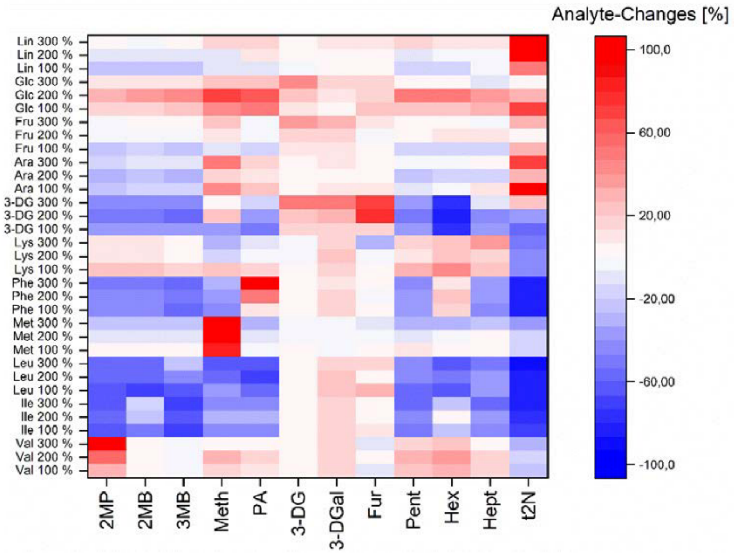

Figure 5. Relative changes [%] in dicarbonyl and aldehyde concentrations by artificially spiked various precursor substances after model wort boiling.

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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3.5 Influence of 3-DG as a Key Precursor Compound on Aging of Lager Beers

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Influence of 3-DG as a Key Precursor Compound on Aging of Lager Beers

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ABSTRACT: 3-Deoxyglucosone (3-DG) is a Maillard reaction intermediate, which forms known beer aging compounds such as Strecker aldehydes. However, the role of 3-DG in beer aging stability has not been described yet. To investigate the influence of 3-DG toward beer aging stability, different concentrations of 3-DG were added to the freshly brewed beer at the beginning of storage. Analysis of well-known degradation products of 3-DG such as 3-deoxygalactosone (HPLC-UV), 5-hydroxymethylfurfural (HPLC-UV), Strecker aldehydes (GC-MS), and free glycated amino acids (HPLC-MS/MS) during beer aging revealed that a higher initial 3-DG concentration increases the formation of the products. In this study, the significant importance of 3-DG as a key precursor compound in beer aging has been shown, especially the increase of Strecker aldehydes.

KEYWORDS: *3-deoxyglucosone, wort boiling, 3-deoxygalactosone, Maillard reaction, brewing*

INTRODUCTION

The aroma of the beer is a diverse synergistic impression of a complex mixture of sensory active aroma compounds. This composition of volatile compounds undergoes a dynamic change during the aging process that results in transformations in the sensory profile of aged beer.[1] For exemple, Zufall et al. (2005), based on work by Dalgliesh (1977), showed an increase of bready, caramel, sweet, and cardboard aroma impressions during beer aging leading to beer deterioration.[2,3] These impressions are caused by synergistic effects[1] as well as the formation of single aroma active substances during aging such as 2-methylbutanal (bready, malty), furfural (caramel), phenyl acetaldehyde (flowery, sweet), or (*E*)-2-nonenal (cardboard).[4]

One key reaction for the sensory changes, especially for the appearance of bready, caramel, and sweet impressions, is the Maillard reaction.[5] Dicarbonyl compounds are formed[6,7] during its advanced phase. Regarding this substance class, 3-deoxyglucosone (3-DG) is the major compound in beer, its process intermediates, and malt.[8,9] 3-DG is a reactive precursor substance that can lead to significant changes in the metabolomic profile of beer during aging.[10] Dicarbonyls formed during beer production are only partly stable in beer[11] and react in various pathways such as interconversion to 3-deoxygalactosone (3-DGal),[12] degradation to 5-hydroxymethylfurfural (HMF),[13] or melanoidin formation.[6] Further degradation reactions are the fragmentation to smaller cleavage products such as glyoxal (GO) or methylglyoxal (MGO),[14] Strecker degradation,[15] or advanced glycation end products formation (AGEs).[16] AGEs are formed by the reaction of dicarbonyls with selected amino acids and characterize the final stage of the Maillard reaction.[7] For example, pyrraline is formed from lysine (Lys) during reaction with 3-DG.[17] Further, Lys reacts with MGO to N^{ϵ}-carboxyethyllysine (CEL)[18,19] and with GO to N^{ϵ}-carboxymethyllysine (CML).[18,20] In addition to Lys, arginine (Arg) is also prone to be modified in the late Maillard reaction and forms the methylglyoxal-derived hydroimidazolone 1 (MG-H1) with MGO.[18] Figure 1 shows the above-mentioned metabolites.

Regarding all of the possible reactions originating from 3-DG, Strecker degradation has a key influence on aroma changes during aging.[21] Amino acids are degraded within this reaction to Strecker aldehydes such as 2-methylpropanal (2MP), 2-methylbutanal (2MB), 3-methylbutanal (3MB), phenylacetaldehyde (PE), or methional. These substances are well-known aging compounds in beer, leading to grainy (2MP), bready (2MB, 3MB), flowery (PE), and cooked-potato-like (methional) flavors.[1] Besides the Strecker degradation in a strict sense, the described aldehydes could be also formed by amino acid reaction with α-unsaturated carbonyls like (*E*)-2-nonenal,[22] by degradation with Amadori rearrangement products,[23] or by direct metal ion-catalyzed oxidation.[24]

The concentration of the dicarbonyl in fresh beer is important for the described degradation reactions resulting from 3-DG. The compound is present in beer because it is already formed during malting,[8] mashing, and wort boiling[11] in malt and beer production. Final amounts in beer range between 18 and 54 mg/L.[25] In the literature, it is shown that 3-DG is degraded during a forced beer aging method (105 days at 28 °C),[11] but less is known about the distribution of possible degradation pathways of 3-DG during natural beer

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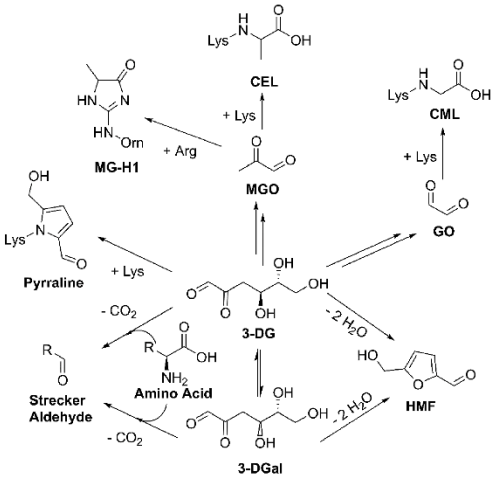

Figure 1. Degradation pathways of 3-deoxyglucosone.

aging. The initial 3-DG content in beer could be used as an aging indicator, which was already suggested in related food matrices such as wine.[26]

We hypothesize that the initial content of 3-DG influences the formation of Strecker aldehydes during beer aging and, therefore, has an influence on the beer stability toward aroma changes. The potential of 3-DG reactivity in fresh beer could be of importance for beer aging stability and could be an additional aging indicator for lager beers. The aim of the study is to investigate the distribution by artificial variation of the initial dicarbonyl concentration of common aging-related 3-DG degradation pathways in beer.

MATERIAL AND METHODS

Chemicals. HMF, 2MP, 2MB, 3MB, PE, methional, ammonium acetate, potassium dihydrogen phosphate, 4-fluorobenzaldehyde, methanol (LC-MS-grade), *o*-phenylenediamine (OPD), hydrochloric acid, nonafluoropentanoic acid, disodium hydrogen phosphate dihydrate, acetic acid, and water (LC-MS-grade) were purchased from Merck (Darmstadt, Germany). Acetonitrile used for liquid chromatography-mass spectrometry (LC-MS) analysis was obtained from VWR (Darmstadt, Germany), and 3-DG (>95%) was obtained from Apollo Scientific Ltd. (Cheshire, UK). 3-DG for spiking experiments was synthesized according to Hellwig et al. (2010).[12] Its purity was checked by the reference standard and matched the described reference quality. 3-DGal was purchased from Carbosynth Ltd. (Compton, UK). Reference material for AGEs quantification was synthesized as published before: CML,[27] CEL,[27] pyrraline,[28] and MG-H1.[27] Prior to use, the water for solutions, buffers, and high-performance liquid chromatography analysis with ultraviolet detection (HPLC-UV) was treated by a micropore water purification system (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Beer Production and Aging Experiments. For the brewing trials, a grist of 100% Pilsen-type malt "Weyermann Pilsner Malz" from Mich. Weyermann GmbH & Co. KG (Bamberg, Germany) was used. Brewing water was obtained from the TU Munich Research Brewery (Freising, Germany). The malt was milled with a Type 16/16 two-roller mill from Künzel (Kulmbach, Bayern). The pilot brew house (80 L scale) consisted of a brew water reservoir, mash kettle, lauter turn, brew kettle, whirlpool, and a plate heat exchanger. Grist (10 kg) was mashed in with 40 L of previously heated water (60 °C). Mashing was conducted by the first rest of 30 min at 62 °C and the second rest of 30 min at 72 °C. At the end of mashing, the mash was heated up to 78 °C and transferred to the lauter turn. Lautering was performed at 78 °C and sparged twice with 15 L followed by one 14 L sparge. After lautering, the wort was boiled for 60 min at atmospheric pressure. At the beginning of boiling, 30.3 g of Hallertauer Magnum pellets (13.2% alpha acid) was added to the brew kettle. After adjusting to 11.5°P, the wort was pumped into the whirlpool for a 15 min rest and afterward cooled down to 10 °C. Fermentation was done in a cylindroconical fermentation vessel with a volume of 50 L. Fermentis Saflager yeast (yeast strain W-34/70) from Fermentis (Marcq-en-Baroeul, France) was pitched at a concentration of 11^6 cells/mL. The fermentation temperature was maintained at 12 °C until the extracted content was below 3°P and followed by maturation at 19 °C until the diacetyl content was below 0.10 mg/L. Cold storage was done for 4 weeks at a temperature of 1 °C. After storage, beer was filtered by Seitz K150 filtration sheets (Pall-Seitz, Bad Kreuznach, Germany) and bottled in brown glass bottles with a volume of 335 mL by a semiautomatic filling unit (Alfred Gruber GmbH, Eugendorf, Österreich). Beer was produced in triplicate.

Each beer was additionally spiked with 3-DG, increasing the initial concentration of 25 mg/L by 50% and 100% to investigate the influence of a varied initial 3-DG content on beer aging. Therefore, 200 (50% increase) or 400 μL (100% increase) of a stock solution of 3-DG (21.4 g/L) was added to the bottle immediately before filling in a carbon dioxide atmosphere. This leads to three types of fresh beer with a varied initial 3-DG concentration: (1) reference beer (25 mg/L); (2) reference beer + 12.5 mg/L 3-DG (37.5 mg/L); (3) reference beer + 25 mg/L 3-DG (50 mg/L).

Each beer type was naturally aged at room temperature (20−25 °C) and sampled at months 1, 2, 3, 6, and 12. The bottles were exposed to light during storage. In addition, the established forced aging method (24 h shaking and 4 days incubation at 40 °C)[29] for beer was used to compare forced and natural aging. The used forcing method is an established procedure to predict beer aging for about 3 months of natural aging.

Quantitation of Strecker Aldehydes. Determination of 2MP, 2MB, 3MB, PA, and methional was applied as described by Lehnhardt et al. (2020)[30] based on Saison et al. (2008).[31] The Thermo Scientific Inc. (Waltham, MA, USA) GC-MS system consisted of a TriPlus RSH autosampler, an Ultra 1300 GC oven, and an ISQ QD mass spectrometer. A DB-5 column (inner diameter, 0.25 mm; film thickness, 0.25 μm; length, 60 m) from Thermo Fisher Scientific Inc. (Waltham, MA, USA) was used for chromatographic separation. The temperature program started at 60 °C for 4 min, was constantly increased by 5 K/min up to 250 °C, and was held at 250 °C for 3 min. Detection was done in full-scan mode (m/z 35−350) after electron ionization. *p*-Fluorobenzaldehyde was used as an internal standard. Beer samples were used directly without any sample preparation before adding the internal standard. Data evaluation was done by Xcalibur 3.1.66.10 (Thermo Scientific Inc., Waltham, MA, USA).

Quantitation of 3-DG and 3-DGal. Derivatization and HPLC conditions were applied as previously performed by Degen et al. (2012).[25] The HPLC system consisted of an UltiMate 3000 Autosampler, an UltiMate 3000 pump module, an UltiMate 3000 column compartment, and an UltiMate 3000 Diode Array Detector. All devices were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Data evaluation was performed by Chromeleon 7.20 software from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

Quantitation of HMF. The concentration of HMF was assessed using the method reported by Rufian-Henares et al.(2006).[32] A Kinetex 5 μm C18, 100 Å, 250 × 4.6 mm column (Phenomenex, Aschaffenburg, Germany) was used. Solvent A was 5% acetonitrile in water, and solvent B was methanol. The gradient mode was modified (0 min 100% A; 9 min, 100% A; 9.5 min, 20% A; 11.5 min, 20% A; 12 min, 100% A; 17 min, 100% A). The flow rate was 1.0 mL/min, the column oven temperature was set to 32 °C, the injection volume was 20 μL, and detection was carried out at 280 nm. The HPLC system was the same as that described earlier. Samples were directly measured after filtration (0.45 μm).

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Table 1. Transitions Recorded during MRM Measurement of Free Glycated Amino Acids in Beer[a]

compound	precursor ion [m/z]	product ion [m/z]	fragmentor voltage [V]	collision energy [eV]	Q/q[b]
MG-H1	229	114	120	10	Q
	229	166	120	10	q
[$^{13}C_6$]MG-H1	235	121	100	12	Q
	235	171	100	12	q
pyrraline	255	148	80	20	q
	255	175	80	10	Q
[$^{13}C_6$,$^{15}N_2$]pyrraline	263	153	75	20	q
	263	182	75	5	Q
CML	205	130	100	20	q
	205	84	100	10	Q
[$^2H_2$]CML	207	130	80	20	q
	207	84	80	10	Q
CEL	219	130	100	20	q
	219	84	100	10	Q
[$^{13}C_3$]CEL	222	130	100	20	q
	222	84	100	10	Q

[a]General conditions:dwell time, 70 ms; positive mode. [b]Q, transition used for quantitation; q, transition used for the confirmation of the presence of the analyte.

Table 2. pH, Color, Alcohol Content, and Extract of Produced Beers at M0, M6, and M12

	M0			
	pH value	color [EBC]	alcohol content [% vol]	extract [% mass]
fresh beer	4.66 ± 0.06	6.04 ± 0.01	4.76 ± 0.07	4.23 ± 0.13
M6				
reference	4.66 ± 0.03	6.67 ± 0.15	4.69 ± 0.06	4.24 ± 0.14
reference + 12.5 mg/L 3-DG	4.64 ± 0.05	7.19 ± 0.23	4.69 ± 0.04	4.22 ± 0.08
reference + 25 mg/L 3-DG	4.64 ± 0.05	7.03 ± 0.24	4.67 ± 0.06	4.25 ± 0.14
M12				
reference	4.63 ± 0.05	7.47 ± 0.12	4.67 ± 0.06	4.20 ± 0.12
reference + 12.5 mg/L 3-DG	4.62 ± 0.05	8.00 ± 0.38	4.66 ± 0.06	4.22 ± 0.11
reference + 25 mg/L 3-DG	4.62 ± 0.03	8.40 ± 0.15	4.66 ± 0.07	4.21 ± 0.14

Quantitation of Glycated Amino Acids. As in the previous works,[18] a 1200 Series high-pressure gradient system (Agilent, Böblingen, Germany), composed of a binary pump, an online degasser, a column oven, and an autosampler, was used with a stainless steel column (Zorbax 100 SB-C18, 50 mm × 2.1 mm, 3.5 mm, Agilent) maintained at a column temperature of 35 °C. As the eluents, a solution of 10 mM perfluoropentanoic acid (NFPA) in double-distilled water (solvent A) and a solution of 10 mm NFPA in acetonitrile (solvent B) were used in the gradient mode at a flow rate of 0.25 mL/min (0 min, 10% B; 15 min, 66% B; 19 min, 66% B; 20 min, 10% B; 28 min, 10% B). The injection volume was 5 μL. Mass spectrometric detection was performed with a 6410 TripleQuad mass spectrometer (Agilent), working in the positive mode with a capillary voltage of 4000 V and a source temperature of 350 °C. The HPLC effluent was transferred to the mass spectrometer between 3 and 21 min, and the transitions given in Table 1 were recorded. A total of 90 μL of degassed beer samples was mixed with 10 μL of isotopically labeled internal standards and measured after centrifugation for 10 min at 10 000 rpm. The final concentrations of isotopically labeled standards were 1 mg/L [$^{13}C_6$]MG-H1, 10 mg/L [$^{13}C_6$,$^{15}N_2$]pyrraline, 10 mg/L [$^2H_2$]CML, and 10 mg/L [$^{13}C_3$]CEL.

Chemical Analysis. Chemical characterization of beer was determined using standard methods according to MEBAK guidelines[33] for pH value (2.13), color (2.12.2), alcohol and extract content (2.9.6.2), carbon dioxide concentration (2.26.1.4), and bitter units (2.17.1).

Statistical Analysis. Statistical analysis was performed with the software JMP Pro 14 (SAS Institute GmBH; Heidelberg, Germany). Results are shown as average ± standard deviation. Differences between averages were checked by ANOVA (Tukey test) at a significance level of 0.05.

RESULTS AND DISCUSSION

Aging of Produced Beers. The study wanted to investigate the influence of a varied initial 3-DG concentration on the aging stability of a pale lager beer. The brewed reference beer showed a pH value of 4.66, a beer color of 6.04 EBC, an alcohol content of 4.76% vol, an extract of 4.23% mass, a carbon dioxide content of 0.42%, bitter units of 17.25 IBU, and an iso-α-concentration of 15.0 mg/L determined by HPLC analytics.[33] Therefore, the produced beer is suitable for common specifications of pale lager beers.[34] This reference beer was spiked with 3-DG and treated by natural and forced aging. Spiking concentrations are within the range that can also be observed in commercial beers.[25] Regarding beer aging, the chemical characteristics pH value, color, alcohol content, and extract were characterized to study the influence of a varied 3-DG concentration on these parameters. The values of fresh beer (M0), 6 months naturally aged beer (M6), and 12 months natural aged beer (M12) are shown in Table 2.

During aging, the pH value, alcohol content, and extract remain constant, which shows that the buffer system of beer is not interfered with by an enhanced 3-DG reactivity and that ethanol plays only a minor role as a reaction partner toward 3-DG. On the basis of melanoidin formation, the color of beer increases during aging.[1] In particular, 3-DG is known as a

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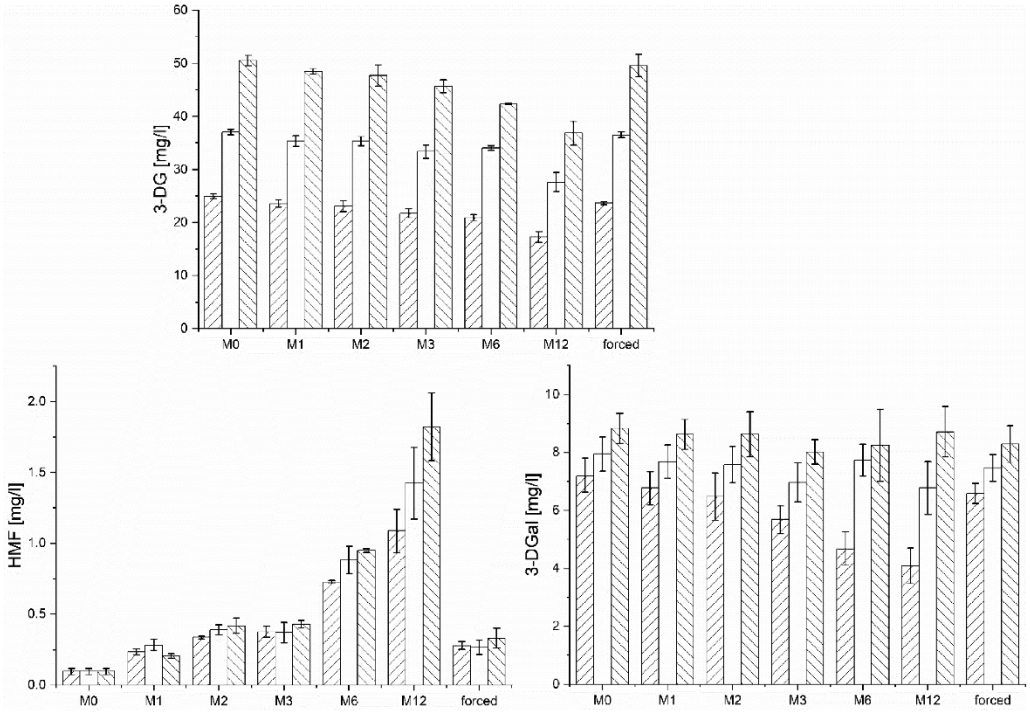

Figure 2. Formation and degradation of 3-DG, HMF, and 3-DGal: (left bar) reference; (middle bar) reference + 12.5 mg/L 3-DG; (right bar) reference + 25 mg/L 3-DG (n = 3).

typical precursor compound for high molecular melanoidin structures.[6] Therefore, 3-DG-spiked samples showed an accelerated beer color increase, which could be caused by an enhanced melanoidin formation. Generally, it is proven that the 3-DG content in fresh beer does not affect the investigated chemical characteristics but does affect beer color during aging.

Interconversion and Caramelization. In addition to the wet chemical characteristics, the study focused on several well-known degradation pathways of 3-DG, namely, the interconversion to 3-DGal, the degradation to HMF, Strecker reaction, and formation of AGEs. Therefore, first, 3-DG degradation was investigated. Fresh beer showed a 3-DG concentration of 24.9 ± 0.4 mg/L. The spiked samples had increased concentrations of 37.0 ± 0.5 (reference + 12.5 mg/L) and 50.5 ± 1.0 mg/L (reference + 25 mg/L), which confirms that the initial concentration of 3-DG could be increased without a remarkable loss during bottling. Figure 2 shows the degradation of 3-DG during natural and forced aging. Both beer aging methods reduce the 3-DG content, as confirmed by the Bravo et al. (2008) study, which also observed 3-DG degradation.[11]

The increased initial concentration of 3-DG caused a higher absolute mass degradation of the dicarbonyl with regard to natural aging. The degradation is linear but not equal for the different spiking levels. Thus, 3-DG is reduced monthly in the reference by 0.60 mg/L, in the first spiking level by 0.75 mg/L, and in the second spiking level by 1.11 mg/L. Bravo et al. (2008) showed a higher reduction at 2.4 mg/L per month in nonspiked beer (commercial) samples.[11] This could be caused by the higher incubation temperature of 28 °C compared to room temperature at about 23 °C in the present study. All three variations showed a comparable reduction of 28% up to 12 months of natural aging owing to relative degradation. It is assumed that the relative rate of the 3-DG degradation pathways does not depend on its initial concentration, which means that a higher 3-DG content in fresh beer causes absolute content of degradation product. This result indicates that 3-DG is potentially suitable as an analytical precursor indicator for beer aging. Regarding the forced aging method, 3-DG is even less degraded in forced aging than during natural aging up to month 1. The relative degradation rate is only about 3%. Further, the absolute degradation is not significant during forced aging. Therefore, the forced aging method cannot simulate the 3-DG reduction reactivity during natural beer aging. It could be assumed that the distribution of the degradation pathways is different for natural and forced aging and that the incubation temperature of 40 °C for 4 days is insufficient to simulate the 3-DG reactivity at room temperature for up to 1 year. The high difference between natural and forced aging indicates that 3-DG degradation in beer depends mainly on storage time and temperature.

The first degradation reaction of 3-DG observed is the interconversion to 3-DGal. The concentrations of 3-DGal are presented in Figure 2 at all sampling points. The initial 3-DGal

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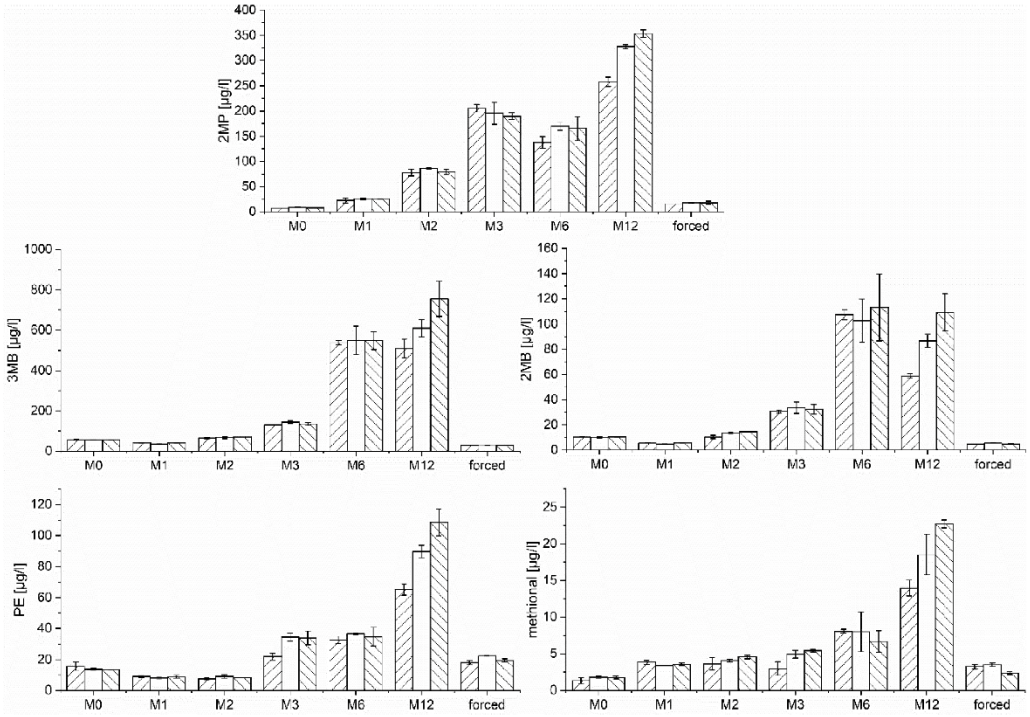

Figure 3. Formation of 2MP, 3MB, 2MB, PE, and methional during natural and forced beer aging;: (left bar) reference; (middle bar) reference + 12.5 mg/L 3-DG; (right bar) reference + 25 mg/L 3-DG (n = 3).

content of the reference beer was 7.2 ± 0.6 mg/L, which is comparable to recent published 3-DGal levels in beer ranging from a none detectable level to 16 mg/L.[9,25] The spiked beers showed a slightly increased initial 3-DGal content (reference + 12.5 mg/L 3-DG, 7.9 ± 0.6 mg/L 3-DGal; reference + 25 mg/L 3-DG, 8.8 ± 0.5 mg/L 3-DGal), which indicates that a small degradation of 3-DG instantly influences the interconversion balance toward 3-DGal. 3-DGal is degraded constantly in the reference sample during natural aging. Here, 43% of the initial concentration of 3-DGal must react in further pathways like degradation to HMF[6] up to 12 months of beer aging. Thus, 3-DGal is less stable than 3-DG in the pale beer used in this study. Regarding absolute degradation, 3-DGal has fewer effects on the reaction pathways than 3-DG, as only 0.26 mg/L is degraded per month, while the relative degradation rate is higher. It is assumed that 3-DG is the more important dicarbonyl for beer aging-related reactions. Surprisingly, the spiked variations showed no significant 3-DGal degradation during natural beer aging, which indicates that spiked 3-DG is constantly interconverted to 3-DGal. As shown in Figure 2, addition of 25 mg/L 3-DG led to the constancy of the 3-DGal concentration. This effect leads to a comparable 3-DG/3-DGal-ratio of 4.2 after 12 months of beer aging. This phenomenon indicates that 3-DG reactivity is mainly influenced by the interconversion balance to 3-DGal. During forced aging, 3-DGal is less degraded with a relative rate of 7% at all variations, which again shows that forced aging may not properly model reactions during "natural" aging.

The second degradation reaction observed in this study is the degradation of 3-DG to HMF as a representative pathway of caramelization. HMF is a beer aging marker mainly increasing by thermal treatment.[5,35] The concentrations during natural and forced beer aging are presented in Figure 2. The HMF concentrations increased linearly during natural beer aging at all variations according to the study of Madigan et al. (1998).[35] An increased 3-DG concentration in fresh beer caused an accelerated HMF formation and consequently a higher HMF content in aged beer. The reference samples showed an HMF formation of 82.0 µg/L per month, the reference + 12.5 mg/L 3-DG variation showed an increase of 110.0 µg/L HMF per month, and addition of 25 mg/L 3-DG in fresh beer caused an increase of 145.0 µg/L HMF per month. The dicarbonyl could be applied as an analytical precursor indicator for beer aging in fresh beer because of the accelerating influence of 3-DG during natural beer aging on the HMF formation. Regarding the forced aging method, the concentration of HMF only slightly increased compared to natural beer aging (Figure 2). An increased initial 3-DG concentration in beer caused no significantly higher contents of HMF in forced aged beer. This effect additionally indicates that the forced aging method cannot simulate 3-DG reactivity toward natural beer aging.

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Strecker Degradation and Maillard Reaction. Further, two degradation pathways were observed, namely, the Strecker reaction and the formation of AGEs, based on the reaction of 3-DG with amino acids. Within Strecker degradation, Strecker aldehydes are formed, which are well-known beer aging indicators.[5] Figure 3 shows the formation of 2MP, 3MB, 2MB, PE, and methional.

Strecker aldehydes are formed during natural and forced beer aging. Surprisingly, during forced aging, 2MB and 3MB showed a slight decrease. This effect also appears in natural beer aging up to 1 month and could probably be caused by the possible conversion of the aldehyde group into masking reactions[36] or oxidation reactions.[5] Comparing both aging methods, it is remarkable that for all Strecker aldehydes besides PE, the levels after forced aging have comparable concentrations to 1 month of natural aging. PE showed a comparable level for forced aging after 3 months of natural beer aging. In this study, natural aging caused a more intensive formation of Strecker aldehydes, although the temperature is increased temporarily (4 days, 40 °C) during forced aging, which predicts about 3 months of beer aging. An increased initial concentration of 3-DG did not affect Strecker aldehyde formation during forced beer aging.

Regarding the more important natural beer aging, it is noticeable that formation of the analyzed Strecker aldehydes is different. 3MB, 2MB, and PE first underwent a reduction and were further formed after 2 months of aging. 2MP showed a local maximum level after 3 months, and methional is formed steadily. These deviations were also reported by Lehnhardt et al. (2018)[21] and could be caused by alternating formation and degradation reactions of the aldehyde compounds. There is no significant influence of the initial concentration of 3-DG on the formation of the Strecker aldehydes up to 6 months of natural aging, but after 12 months, all Strecker aldehydes showed increased levels at the 3-DG-spiked variations. Therefore, in this study, an increased initial concentration of 3-DG in fresh beer leads to an enhanced Strecker aldehyde formation after 6 months of natural beer aging. This indicates that the Strecker aldehydes are first mainly formed by release from their bound form[36] or alterative formation ways such as amino acid reaction with other dicarbonyls like methylglyoxal,[37] α-unsaturated carbonyls,[22] or Amadori compounds.[23] It is assumed that formation of amino acids out of the reaction with 3-DG occurred simultaneously, but alternative formation pathways are advantageous in the storage period up to 6 months. Because 3-DG is described as a precursor compound for Strecker reaction[15] in this study, de novo formation of Strecker aldehydes out of 3-DG reactivity is assumed to be delayed and increased in the advanced phase (after 6 months) of natural beer aging. Recent studies observed also a delayed de novo formation of Strecker aldehydes from deuterated spiked amino acids after a aging period of 3 months at an increased storage temperature of 30 °C.[38] The formed Strecker aldehydes are analytical and aroma-relevant beer aging indicators. In particular, because of common shelf lifes up to 12 months for pale lager beers, it is shown that the initial concentration of 3-DG has an influence on prolonged natural beer aging.

In addition to Strecker degradation, 3-DG can also react with amino acids to AGEs in the Maillard reaction. The concentrations of MGH1, pyrraline, CML, and CEL are presented in Table 3. The concentration of selected AGEs remained mainly constant up to 6 months of natural beer

Table 3. Concentration of Selected AGEs

	MGH1 [μg/L]			pyrraline [μg/L]			CML [μg/L]			CEL [μg/L]		
aging	reference	reference + 12.5 mg/L 3-DG	reference + 25 mg/L 3-DG	reference	reference + 12.5 mg/L 3-DG	reference + 25 mg/L 3-DG	reference	reference + 12.5 mg/L 3-DG	reference + 25 mg/L 3-DG	reference	reference + 12.5 mg/L 3-DG	reference + 25 mg/L 3-DG
M0	601.3 ± 73.2	526.7 ± 8.7	567.6 ± 81.5	302.4 ± 19.1	291.5 ± 17.5	295.2 ± 15.9	122.8 ± 6.4	124.4 ± 12.2	127.5 ± 8.6	94.4 ± 10.8	91.8 ± 2.3	97.5 ± 6.3
M1	627.44 ± 10.8	685.0 ± 10.7	664.9 ± 3.7	307.2 ± 9.9	298.3 ± 7.9	286.1 ± 10.9	142.3 ± 6.9	142.8 ± 1.6	138.3 ± 6.7	109.9 ± 2.1	100.5 ± 2.1	91.7 ± 1.3
M2	623.2 ± 20.4	638.1 ± 3.2	635.3 ± 23.3	297.8 ± 1.3	323.4 ± 16.9	322.1 ± 19.8	137.6 ± 9.6	137.9 ± 9.0	138.7 ± 10.1	97.3 ± 2.1	98.1 ± 5.4	95.4 ± 3.6
M3	684.0 ± 32.8	635.1 ± 4.5	620.9 ± 76.1	307.8 ± 7.9	300.8 ± 11.2	307.4 ± 12.2	130.0 ± 1.9	140.9 ± 5.5	140.8 ± 15.0	98.0 ± 2.1	98.1 ± 5.4	95.4 ± 3.6
M6	446.5 ± 73.0	671.0 ± 4.3	650.7 ± 30.2	212.6 ± 40.2	271.1 ± 7.1	307.3 ± 11.8	101.6 ± 4.0	138.3 ± 5.8	140.8 ± 8.8	69.1 ± 4.5	91.6 ± 5.1	89.4 ± 1.1
M12	674.6 ± 53.8	688.3 ± 84.9	736.6 ± 40.2	282.0 ± 23.7	294.2 ± 4.4	330.4 ± 23.7	173.1 ± 3.6	182.3 ± 16.0	181.0 ± 5.6	103.6 ± 0.2	107.5 ± 4.1	108.3 ± 7.0
forced	544.0 ± 0.5	662.4 ± 110.9	631.3 ± 112.3	297.5 ± 13.5	301.5 ± 6.5	323.2 ± 17.5	122.0 ± 0.5	132.1 ± 6.7	129.0 ± 1.9	95.7 ± 7.3	102.9 ± 2.4	100.8 ± 4.1

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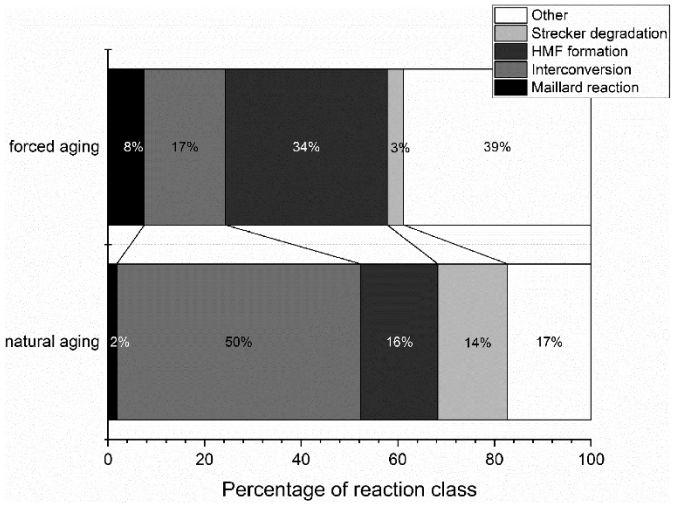

Figure 4. Distribution of degradation reactions of spiked 3-DG at natural (M12) and forced aging.

aging, which indicates that the Maillard reaction is already in the final stage in fresh beer.[7] Surprisingly, the reference beer showed reduced contents of MGH1 and pyrraline after 6 months of natural beer aging. It is assumed that both substances may have been degraded by reactions such as Strecker degradation or nucleophilic additions of their free amino groups during aging. Contrary to the reference samples, spiked variations showed slightly increased (pyrraline, MGH1) or indistinguishable (CML, CEL) levels after 6 months of natural beer aging. The slightly increased levels are caused by an enhanced formation owing to an increased initial 3-DG concentration. After 6 months of beer aging, MGH1 and CML are formed more strongly, whereas pyrraline showed levels comparable to fresh beer. Formation of MGO owing to the cleavage of 3-DG or other dicarbonyls is thermally induced.[14] As a result, the formation of MGH1 could be strongly delayed during natural beer aging, because 3-DG first needs to be converted to MGO. Regarding the effect of an increased initial 3-DG concentration in fresh beer, only pyrraline showed a significantly increased concentration after 12 months of natural beer aging. The increase is probably caused by an enhanced reaction of 3-DG and lysine to pyrraline.[17] Forced aging showed no significant effect on the selected AGE compounds. Generally, formation of AGEs in natural and forced beer aging plays only a minor role in 3-DG reactivity.

Distribution of 3-DG Degradation Pathways. To evaluate the observed 3-DG reaction pathways, the percentile distribution of Strecker degradation, HMF formation, interconversion to 3-DGal, AGEs formation (Maillard reaction), and other reactions was calculated in forced and natural aged beer. The calculation is based on the difference of the concentration of the degradation products between the reference beer and the spiked variation, where 25 mg/L 3-DG was added. As compared to the reference sample, 100% conversion was set by the absolute increased molar content of degraded 3-DG at the spiked variation (reference + 25 mg/L). The distribution of the observed degradation pathways of forced aging and the natural aging of 12 months is presented in Figure 4. M12 was decided because commercial samples in Germany show shelf lifes up to 12 months.

Regarding forced aging, other reactions (39%) such as melanoidin formation or protein modification[6] are the major degradation reactions. Second, the conversion to HMF is the most important reaction in which 34% of initial 3-DG is degraded to HMF during forced beer aging, which is mainly thermally treated. Thus, the increased thermal treatment during forced aging catalyzes thermal focused reaction pathways.

Natural beer aging showed a different distribution. In this study, the interconversion to 3-DGal (50%) is the major degradation pathway of 3-DG. An increased initial concentration of 3-DG mainly reacts to 3-DGal owing to a balanced ratio of 3-DG and 3-DGal. Compared to the forced aging, the Strecker reaction percentage is strongly increased from 3% to 14%, which indicates that the 3-DG reactivity toward abolute formation of Strecker aldehydes is more influenced by time than by temperature. Formed Strecker aldehydes during forced aging could be degraded further because of the increased incubation temperature. This effect could also explain the increased percentage of other degradation reactions. The effect confirms the results of the Strecker aldehydes, where it is concluded that the de novo formation of Strecker aldehydes is strongly delayed during natural beer aging. Further, the percentages of AGEs (Maillard reaction) and HMF formation were decreased at natural aging compared to forced aging. This could be mainly due to less intense thermal treatment during natural aging. Other degradation reactions covered only 17% of the absolute 3-DG degradation. Therefore, 83% of the 3-DG degradation was described by the observed degradation reactions.

Generally, the distribution of the degradation pathways was very different in forced and natural beer aging. Thus, the forced aging method could not predict natural beer aging due to 3-DG reactivity because 3-DG underwent different degradation reactions in both methods. In summary, the study showed for the first time that an artificially increased content of 3-DG

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leads to an accelerated beer aging, especially the enhanced Strecker degradation, which causes common sensory active beer aging indicators. The hypothesis that the initial concentration of 3-DG in beer has an influence on beer aging toward Strecker aldehyde formation is confirmed during natural aging compared to forced beer aging. Therefore, 3-DG concentration has an influence on the beer aging stability in prolonged storage conditions (>6 months storage), and it is concluded that this dicarbonyl could be used as a key precursor indicator for beer aging.

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ABBREVIATIONS USED

3-DG, 3-deoxyglucosone; 3-DGal, 3-deoxygalactosone; HMF, 5-hydroxymethylfurfural; GO, glyoxal; MGO, methylglyoxal; AGEs, advanced glycation end products; Lys, lysine; CEL, N^{ε}-carboxyethyllysine; CML, N^{ε}-carboxymethyllysine; Arg, arginine; MG-H1, methylglyoxal-derived hydroimidazolone 1; HPLC, high-pressure liquid chromatography; 2MP, 2-methylpropanal; 2MB, 2-methylbutanal; 3-MB, 3-methylbutanal; PE, phenyl acetaldehyde/phenyl ethanal; OPD, *o*-phenylenediamine; LC-MS, liquid chromatography-mass spectrometry; NFPA, perfluoropentanoic acid; M, month; MRM, multiple reaction monitoring

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3.6 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

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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).

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

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.

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:

Weighted overall DLG score = [(2 × purity of smell) + (2 × purity of taste + palate fullness + freshness) + (2 × 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:

c(bound) (%]) = (c(released) − c(free))/(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)).

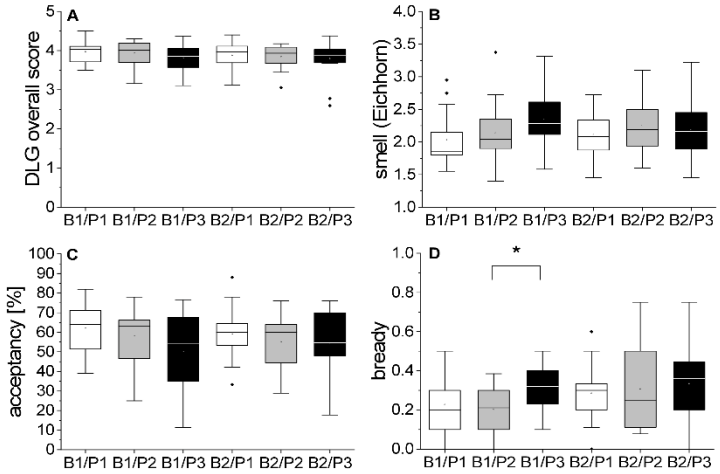

Figure 1. Sensory analysis of beers: (**A**) DLG overall score, (**B**) smell according to the Eichhorn scheme, (**C**) acceptancy (%), and (**D**) attribute bready. Boxplots show values of all aging points ($n = 18$). Linked boxplots showed a significant difference (*: $p < 0.05$). (B1: barley variety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

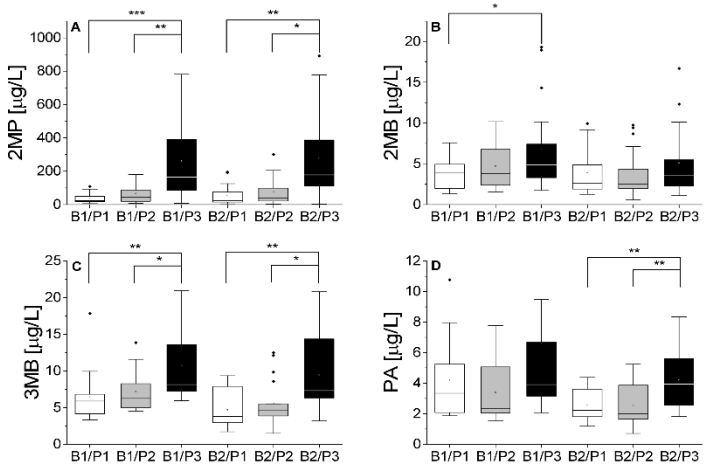

Figure 2. Boxplots of all aging points of chosen aldehydes ((**A**) 2MP; (**B**) 2MB; (**C**) 3MB; (**D**) PA) by malt modification level (B1: barley variety 1; B2: barley variety 2; P1: low proteolytic malt modification; P2: medium proteolytic malt modification; P3: high proteolytic malt modification).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

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].

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 release-able 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.

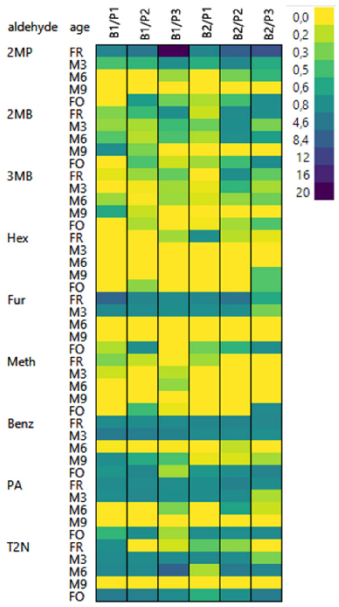

Figure 3. 4VP-releasable aldehydes (n = 6): the heatmap shows the relative concentration of bound state aldehydes in comparison to the free form (B1: barley variety 1; B2: barley variety 2; P1: low proteolytic malt modification; P2: medium proteolytic malt modification; P3: high proteolytic malt modification).

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.

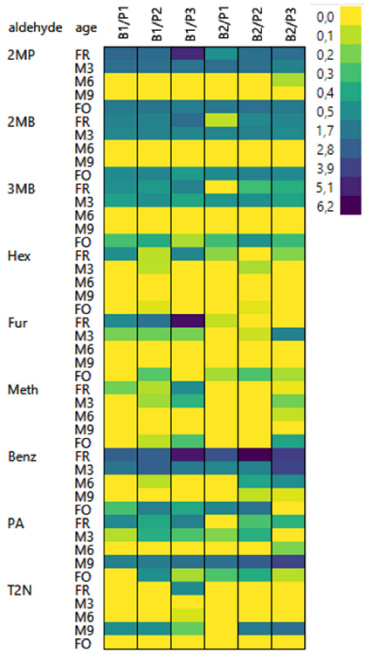

Figure 4. ACA-releasable aldehydes ($n = 6$): heatmap shows relative concentration of bound state aldehydes in comparison to the free form (B1: barley variety 1; B2: barley variety 2; P1: low proteolytic malt modification; P2: medium proteolytic malt modification; P3: high proteolytic malt modification).

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 ($K_a = 485 \times 10^3$), Meth ($K_a = 50 \times 10^3$) and 3MB ($K_a = 29 \times 10^3$) showed higher affinity toward HSO_3^- compared to PA ($K_a = 17 \times 10^3$), 2MP ($K_a = 2.8 \times 10^3$), 2MB ($K_a = 2.6 \times 10^3$), or Fur ($K_a = 0.1 \times 10^3$). 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.

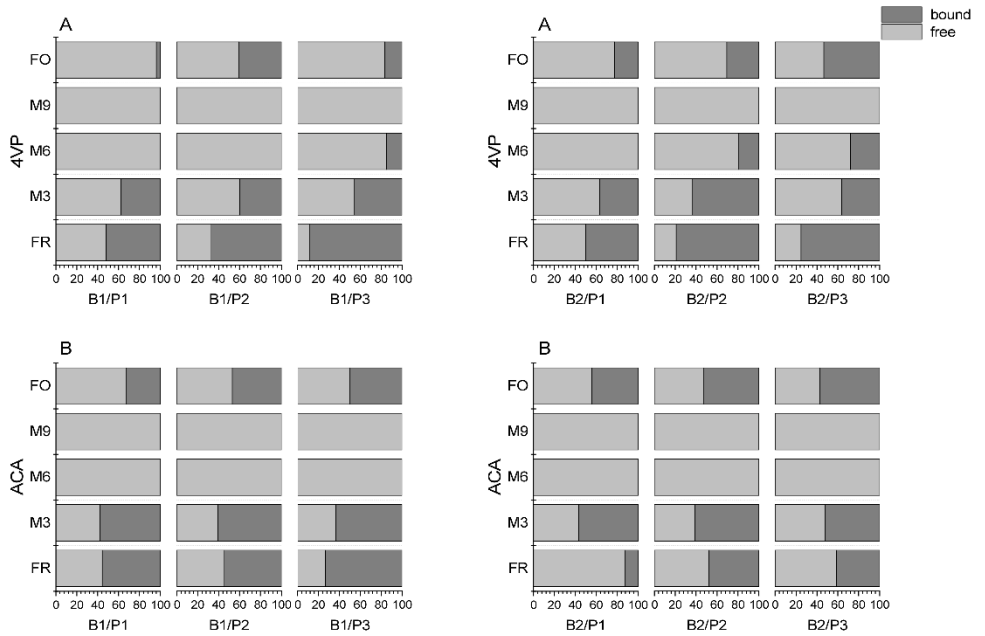

Figure 5. Ratio of sum of free and bound state aldehydes assessed by (**A**) 4VP and (**B**) ACA (B1: barley variety 1; B2: barley variety 2; P1: low proteolytic malt modification; P2: medium proteolytic malt modification; P3: high proteolytic malt modification).

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.

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 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.

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3.7 A comprehensive evaluation of flavor instability of beer – part 2: Influence of de novo formation of aging aldehydes

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A Comprehensive Evaluation of Flavor Instability of Beer (Part 2): The Influence of *De Novo* Formation of Aging Aldehydes

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Abstract: Flavor instability of beer is affected by the rise of aroma-active aldehydes during aging. Aldehydes can be either released from bound-state forms or formed *de novo*. This second part of our study focused on the *de novo* formation of aldehydes during the Maillard reaction, Strecker degradation, and oxidation reactions. Key precursor compounds for *de novo* pathways are free amino acids. This study varied the potential for reactions by varying free amino acid content in fresh beer using different proteolytic malt modification levels (569–731 mg/100 g d. m. of soluble nitrogen) of the used malt in brewing trials. Overall, six pale lager beers were produced from three malts (different malt modification levels), each was made from two different barley varieties and was naturally and forcibly aged. It was found that higher malt modification levels in fresh beer and during beer aging increased amino acid and dicarbonyl concentrations as aging precursors and Strecker aldehyde contents as aging indicators. Dicarbonyls were degraded during aging. Advanced glycation end products as possible degradation products showed no consistent formation during aging. Therefore, Strecker reactions were favored during beer aging. No alternative oxidative formation of Strecker aldehydes from their corresponding alcohols could be confirmed. Along with the preceding part one of our investigation, the results of this study showed that *de novo* formation and release occur simultaneously. After 4 months of natural aging, aldehyde rise is mainly accounted for by *de novo* formation.

Keywords: beer aging; Maillard reaction; dicarbonyls; aldehydes; proteolytic malt modification; Strecker degradation

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1. Introduction

After bottling, beer flavor is affected by various chemical reactions, leading to flavor instability. One main reason for a change in flavor in beers is the rise of aging indicators such as sensory-active aldehydes. In our preceding study (part one), the sensory and chemical effects of beer aging in lager beer were introduced [1]. The concentration of aging-relevant aldehydes increases during storage, and these aldehydes can be either formed by their release from bound-state aldehydes forms or by *de novo* formation pathways [2]. The preceding study focused on the release of bound-state aldehydes (part one). Important reactions for *de novo* formation of aging aldehydes are the Strecker degradation, the Maillard reaction, and oxidation reactions such as lipid oxidation or oxidation of the corresponding alcohols [3,4].

Despite moderate storage temperatures of approximately 20 °C, *de novo* formation of aging aldehydes can occur with low reaction rates, starting in fresh beer. Here, the concentrations of precursor substances are a critical factor for reaction kinetics [4]. Figure 1

summarizes the reaction pathways for the *de novo* formation of aldehydes observed in this study.

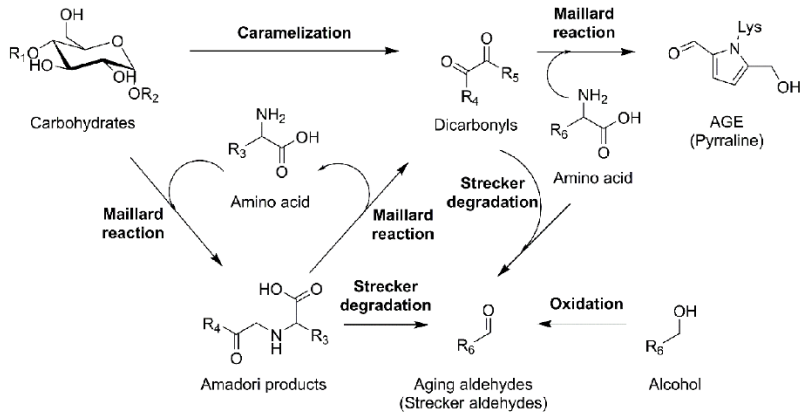

Figure 1. Overview of observed *de novo* formation pathways of aging aldehydes.

Regarding Strecker degradation and the Maillard reaction, amino acids, Amadori products, and dicarbonyls are important precursor substances [5–7]. During Strecker degradation, dicarbonyls and amino acids react to Strecker aldehydes. Here, they form an unstable hemiaminal adduct by water elimination, which further undergoes irreversible decarboxylation. After water addition, the adduct is decomposed into Strecker aldehydes and an α-ketoamine compound [4]. Important aging-relevant Strecker aldehydes are 2-methylpropanal (2MP) from valine, 2-methylbutanal (2MB) from leucine, 3-methylbutanal (3MB) from isoleucine, phenylacetaldehyde (PA) from phenylalanine, and methional (meth) from methionine. Alternatively, Strecker aldehydes could be directly formed from Amadori products [8] or by oxidation of their corresponding alcohols, e.g., 2MP from the oxidation of 2-methylbutanol [9]. Regarding the direct oxidation of higher alcohols, the pathway could be deemed insignificant due to minimal O_2 levels after bottling (<0.1 mg/L) [10]. Further, a study on wine recently found that oxidation of higher alcohols to their respective aldehydes is significantly less relevant than the degradation of amino acids during the Strecker reaction [11].

A competing reaction pathway for dicarbonyls and amino acids is the final stage of the Maillard reaction [12]. Here, the amino groups of arginine or lysine can react with dicarbonyls forming "advanced glycation end products" (AGEs). For example, pyrraline and N^{ε}-carboxyethyllysine (CEL) can be formed via the reaction of the ε-amino group of lysine with the dicarbonyl 3-deoxyglucosone (3-DG) [13] or with methylglyoxal [14], respectively. Nobis et al. demonstrated an 8% conversion from artificially spiked 3-DG to AGEs during aging [15]. Therefore, AGE formation can influence dicarbonyl reactivity during aging.

Thus far, several studies have shown a negative sensory effect of artificially increased precursors. Lund et al. pointed out that an elevated content of amino acids increased protease activity during the mashing procedure had a negative effect on beer sensory quality during aging [16]. Vesely et al. reported negative sensory effects by spiking phenylalanine and methionine to fresh beer [17]. Nobis et al. showed enhanced Strecker aldehyde formation after prolonged storage by adding 3-DG to fresh beer [15]. However, there is a lack of knowledge about the effect of technologically varied initial precursor content on *de novo* aging aldehyde formation during beer aging (forced and natural). As introduced in our preceding study (part one), accelerated proteolytic malt modification, in particular, is likely to be a good adjusting tool because it increases the levels of amino acid and dicarbonyls in malt [18] and wort [19].

In this second part of our study, we hypothesized that an increased proteolytic malt modification level elevates flavor instability by promoting *de novo* formation of aldehydes due to an enhanced initial concentration of amino acids and dicarbonyls in fresh beer. Therefore, the aim of part two was to investigate *de novo* formation pathways of aging aldehydes due to different proteolytic malt modification levels using Strecker degradation and the Maillard reaction. Furthermore, the application of precursor concentration for the early stage assessment of aging stability was evaluated.

2. Materials and Methods

2.1. Chemicals

All amino acids (L-form), (13C, 15N) labeled amino acids, D-glucose, potassium dihydrogen phosphate, methanol (LC-MS grade), o-phenylenediamine, furfural, pentanal, hexanal, heptanal, (E)-2-nonenal, hydrochloric acid, 4-fluorobenzaldehyde, ethanol (absolute), disodium hydrogen phosphate dihydrate, butanol, methyl caproate, 2-methypropanol, 3-methylbutanol, 2-methylbutanol, acetic acid, 2-methylbutyraldehyde (2MB), 3-methylbutyraldehyde (3MB), isobutyraldehyde (2MP), phenylacetaldehyde (PA), O-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine (PFBHA), nonafluoropentanoic acid (NFPA), pepsin (3839 U/mg protein), pronase E (4000 PU/mg), leucine amino-peptidase (18 U/mg protein), prolidase (553 U/mg protein), o-phenylenediamine, quinoxaline, 2-methylquinoxaline, 2,3-dimethyl-quinoxaline, and water (LC-MS grade) were obtained from Merck (Darmstadt, Germany). Acetonitrile used for liquid chromatography–mass spectrometry (LC-MS) analysis was purchased from VWR (Darmstadt, Germany). Methanol was acquired from Fisher Scientific (Loughborough, UK). Before use, the water for analytics was purified using a micropore water purification system (Thermo Fisher Scientific Inc., Waltham, MA, USA).

The following substances were synthesized according to literature: N-ε-fructosyllysine [20], N-ε-maltulosyllysine [20], pyrraline [13,21], and quinoxaline derivatives of 3-DG [22], 3-DGal [22], and 3-DM [23].

2.2. Malt, Wort, and Beer Production

The production of malt, wort, and beer samples has been described in our preceding study [1]. As presented in part one, the sample set comprised six beers from two barley varieties (B1 and B2) each malted at three different malt modification levels (P1 < P2 < P3) [1]. Table 1 summarizes the sample set and the malt modification level of the used malt assessed by the soluble nitrogen content [1].

Table 1. Malt variation of the sample set: steeping degree, soluble N (target and real) [1].

Malt Variation	Steeping Degree (%)	Target Soluble N (mg/100 g malt d. m.)	Real Soluble N (mg/100 g malt d. m.)
B1P1	38	550 ± 25	573 ± 10
B1P2	41	625 ± 25	601 ± 1
B1P3	44	700 ± 25	660 ± 1
B2P1	39	550 ± 25	569 ± 3
B2P2	43	625 ± 25	620 ± 14
B2P3	47	700 ± 25	731 ± 1

For reaching the target values of soluble N, both barley varieties were malted at different steeping degrees (moisture content during germination).

2.3. Aging and Sample Treatment

Forced (FO) and natural beer aging from fresh beer (FR) has been described in part one of our study [1]. Sampling during natural beer aging was performed after one month (M1), two months (M2), three months (M3), four months (M4), five months (M5), six months (M6), and nine months (M9). The sampling at each sampling point was performed by filtration through a folded paper filter. After filtration, the samples were quickly homogenized,

collected in 50 mL plastic tubes, and frozen. Prior to high-performance LC (HPLC) analytics, beer samples were used directly after filtration through a membrane filter (0.45 µm) from VWR (Radnor, PA, USA).

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

The procedure was performed according to Lehnhardt et al., with minor modifications [24]. The cooled wort sample (5 mL) and 50 µL internal standard (2 mg/L 4-fluorobenzaldehyde in ethanol) were transferred to a 20 mL headspace vial and stored in a cooled autosampler tray (17 °C). The extraction was performed using a Supelco® 50/30 µm coating CAR-PDMS-DVB fiber from Merck (Darmstadt, Germany). 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. 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 of 60 m; inner diameter of 0.25 mm; film thickness of 0.25 µm; Thermo Scientific Inc., Waltham, MA, USA). The carrier gas was helium (flowrate 1.85 mL/min). The starting temperature was held 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 used 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).

2.5. Quantitation of 1,2-Dicarbonyl Compounds

For this study, 1,2-dicarbonyl compounds were analyzed as quinoxaline derivatives using a high-pressure gradient system series 1200 (Agilent Technologies, Böblingen, Germany) consisting of an online degasser, autosampler, a pump, a column oven, and a diode array detector. Quantitation was performed according to Degen et al. [25].

2.6. Quantitation of Amino Acids

A total of 19 amino acids were determined according to the method reported by Sonntag et al. [26] by high-performance LC tandem mass spectrometry (HPLC MS/MS) in a multiple reaction monitoring (MRM) mode, as previously published by Nobis et al. [18].

2.7. Quantitation of Glycated Amino Acids

By using a high-pressure gradient system series 1200 (Agilent Technologies, Böblingen, Germany), including a binary pump, an online degasser, a column oven, an autosampler, and a diode array detector, as well as the triple-quadrupole mass spectrometer 6410, glycated amino acids were analyzed according to a previously published protocol [23].

2.8. Determination of Higher Alcohols and Esters

Higher alcohols and esters were quantified according to MEBAK WBBM 2.21.1 [27] as volatile fermentation byproducts with a gas chromatography flame ionization technique (GC-FID). The system comprised an HP 7694 Headspace Sampler and an HP 6890 Series GC from Agilent (Waldbronn, Germany). An HP-Ultra 2 capillary column (50 m × 0.32 mm × 0.52 µm) was used for chromatographic separation from Agilent. Samples were used directly without any treatment. Then, 5 mL beer and 0.1 mL of the internal standard (100 mg/L methyl caproate and 2 g/L butanol in 10 v/v% ethanol in water) were transferred in a 22 mL headspace vial and directly analyzed.

2.9. Statistical Analysis

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

3. Results and Discussion

Flavor instability of beer is mainly caused by increasing aldehyde concentrations in freshly bottled beer. Since the preceding study observed the release of aging-relevant aldehydes, this study focused on *de novo* formation pathways. In the following section, the oxidation of higher alcohols, the Maillard reaction, and the Strecker degradation are discussed because of their contribution to Strecker aldehyde formation during beer aging.

3.1. Oxidation of Higher Alcohols

The first pathway studied is the oxidation of higher alcohols. As already introduced, these compounds can be oxidized in the presence of reactive oxygen species, forming their corresponding aldehydes [2]. The degradation is an alternative pathway for Strecker aldehyde formation. Figure S1 shows the concentrations of 2-methylpropanol (2MP precursor), 2-methylbutanol (2MB precursor), and 3-methylbutanol (3MB precursor) during natural and forced beer aging at two barley varieties (B1 and B2), each with three different malt modification levels (P1 < P2 < P3).

There was no consistent trend in the behavior of natural beer aging at all variations. Therefore, the most constant trend did not indicate an oxidation reaction. The oxygen level was below 0.1 mg/L in fresh beer samples at all variations [1]. Thus, the oxidation reaction was inhibited and no contribution to the aging aldehyde formation could be observed. However, only the P1 levels of 2-methylbutanol and 3-methylbutanol at B2 showed a significant reduction during aging. The degradation could indicate the oxidative formation of 3MB or alternative degradation pathways of 3-methylbutanol favored such as esterification, acetal formation, or elimination reaction. Further, the aging of B2P1 is more influenced by oxygen because it showed the lowest precursor levels (Figure 2), as well as the lowest SO_2 levels [1], but oxygen concentrations were low, compared with the other variations in this study. Regarding the effect of malt modification, there was also no consistent trend observable. Surprisingly, the medium malt modification level (P2) showed the highest concentrations for 2-methylpropanol and 2-methylbutanol. The higher alcohol concentration measured in fresh beer is mainly formed during fermentation from their corresponding amino acids via the Ehrlich pathway [28]. Although the highest amino acid concentrations of valine, leucine, and isoleucine were found in the highest malt modification level (P3) after boiling [19], a differentiation of the corresponding higher alcohols due to the order in boiled wort (P1 < P1 < P3) was not detected. That confirms that the Ehrlich pathway is affected by various parameters such as yeast strain, pH value, aeration, or additional glucose content, which was not monitored in this study, in boiled wort [28,29]. Exemplarily, 3-methylbutanol showed no trend within the different malt modification levels.

Generally, there was no consistent indication for oxidative Strecker aldehyde formation during beer aging for the observed analytes in this study, which confirms the literature study on wine [11], as already elucidated in the Introduction Section.

3.2. Reactivity of Precursor Compounds in the Maillard Reaction

The possible precursor compounds for flavor-active Strecker aldehydes are primarily the corresponding amino acids and dicarbonyl compounds. The latter can be formed because of direct caramelization, or indirectly via the Amadori product degradation as part of the Maillard reaction from carbohydrates [5]. Figure 2 shows the free amino acids depending on the malt modification level (P1 < P2 < P3) for two barley varieties (B1 and B2) during natural and forced aging. Valine, methionine, leucine, isoleucine, and phenylalanine were selected because their corresponding Strecker aldehydes are sensory-active beer aging indicators. Furthermore, lysine is shown to be an important reactive and often modified amino acid within the Maillard reaction.

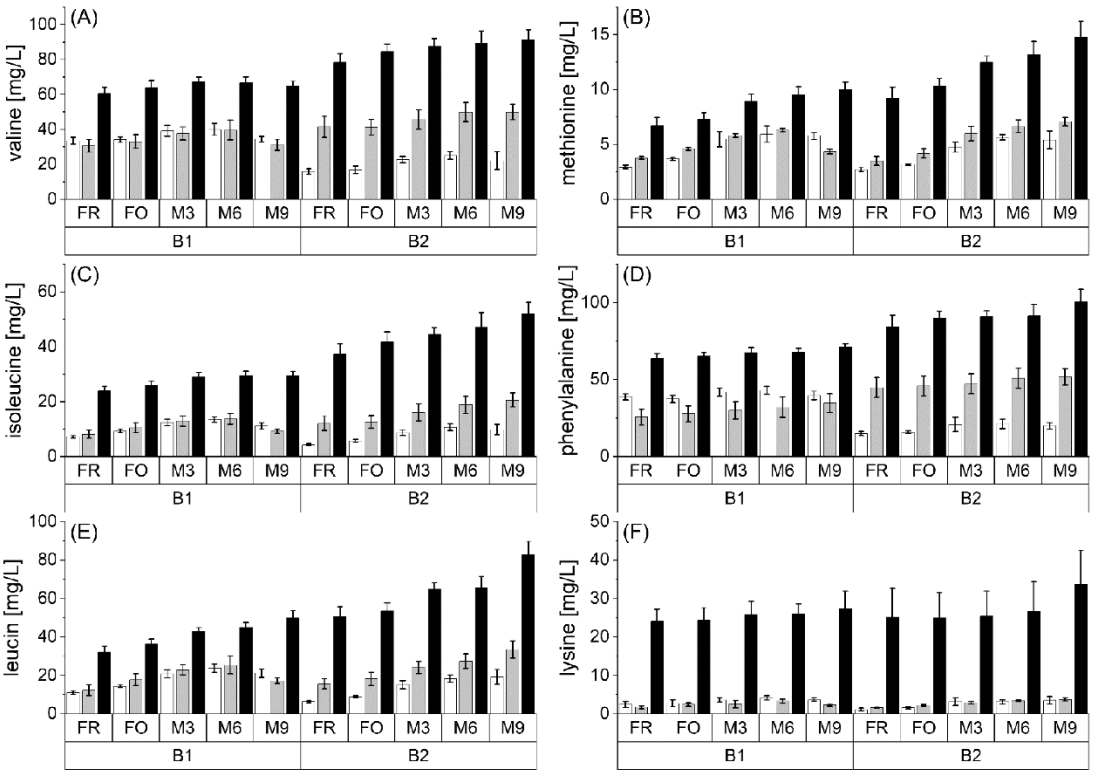

Figure 2. Concentrations of (**A**) valine, (**B**) methionine, (**C**) isoleucine, (**D**) phenylalanine, (**E**) leucine, and (**F**) lysine at fresh (FR), forcibly aged (FO), and naturally aged (M3, M6, and M9) beer at different malt modification levels (P1: white bar; P2: grey bar; P3: black bar) at two barley varieties (B1 and B2); $n = 3$.

The highest contents of the selected amino acids were determined at the highest malt modification level. The observed effect confirmed the ratio of the amino acids in the final wort [19]. Although amino acids were metabolized during yeast fermentation [9], their ratio between different malt modification levels remained constant. It is evident that this ratio differed between the observed amino acids. Thus, the difference in free lysine concentration between the lowest and highest malt modification level in fresh beer was at factor 20, whereas methionine and valine concentrations showed factor 2–5. This may be due to biological differences in protein composition and enzyme activity of barley varieties [30]. Generally, an increased malt modification level provides higher reaction potential for *de novo* Strecker aldehyde formation due to increased amino acid content.

The forced aging of the beers did not lead to significant changes in amino acid concentrations. This is partly due to the low reactivity of amino acids and, further, the relatively short reaction time of 4 days, compared with natural aging. However, natural aging increased Strecker-active amino acids. This could be explained, on the one hand, by the release from imines or Amadori products and, on the other hand, the degradation of peptides and proteins. Thus, it seems likely that the degradation of Strecker aldehydes occurred simultaneously with the release of amino acids. Therefore, an increasing amino acid concentration during aging could additionally increase the *de novo* formation potential for Strecker aldehydes.

Additional precursor compounds that can be formed during the Maillard reaction are Amadori products and the resulting dicarbonyl compounds. Strecker aldehydes can be

formed from both substance classes [7]. Exemplarily, the Amadori products of lysine (ML and FL) and the dicarbonyl compounds 3-DG, 3-DGal, and 3-DM are discussed. Figure 3 shows the mentioned analytes in their free form, depending on the malt modification level (P1 < P2 < P3), at two barley varieties (B1 and B2) during natural and forced aging.

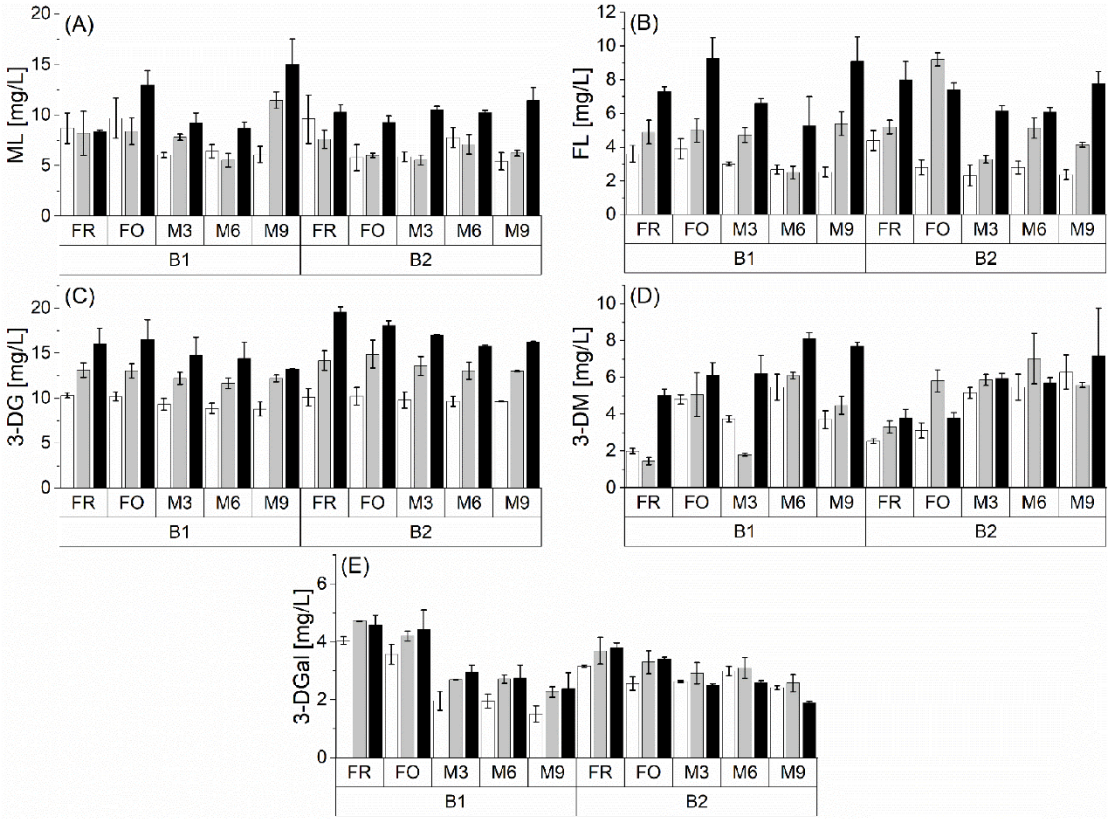

Figure 3. Concentrations of Amadori products ((**A**) ML and (**B**) FL) and dicarbonyls ((**C**) 3-DG, (**D**) 3-DM, and (**E**) 3-DGal) at fresh (FR), forcibly aged (FO), and naturally aged (M3, M6, and M9) beer at different malt modification levels (P1: white bar; P2: grey bar; P3: black bar) at two barley varieties (B1 and B2); $n = 3$.

FL ranged from 3.0 to 11.4 mg/L. ML was determined at 5.5–15.0 mg/L. No significant differences could be seen between the barley varieties with regard to the Amadori product concentration. During forced and natural beer aging, there was also no consistent trend of the Amadori product levels. Note that this study focused on ε-terminal Amadori products of lysine (FL and ML). The α-terminal Amadori products of Strecker-active amino acids might undergo degradation and, therefore, can contribute to Strecker degradation [7]. Regarding the influence of the malt modification level, the highest level mainly showed the highest concentrations of Amadori products. The effect could be caused by the increased concentration of amino acids (Figure 2), which was already observed during wort boiling [19]. This fact indicates that the FL and ML levels could have already been increased in the final wort before fermentation.

As main dicarbonyl compounds, 3-DG (7.9–19.5 mg/L), 3-DGal (1.5–4.7 mg/L), and 3-DM (1.5–9.9 mg/L) were found in concentrations comparable to previous studies [31,32]. B2 showed slightly higher contents of dicarbonyl compounds. Regarding the 3-DG and 3-DGal content, a decreasing trend during natural beer aging could be observed, as has

been previously reported [15,31]. This decrease is caused by the degradation of dicarbonyls, indicating the *de novo* formation of Strecker aldehydes. Furthermore, dicarbonyl compounds can undergo conversion to 5-hydroxymethylfurfural (HMF), as previously reported during beer aging [15]. Nevertheless, the formation of both dicarbonyls cannot be excluded during aging. Exemplarily, Rakete et al. observed the formation of 3-DG and 3-DGal by forced aging model experiments of maltotetraose [31]. Therefore, the degradation trend of both compounds tends to be underestimated. A higher malt modification level showed a consistent increase in 3-DG concentration during natural and forced aging, whereas 3-DGal content was less affected. However, higher 3-DG content could be caused by a stronger formation during wort boiling at higher malt modification levels [19,33]. The resulting higher absolute degradation of 3-DG promotes the *de novo* formation of Strecker aldehydes during natural beer aging. Furthermore, this effect can decrease flavor instability, as already shown by artificially increased 3-DG content in pale lager beers [15]. Dicarbonyl 3-DM showed a slightly increasing tendency during natural beer aging. This could be caused by a new formation from maltose. This phenomenon has already been described in the aging of pilsner beer [31]. Thus, the participation of 3-DM in Strecker degradation can be confirmed, but the degradation of α-dicarbonyl can occur simultaneously with the dominant formation during beer aging.

In summary, no clear indication for the formation of Strecker aldehydes from 3-DM and Amadori products could be observed. It should be considered that only free dicarbonyls were used for the observation and that only ε-terminal Amadori products were quantified. In contrast, the occurring degradation of 3-DG and 3-DGal indicated a possible *de novo* formation of Strecker aldehydes.

3.3. Formation of Free Aging Aldehydes during Beer Aging

The elucidated precursor compounds react further during beer aging and form aging aldehydes, causing sensory deterioration. This study focused on the Strecker degradation reaction because this is a typical reaction of the observed precursors amino acids, dicarbonyl compounds, and Amadori products. Figure 4 shows the concentration of 2MP, 2MB, 3MB, meth, and PA during natural and forced aging in two barley varieties (B1 and B2) and three different malt modification levels (P1 < P2 < P3). Data of all sampling points are provided in the Supplementary Excel file (Table S1).

The barley variety showed no effect. The formation of the aging aldehydes was comparable for both barley varieties. During aging, aldehydes were formed. As already shown in our preceding study, the rise was caused by the release from bound-state forms [1]. However, due to the observed decreasing levels of 3-DG and 3-DGal as Strecker aldehyde precursors during aging, *de novo* formation was suggested to be occurring simultaneously. In what follows, the aging behavior of the observed Strecker aldehydes is discussed separately.

The 2MP concentration showed a strong increase during natural and forced aging. The concentration after forced aging represented levels comparable to the natural aging of 0–3 months. During natural aging, the 2MP concentration increased up to 6 months and was then reduced up to 9 months. A shift to masked forms can be excluded because, after 9 months of natural aging, no bound-state form was observed in the preceding study [1]. It can be assumed that aldehydes are degraded by alternative reactions such as aldol condensation or further oxidation of the carbonyl group. Furthermore, 2MP surpassed its threshold (86 µg/L) [34] after 3 months of natural aging at the highest malt modification level (P3) and after 5 months at low (P1) and medium (P2) levels. Sensory attributes showed significant changes after natural aging ranging from 3 to 5 months [1]. The conformity indicates that 2MP played a key role in the sensory changes during beer aging in the study. It is worth noting that 2MB and 3MB were also formed during natural and forced aging but less strongly than 2MP. The concentration of both compounds after forced aging was comparable to the concentration after 3–6 months of natural aging. As already discussed for the 2MP concentration, the degradation of both aldehydes occurred from 6 to 9 months

of natural aging. This effect could also be caused by alternative degradation pathways such as aldol condensation or oxidation reactions. In this study, 2MB and 3MB did not surpass their flavor thresholds (2MB: 45 µg/L; 3MB: 56 µg/L) [34] but could still contribute to sensory flavor changes by synergistic effects. The limit of detection of meth is 0.8 µg/L. Therefore, the Strecker aldehyde was majorly quantifiable after forced aging in B1 and after 3 months of natural aging in both barley varieties. For meth, forced aging was comparable to the natural aging of 3–6 months. After 3 months of natural aging, a constant increase in meth concentration was observed. The aldehyde surpassed its threshold of 4.2 µg/L [34] after 9 months of natural aging. This result indicates that meth critically contributes to sensory deterioration in an advanced stage of beer aging (>6 months of natural aging). PA concentration increased constantly after 3 months of natural beer aging. The forced method yielded PA concentrations comparable to 6–9 months of natural beer aging, with an exception of B2P1 and B2P2 (3–6 months of natural beer aging). The aldehyde did not surpass its threshold at 105 µg/L [34] but could contribute to sensory deterioration by synergistic effects such as 2MB and 3MB.

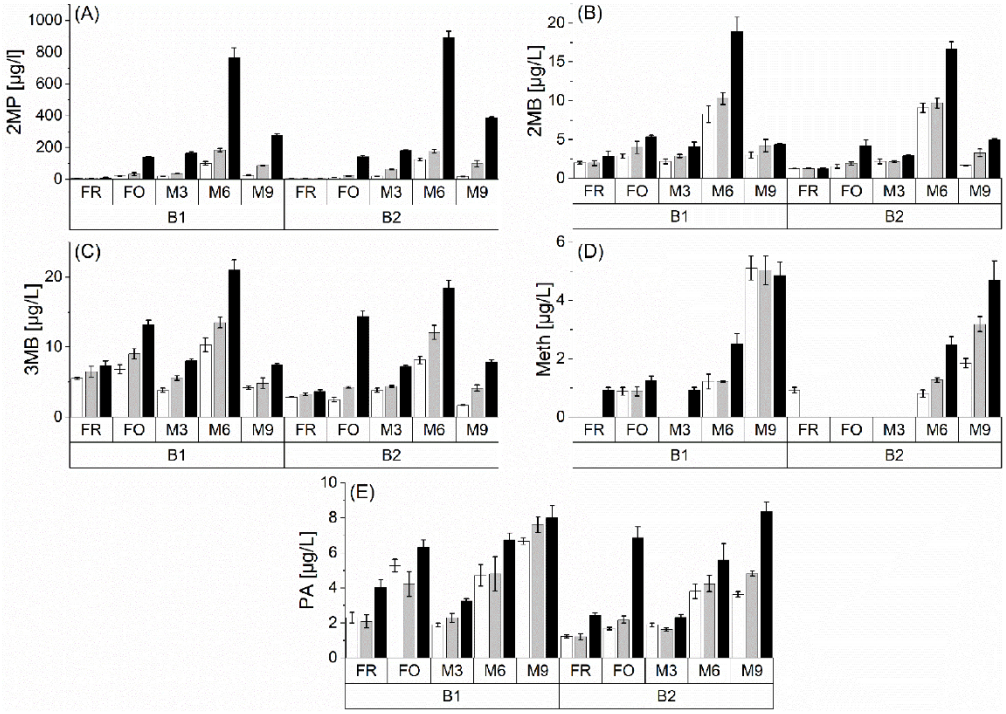

Figure 4. Concentrations of free Strecker aldehydes ((**A**) 2MP, (**B**) 2MB, (**C**) 3MB, (**D**) Meth, and (**E**) PA) at fresh (FR), forcibly aged (FO), and naturally aged (M3, M6, and M9) beer at different malt modification levels (P1: white bar; P2: grey bar; P3: black bar) at two barley varieties (B1 and B2); $n = 3$.

Generally, an increasing malt modification level caused an enhanced formation of all Strecker aldehydes during natural and forced aging. This effect indicated an accelerated beer aging by both increased release and *de novo* formation of Strecker aldehydes at higher malt modification levels. Therefore, *de novo* formation is promoted by a higher content of amino acids and dicarbonyls as Strecker aldehyde precursors due to an increased modification level, as presented in the previous section.

3.4. Formation of AGEs

Glycated amino acids are formed in the late phase of the Maillard reaction and are considered their end products [12]. Since the formation also occurs in dicarbonyl compounds, it represents a concurrence reaction to the Strecker degradation. In addition to pyrraline, AGEs MG-H1, CEL and CML were investigated. Here, only the free amino acids were determined. Figure 5 shows the contents of these AGEs depending on the malt modification level (P1 < P2 < P3) in two barley varieties (B1 and B2) during natural and forced aging.

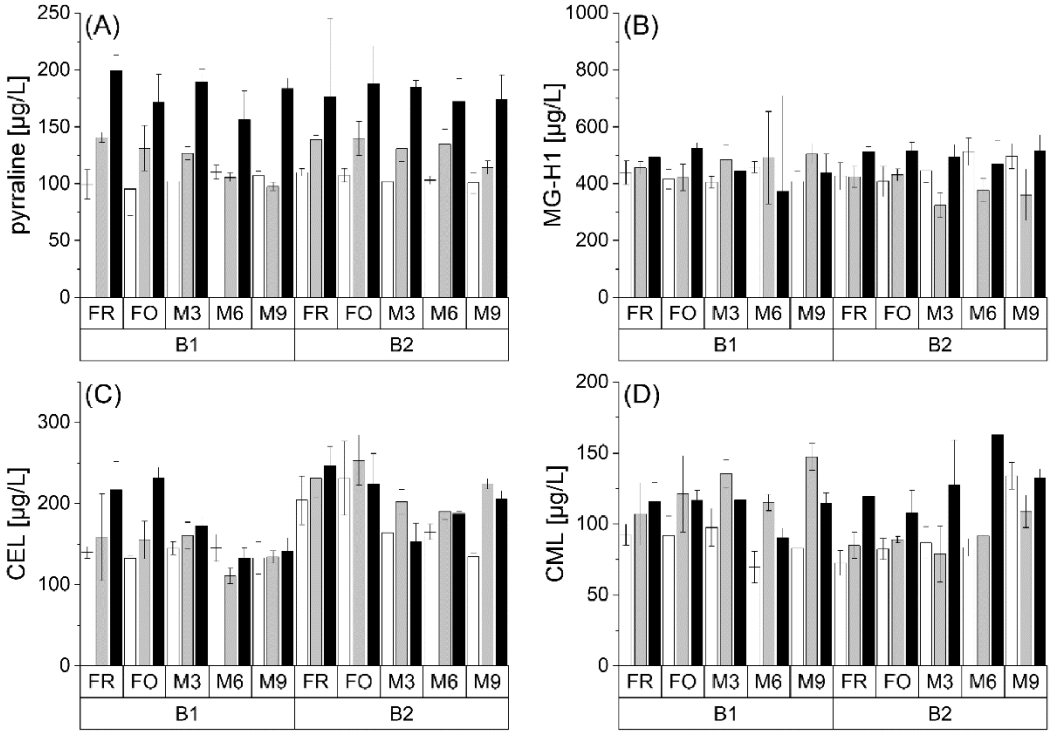

Figure 5. Concentrations of free AGEs ((**A**) pyrraline, (**B**) MG-H1, (**C**) CEL, and (**D**) CML) at fresh (FR), forcibly aged (FO), and naturally aged (M3, M6, and M9) beer at different malt modification levels (P1: white bar; P2: grey bar; P3: black bar) in two barley varieties (B1 and B2); $n = 3$.

Pyrraline, formed from 3-DG and lysine, was analyzed within 81–188 µg/L. The reaction of MGO with the guanidino group of arginine produces MG-H1, which was quantified at 325–515 µg/L in all samples. In comparison to previous studies, both concentration ranges could be considered low [15,35]. CEL and CML are derived from the reaction of MGO and GO with lysine, and thus, similar to MG-H1, are only indirectly involved in the degradation of 3-DG. CEL ranged between 111 and 253 µg/L, and CML was found between 69 and163 µg/L. Here, the contents were in the range described for pale lager beer [15].

In general, no influence of barley variety on the investigated AGEs could be observed. Higher malt modification tended to result in higher content of AGEs. Natural and forced beer aging revealed no consistent trend on the analytes. This confirmed the results of a study by Nobis et al., in which the concentration of AGEs also remained constant during beer aging. The authors suspected AGE formation during beer aging as a minor reaction [15]. Furthermore, Rakete et al. observed only a minimal increase in AGEs

derived from proline (N-formylproline and N-carboxymethlyproline) during a forced aging experiment [31]. Thus, the formation of the selected AGEs from the precursor compound 3-DG could be considered as subordinate.

3.5. Influence of the Proteolytic Malt Modification Level on De Novo Formation

The previous sections focused on several individual precursor compounds and aging aldehydes. However, the contribution to *de novo* formation should be further regarded for each substance class in a holistic perspective. Hereby, the influence of malt modification level on the aging potential is focused in the following lines. Figure 6 shows the molar distribution of the sum of free aldehydes, bound aldehydes (4-VP releasable aldehydes) [1], and precursor compounds (amino acids, dicarbonyls, and Amadori products) during aging due to different malt modification levels (P1 < P2 < P3) and barley varieties (B1 and B2). For amino acids, the sum of Strecker-relevant amino acids (leucine, isoleucine, valine, methionine, and phenylalanine) was calculated. Dicarbonyls comprised the sum of 3-DG, 3-DGal, and 3-DM concentrations. Amadori products were calculated as the sum of FL and ML content. All three substance classes are precursor compounds contributing to Strecker degradation. The 4-VP releasable aldehydes from the preceding article were included in the evaluation to further elucidate comprehensively the influence of *de novo* formation and release for the formation of flavor-active volatiles. All observed compounds contribute to the actual aging potential at several aging stages [19].

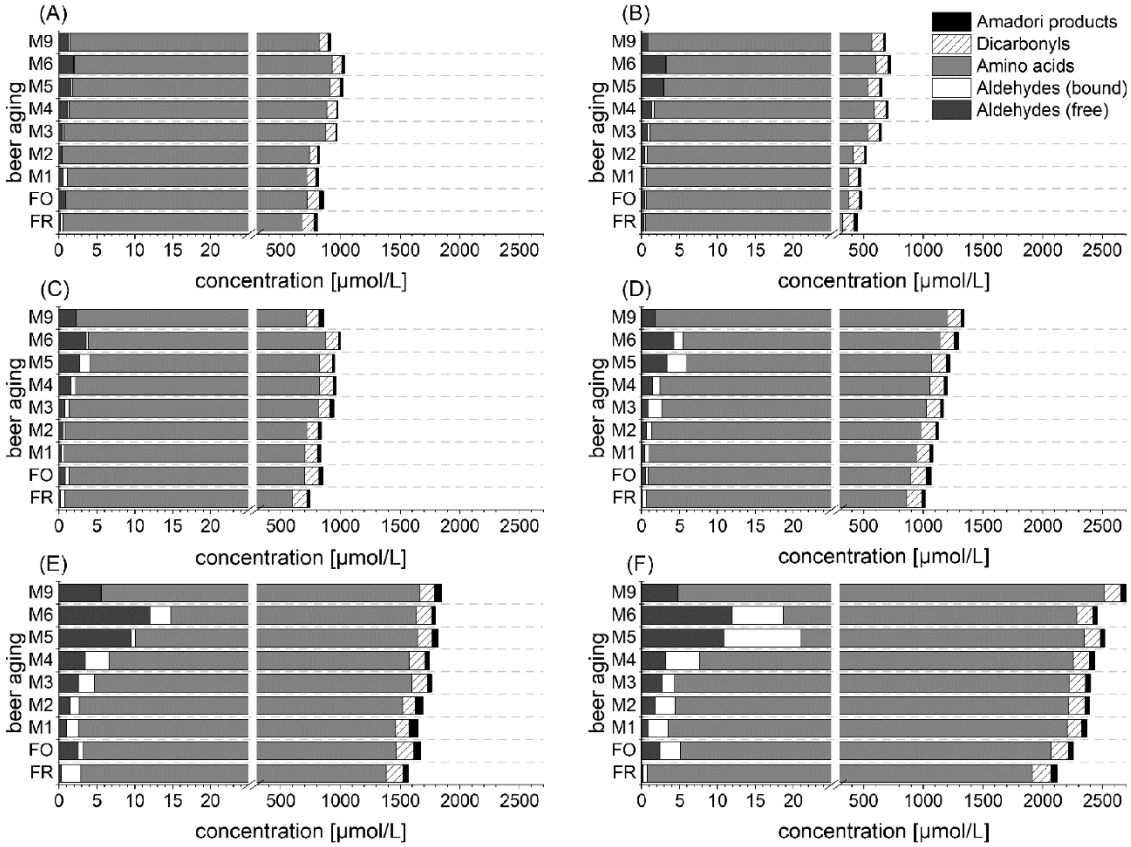

Figure 6. Molar distribution of free and bound aldehydes and precursor compounds (amino acids, dicarbonyls, Amadori products) during aging in beer at three different malt modification levels (P1 < P2 < P3) and two barley varieties (B1 and B2); (**A**) B1P1; (**B**) B2P1; (**C**) B1P2; (**D**) B2P2; (**E**) B1P3; (**F**) B2P3.

In what follows, all substance classes are considered, starting with precursors. Strecker-active amino acids represented the highest percentage of the overall sum of the calculated compounds. Notably, 73–93% of the sum belonged to this substance class. Furthermore, the concentration of amino acids was 5–16 times higher than that of dicarbonyls, accounting for only 5–20% of the aging potential (sum of all compounds). The ratio of amino acids and α-dicarbonyls indicated that the dicarbonyl concentration was the critical factor for *de novo* Strecker degradation from both classes. This fact is further substantiated by the higher reactivity of dicarbonyl compounds [5]. Dicarbonyls were the only precursor substance class in which degradation was observed for almost all variations. This fact could be shown by the changes in the sum from fresh beer to 9 months naturally aged beer (B1P1: 95 to 75 µM; B1P2: 114 to 103 µM; B1P3: 143 to 120 µM; B2P1: 90 to 94 µM; B2P2: 120 to 113 µM; B2P3: 156 to 134 µM). The degradation indicated Strecker degradation and confirmed previous references that also observed dicarbonyl degradation during beer aging [15,36]. The Amadori products accounted for only a minor percentage of 1–8% of the aging potential at all aging stages. Here, it should be considered that only two Amadori products as direct precursors of dicarbonyls were observed. Amadori products formed at the α-amino group of amino acids as direct Strecker aldehyde precursor compounds [8] might be more important. For example, Wittmann and Eichner (1989) determined fructose-derived Amadori products from valine, leucine, and isoleucine in the sum of 105 µM in fresh beer [37].

Regarding free and bound-state aldehydes, it was revealed that both forms in sum accounted for only 0.7% of the aging potential. The precursor substance classes were highly elevated, compared with the free aldehydes in this study, whereby the ratio increased toward free aldehydes during aging. In fresh beer, the precursor concentration (sum of amino acids, dicarbonyls, and Amadori products) was 3050–13,790 times higher than free aldehyde concentration. After natural aging of 9 months, the ratio decreased to only 334–787 times. The shift and ratio indicate that *de novo* formation of aging aldehydes can occur directly starting from freshly bottled beer. The concentration of bound aldehydes increased with higher amino acid concentration, indicating imine formation.

The malt modification level had a strong influence on the entire aging potential. During all stages of natural and forced aging, the sum of free aldehydes, bound-state aldehydes, and precursors was increased by a higher malt modification level. The difference between B1P1 and B1P2 was low because both samples had the lowest difference between the soluble nitrogen content of the used malts [1]. However, it could be stated that a higher proteolytic malt modification level increases the *de novo* formation of free aging aldehydes by an elevated pool of precursors in fresh beer.

Regarding the result of the preceding article [1] and this study, it could be concluded that the release of the bound-state and *de novo* formation of aging aldehydes occurred simultaneously. However, both studies aimed to comprehensively elucidate whether one of both pathways was dominant at a certain aging period. Therefore, a calculation was performed to evaluate the importance of the release of aging aldehydes. The sum of the concentrations of free and bound (4-VP releasable) aging aldehydes [1] in fresh beer was compared with the concentration of free aldehydes of the following sampling points during natural aging for each barley variety and malt modification level. The aging period, where the calculated sum in fresh beer exceeded the concentration of free aldehydes in the aged beer, was defined as the release-dominated stage. Neglecting the simultaneously occurring *de novo* formation, within these months of aging, the free aldehydes were mainly formed by their observed bound-state concentrations in fresh beer. Table 2 summarizes the release-dominated aging period of each variation. Longer ranges indicate that *de novo* formation of aging aldehydes is less important.

Table 2. Release dominated aging period of aldehydes of pale lager beer from two barley varieties (B1 and B2) and three different malt modification levels (P1 < P2 < P3).

Variation	Release Dominant Period of Natural Aging
B1P1	0–4 months
B1P2	0–4 months
B1P3	0–4 months
B2P1	0–3 months
B2P2	0–2 months
B2P3	0–1 months

A higher malt modification level at B2 tended to favor earlier importance of *de novo* formation pathways, whereas no influence was observed for B1 variations. Generally, it could be shown that the 4-VP releasable bound-state aldehyde was predominant toward aging aldehyde formation up to 4 months of natural aging. The result confirmed the findings of the study by Nobis et al., in which Strecker degradation by 3-DG spiking was observable after prolonged storage (>6 months) [15]. The sample set showed varied release-dominated aging periods. Therefore, *de novo* formation became important starting from 1 to 4 months of natural aging in this study.

4. Conclusions

The objective of the study was to investigate the *de novo* formation of free aldehydes during beer aging due to different malt modification levels. Thus, the investigation focused on the oxidation of higher alcohols, Strecker degradation, and the Maillard reaction. No formation of Strecker aldehydes from their corresponding alcohols could be confirmed. A higher malt modification level increased the concentration of amino acids, dicarbonyls, and free Strecker aldehydes. This increased aging potential explains the decreased sensory evaluation in the preceding article by a higher malt modification level [1]. The dicarbonyl compounds 3-DG and 3-DGal were degraded during aging, indicating Strecker degradation. Amadori products showed no consistent trend during aging. The effect might be caused by a balance between aldehyde formation and degradation, which could further lead to observed increased 3-DM concentrations during aging. AGEs remained constant during beer aging. Therefore, dicarbonyls reacted more strongly to Strecker aldehydes or alternative pathways such as HMF or melanoidin formation. For Strecker degradation, dicarbonyl concentrations were found to be more critical than the amino acid concentration. Finally, it was concluded that *de novo* formation and release of bound-state aldehydes occurred simultaneously. Up to four months of natural aging, the release of bound-state aldehydes was the predominant way for aging aldehyde formation. Significant sensory changes occurred at a range of 3–5 months of natural aging. Therefore, it can be assumed that the release of aldehydes basically provided the potential for sensory changes and needs to be regarded as a separate aging mechanism. In conclusion, *de novo* formation mainly reinforced and accelerated sensory beer aging after 3–5 months in this study.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10112668/s1, Figure S1: Concentrations of (A) 2-methylpropanol, (B) 2-methylbutanol, and (C) 3-methylbutanol in fresh (FR), forcibly aged (FO), and naturally aged (M3, M6, and M9) beer at different malt modification levels (P1: white bar; P2: grey bar; P3: black bar) at two barley varieties (B1 and B2); n = 3, Table S1: All data from amino acids, aldehydes, Amadori products, dicarbonyls, and AGEs.

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

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4. Discussion

Currently, the established forced aging methods show some critical disadvantages. Although, the precursor potential of 3-DG in fresh beer seems to be promising for an early prediction of flavor instability, it was not regarded until now. Therefore, the formation of 3-DG during malt and beer production shall be discussed in a technological chronological order in the following. This discussion for 3-DG formation is based on part I, part II, and part III of the cumulative thesis. Subsequently, the influence of the formed 3-DG on the flavor instability of fresh beer will be discussed. In this context, the 3-DG content was varied first artificially (part IV) by spiking and second technologically (part V and part VI) by a varied reactant (3-DG precursor) level via the malt modification level of the used malt. The influence of 3-DG reactivity on flavor instability will firstly be discussed in an analytical (formation of follow up products) and in a sensory way.

4.1 The formation of 3-DG during the malt production

First, 3-DG formation during the malting process was investigated. Part I revealed that the malt modification level and the final kilning temperature are the main influencing parameters on 3-DG formation during malt production. Therefore, the following discussion is mainly based on part I, which focused on malting with different malt modification levels (soluble nitrogen content in mg/100 g d. m.: 941.3 (M1), 732.5 (M2), and 683.8 (M3)) and final kilning temperatures (60–100 °C). Figure 2 shows the final concentration in malt varied by the described technological modifications and the formation of 3-DG during the process at a final kilning temperature of 100 °C.

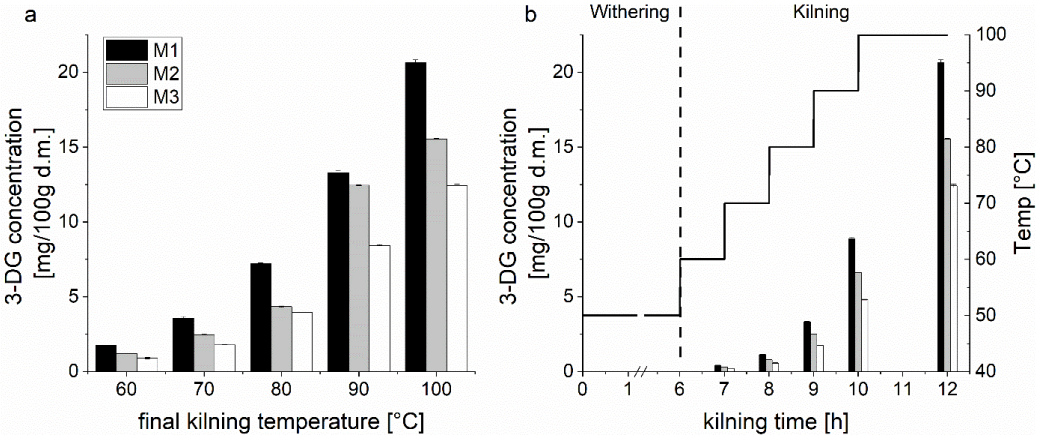

Figure 2 3-DG concentration (a) in malt after kilning due to different malt modification levels (M1, M2, and M3) and different final kilning temperatures and (b) during withering and kilning at the final kilning temperature of 100 °C (n=3)

A higher final kilning temperature promotes the 3-DG formation during the kilning process. The enhanced formation is caused by a higher thermal load and decreased water activity at higher final kilning temperatures (34). Kilning temperatures in the range of 70 to 100 °C show a linear dependency to the observed 3-DG concentration of final malt (see Table 4). In average the 3-DG concentration is enhanced with 4.7 mg/100 g d. m. while increasing of the final kilning temperature by 10 °C. Beyond this, a higher malt modification level enhances the 3-DG formation by temperature (Table 4). The typical final kilning temperature of pale lager malts ranges between 80–85 °C. Within these specifications, the influence of the final kilning temperature on the 3-DG formation was estimated. Table 4 summarizes the calculated values.

Table 4 Evaluation of the influence of the final kilning temperature onto the 3-DG concentration in malt: calculation of the relative variation of 3-DG content in malt determined by common final kilning temperatures (80-85°C) of pale malts

Malt modification level	Formula (linear fit); x-temperature, y-3-DG conc.	R^2 (linear fit)	$c_{3\text{-DG}}$ (82.5 °C) [mg/100 g d. m.]	$\Delta c_{3\text{-DG}}$ (80–85 °C) [mg/100 g d. m.]	$\frac{\Delta c_{3-DG}\,(80-85\,°C)}{c_{3-DG}(82.5\,°C)}$
M1	0.57x-37.45	0.98	9.75	2.85	29.2%
M2	0.47x-31.53	0.94	7.51	2.35	31.3%
M3	0.36x-24.22	0.98	5.65	1.80	31.8%

Based on a linear fit, first the 3-DG concentration at 82.5 °C was calculated. Further, the difference between the calculated 3-DG concentrations at 80 °C and 85 °C was determined. These differences were then normalized using the calculated 3-DG content at 82.5 °C which results in an average percentage of 30.8% over all malt modification levels. That outcome indicates, that based on the data set of Nobis et al. (2019) the setting of the final kilning temperature allows varying the 3-DG content in arrange up to 30% within the standard specifications (80–85 °C) (105).

Considering the time dependency, 3-DG was first observable after 7 h of kilning after the withering phase (Figure 2). This could be explained by the behavior of reactants such as glucose and lysine during malting. Figure 3 shows the concentration of lysine and glucose after the second step of wet steeping (S1d4h), after two days of germination (G2d) and during kilning (K0h–K12h) following the temperature procedure of Figure 2.

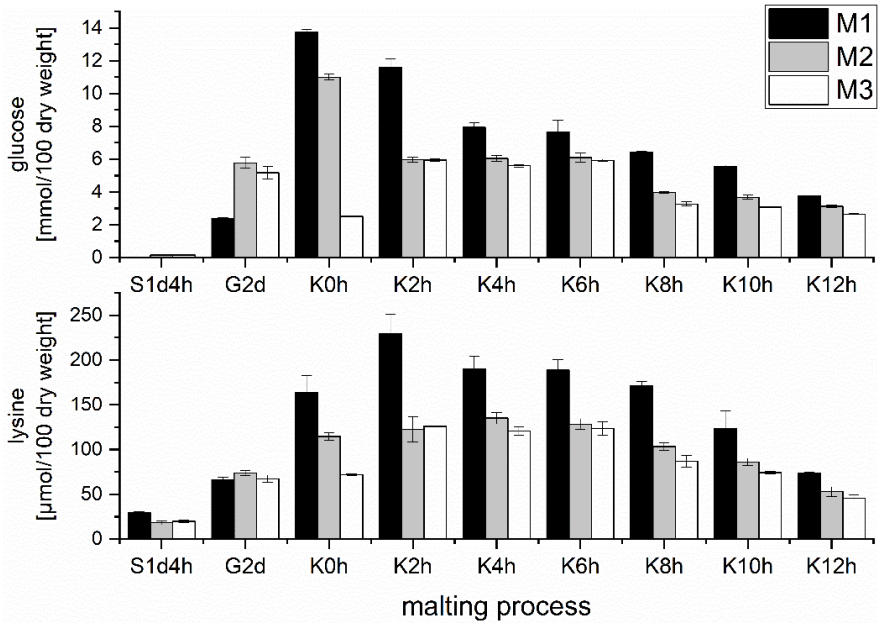

Figure 3 Formation of glucose and lysine during the malting process (steeping: S; germination: G; kilning: K) at different malt modification levels (M1 < M2 < M3) (105)

The reactants (glucose and lysine) are formed during germination and, hence, their concentration is maximal at the beginning of withering. During withering and kilning both are degraded and allow to form 3-DG through Maillard reaction and caramelization (18). This seems to be the reason why 3-DG is first observable after the withering phase during kilning.

The malt modification level also shows a strong enhancing effect on the 3-DG formation during kilning (Figure 2). The effect could be explained by promoted formation of reactants by a higher malt modification level during germination (Figure 3). According to the estimation of the influence of the final kilning temperature, the influence of the malt modification level assessed by the parameter soluble nitrogen was calculated as well. Here, a set of 41 pale malt samples (produced at the chair of Brewing and Beverage Technology) was used where all were kilned at 80 °C, because part I comprised only 3 malts at 80 °C. The sample set ranged from 470–670 mg/100 g d. m. soluble nitrogen and 0.7–2.5 mg/100 g d. m. 3-DG. Figure 4 shows the linear fit of the 3-DG concentration in dependence to the soluble nitrogen content of the pale malt samples.

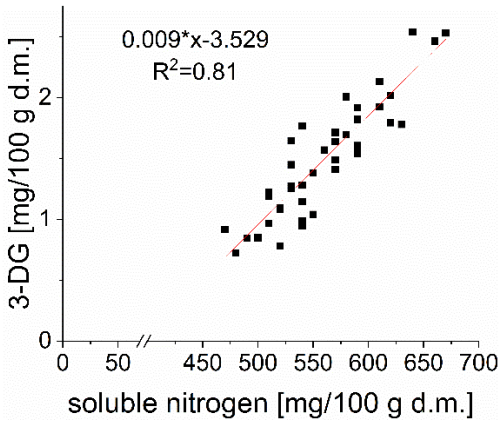

Figure 4 Linear fit between 3-DG concentration and soluble nitrogen content for 41 pale malt samples

A correlation coefficient of 0.81 indicates that the variation of 3-DG concentration of pale malt is clearly influenced by the proteolytic malt modification level assessed by the soluble nitrogen content. The specifications of soluble nitrogen for pale malts are 580–680 mg/100 g d. m. for brewing purposes (114). According to the linear fit, the calculated 3-DG concentration for the limits are 1.7 (lower limit of soluble nitrogen) and 2.6 mg/100 g d. m. (higher limit of soluble nitrogen). The difference between both limits is 0.9 mg/100 g d. m. 3-DG. The average of the soluble nitrogen content within the given specifications is 630 mg/100 g d. m., which leads to a 2.1 mg/100 g d. m. 3-DG concentration in malt. Normalizing the difference of 0.9 mg/100 g d. m. 3-DG within the malting specifications by the computed average 3-DG concentration of 2.1 mg/100 g d. m. yields the variable percentage of 3-DG due to the specifications of soluble nitrogen of 42.3%.

In conclusion, it is shown that 3-DG is formed after the withering phase during kilning mainly influenced by the final kilning temperature and the malt modification level. Comparing both adjustment tools within the specifications for pale malts, the malt modification level (assessed by soluble nitrogen content) allows to vary the 3-DG concentration in pale malts by about 40% which is significantly more impact than the final kilning temperature (about 30%). Therefore, the 3-DG content as well as formation potential for beer production was varied by the malt modification level for the following approaches.

4.2 The formation of 3-DG during beer production

After studying the formation of 3-DG during malting, this thesis (part II) investigated the 3-DG formation during the beer production. The contribution of each single process step will be discussed in the following.

Quantitative contribution of malt, mashing and wort boiling on final wort 3-DG formation

The mentioned part II investigated the influence of a varied mashing procedure. However, no effect on an additional proteolytic rest during mashing on 3-DG formation during beer production could be observed. Although amino acids content was increased by an additional proteolytic rest, 3-DG was not formed more intensively during boiling (109). This fact indicates that only the amino acid content cannot be used as prediction for dicarbonyl formation. Assumably, the formation of Amadori product which are direct precursors of 3-DG is more important.

Regarding the contribution of malt, mashing and wort boiling on the 3-DG content after boiling, Figure 5 quantitatively and qualitatively summarizes the proportion of the single process steps according to Nobis et al. 2021 (109). According to section 4.1, the malt modification level determines the level of 3-DG in malt which is here used for the brewing process. The study is based on the malts in the previous section, but comprises only M2 and M3 malt modification level. The focus of part II is on a combination of varied malt modification levels and mashing procedures as well as investigation of degradation pathways of 3-DG by spiking the dicarbonyl.

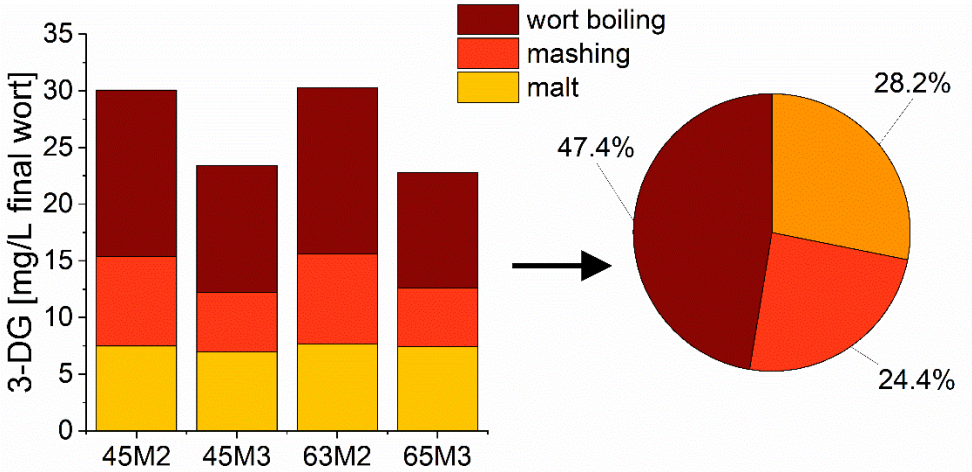

Figure 5 Contribution of malt, mashing and wort boiling onto final 3-DG content after boiling due to different malt modification levels and (M2 and M3) and different mashing procedures (additional proteolytic rest: 45 and reference procedure: 63) (109)

The relative percentages of the process steps are comparable for all variations. Malt provides about 28.0%, mashing 24.5% and wort boiling 47.5% of the 3-DG content in final wort. Therefore, the wort boiling process was found to be the most contributing process step for 3-DG formation in beer production rather forming 50% of the 3-DG content in final wort. As already discussed in the previous section, the malt modification level influences the absolute 3-DG concentration significantly. A higher malt modification level increases the 3-DG concentration after mashing and after wort boiling (Figure 5). The elevated formation potential is explained by an increased content of amino acids as 3-DG precursors as well as a higher initial 3-DG content from malt as a consequence of an enhanced malt modification level.

Qualitative formation of 3-DG during wort boiling

The wort boiling process was identified to be the most important process and the malt modification level assessed by the soluble nitrogen content as the most important technological influence on 3-DG formation. Thus, both these aspects will be discussed in more detail.

First, the qualitative formation of 3-DG during the process is considered. Figure 6 shows the differences onto the formation of 3-DG in a closed (a) and an open boiling system (b). The closed boiling system comprises two stages of malt modification (M2 and M3) (109), while the open boiling system includes three different levels of malt modification (B2P1, B2P2, and B2P3) (110).

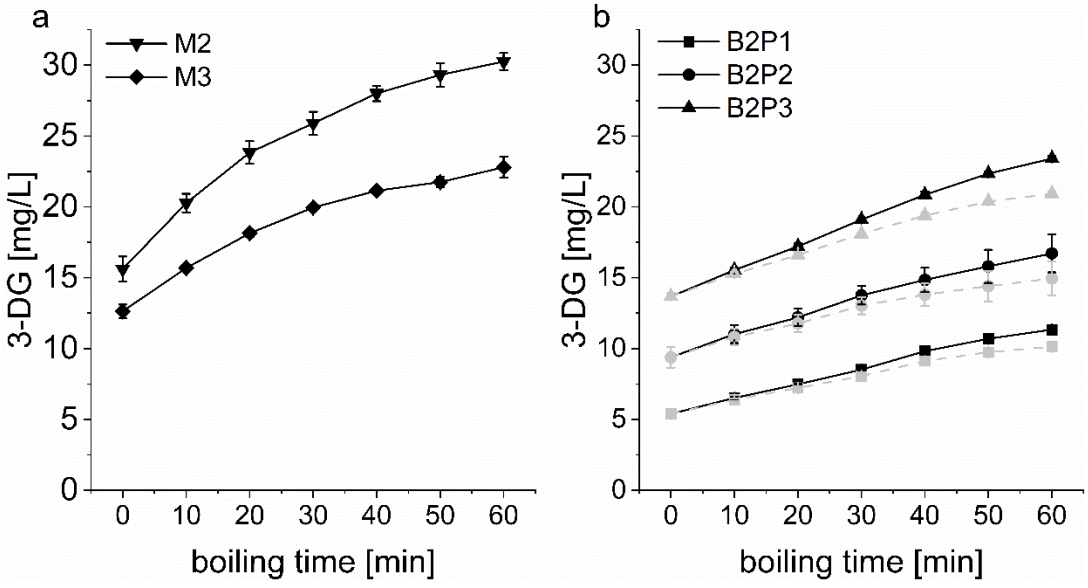

Figure 6 Formation of 3-DG during wort boiling in (a) a closed boiling system at lab scale (109) and (b) in a open boiling system at pilot scale (110); black: determined contents, grey: calculated contents without evaporation effect (n = 3)

In a closed system, the formation rate of 3-DG is high at the beginning of boiling and decreases constantly towards the end. This behavior indicates that 3-DG is strongly formed at the beginning of boiling and that the degradation of 3-DG partly rises up to the end of boiling. Therefore, the Maillard reaction proceeds to its advanced stage (18). These findings are confirmed by a decrease of the Amadori product FL as 3-DG precursor, which also indicates the advanced stage of the Maillard reaction (109). In contrast, the open boiling systems, which covers the majority of applications in the brewing industry, forces linear 3-DG formation during wort boiling. This effect is explained by the additional evaporation occurring in open boiling systems. In order to evaluate this effect, the concentration of 3-DG was computed (grey line in (b) at Figure 6) eliminating the evaporation rate of 10.6% in the used boiling system. In this case, a decreased formation rate is also observed at the end of boiling. This confirms that the degradation of 3-DG partly rises up at the end of the boiling period.

Second, the effect of the malt modification level is to be considered. Higher malt modification levels enhance the formation of 3-DG during wort boiling. This effect refers to the initial content of 3-DG, its formation rate, and its final content during wort boiling. A comparable effect was already observed during malting in the previous section. Therefore, the enhanced 3-DG formation during boiling could be the consequence of an already higher 3-DG forming potential during malting. This potential is provided by an enhanced present 3-DG content (105), the present amino acid, and Amadori product concentration (105) as well as the enzyme potential (proteolytic and amylolytic enzymes) (82) in malt included by a higher malt modification level. An enhancement of amino acids and Amadori products as reactants by a higher proteolytic malt modification at the beginning of wort boiling could be also confirmed in part II and part III of the thesis.

Reactivity of 3-DG during wort boiling

3-DG is considered less stable (115, 116). The C4-β-hydroxyl group is prone to undergo elimination forming a mesomeric stabilized α,β-unsaturated carbonyl sub-structure (3,4-DGE formation) (22). Model experiments in part II confirm that 16% of the initial 3-DG were degraded during wort boiling. However, part II shows that only 4% of the degraded 3-DG is converted to HMF and 25% of 3-DG is interconverted to 3-DGal. A reaction to Strecker aldehydes by 3-DG spiking was not observed during wort boiling in the closed system. This allows to assume, that a reaction of 3-DG to

volatiles like Strecker aldehydes depends strongly on the gas volume above the liquid phase. A small gas volume in a closed boiling system disadvantages the continuous formation of volatiles as well as CO_2 as by-product in Strecker degradation due to the increasing partial pressure in the gas phase. Part III confirms this assumption, as the increased content of 3-DG at the beginning of wort boiling resulted in increased Strecker aldehyde contents at the end of boiling. On the one hand, the aldehydes can be formed by reaction of other dicarbonyls such as MGO or GO or alternatively from Amadori products (39). On the other hand, 3-DG is the major dicarbonyl during wort boiling. Therefore, a continuous formation of Strecker aldehydes from 3-DG degradation can take place and is additionally advantaged because of the evaporation effect. In conclusion, the formation of Strecker aldehydes from 3-DG in dependence of the boiling system (closed or open) cannot be neglected.

The effect of fermentation on 3-DG content

The investigation of the fermentation step was not part of this thesis but needs to be considered. In this thesis, standardized fermentation processes and unvaried fermentation parameters were used. Figure 7 shows the concentrations of 3-DG comparing final wort and fresh beer for six pale lager beers according to part III and part V of the thesis.

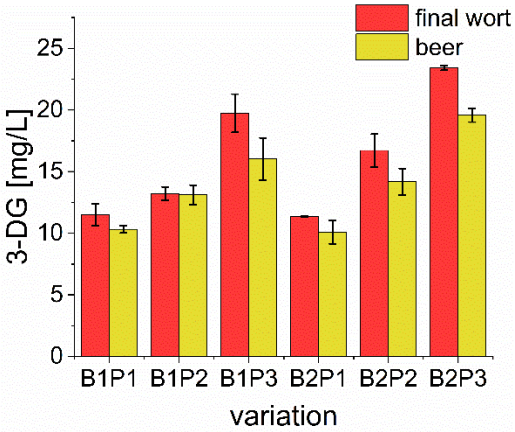

Figure 7 Comparison of 3-DG content of final wort and fresh beer at two different barley varieties (B1 and B2) and three different malt modification levels (P1 < P2 < P3) (n=3)

In general, for all variations (except B1P2) a reduction of 3-DG was observed from final wort to fresh beer. However, an explanation could be degradation during the whirlpool rest, while an adsorption during filtration would also cause a reduction of 3-DG content. Furthermore, Hellwig et al. (2021) reported an enzymatic 3-DG reduction of 17.8% during fermentation in model studies (98). The thesis shows a relative reduction

ranging from 0.8%–18.9% at an average of 12.1%. This finding indicates an enzymatic reduction of 3-DG during fermentation. However, it needs to be considered that the fermentation parameters (temperature and substrate concentration) are significantly different between the study of Hellwig et al. (2021) (98) and the brewing trials in part V. Therefore, the reduction is only partly explained by enzymatic conversion to 3-deoxyfructose (98).

In conclusion, the observed reduction in the study could be an indication for the metabolic conversion of 3-DG by yeast.

4.3 Influence of 3-DG on flavor instability during beer aging

Up to now, the thesis focused the formation of 3-DG during malt and beer production. As stated in the hypothesis, the formed concentration of 3-DG in fresh beer during beer production can be degraded during aging and, therefore, influence the flavor instability of beer. The hypothesis should be discussed in the following including part IV, part V, and part VI.

3-DG reactivity during natural and forced aging

In order to evaluate the degradation of 3-DG, several aging-relevant reactions were observed in part IV and their contribution was determined by 3-DG spiking. Figure 8 shows the distribution of 3-DG degradation reactions including Strecker degradation, HMF formation, interconversion to 3-DGal, and AGE formation (Maillard reaction) after 3 months (3M), 6 months (6M) and 12 months (12M) of natural aging, as well as after forced aging.

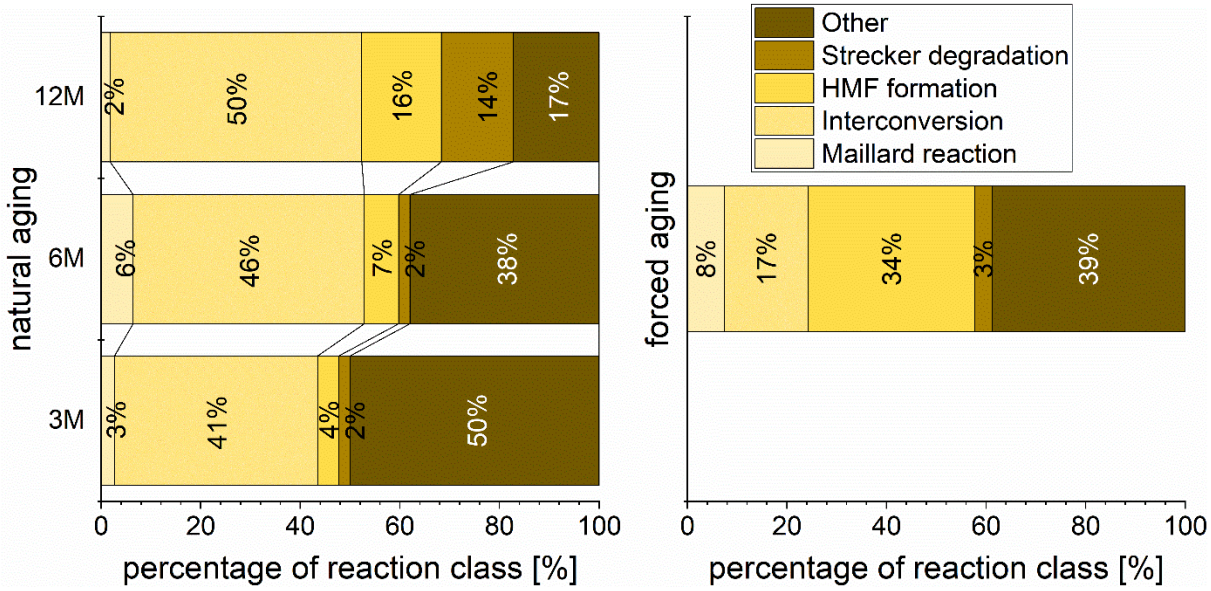

Figure 8 Distribution of 3-DG reaction classes after 3M, 6M, and 12M of natural and forced aging (111)

Regarding natural aging, an increase of the 3-DG degradation within interconversion, HMF formation and Strecker degradation by progressive natural aging on the cost of the share of other reactions was observed. The results indicate that the reactions require time to reach their activation energies allowing 3-DG to be converted to 3-DGal, HMF, or Strecker aldehydes. With interconversion and HMF formation, 3-DG must first react to 3,4-DGE, which can then be dehydrated to HMF or react to 3-DGal by water addition (22). With Strecker degradation, 3-DG needs to form a α-imino carbonyl with the amino acids first, which can further be decarboxylated and react to Strecker aldehydes (5). The variations between 3M to 12M measurement indicate that degraded 3-DG stays majorly at these intermediate stages up to 6 months and is then metabolized to the final degradation products. An exception is the interconversion to 3-DGal which covers already 41% of degraded 3-DG after 3 months of natural aging. Here, it can be assumed that a degradation of 3-DG to 3,4-DGE directly shifts the equilibrium towards 3-DGal because the rehydration of 3,4-DGE is advantaged in the watery matrix beer. The reaction of 3-DG to AGEs within the Maillard reaction could be only minorily observed (max. 6%) during natural aging.

With forced aging, it was observed that 3-DG is mainly degraded to HMF or to other reactions such as fragmentation or staying in intermediate stages as discussed before. The degradation to HMF is thermally catalyzed (117) and, therefore, the higher incubation temperature (40 °C) during forced aging favors the HMF formation. The distribution of the degradation pathways after forced aging seem comparable with 6 months of natural aging, but the interconversion is underestimated as the HMF

formation is overestimated. In conclusion, it could be stated that the forced aging method is not suitable to predict the 3-DG reactivity of fresh beer.

Influence of 3-DG on the flavor instability of beer by a technologically varied initial 3-DG content

Part IV shows that an artificially increased 3-DG content in fresh beer enhances the concentrations of Strecker aldehydes after prolonged storage (> 6 months). This result needs to be verified in part V and part VI by modifying the 3-DG content in fresh beer technologically. Furthermore, the question needs to be answered whether a technologically enhanced 3-DG content in fresh beer also causes increased sensory deterioration. Figure 9 shows a summary of the analytical (sum of Strecker aldehydes) and the sensory (DLG *smell* and DLG *taste*) changes and the 3-DG degradation during fresh and forced beer aging due to varied malt modification levels (P1 < P2 < P3).

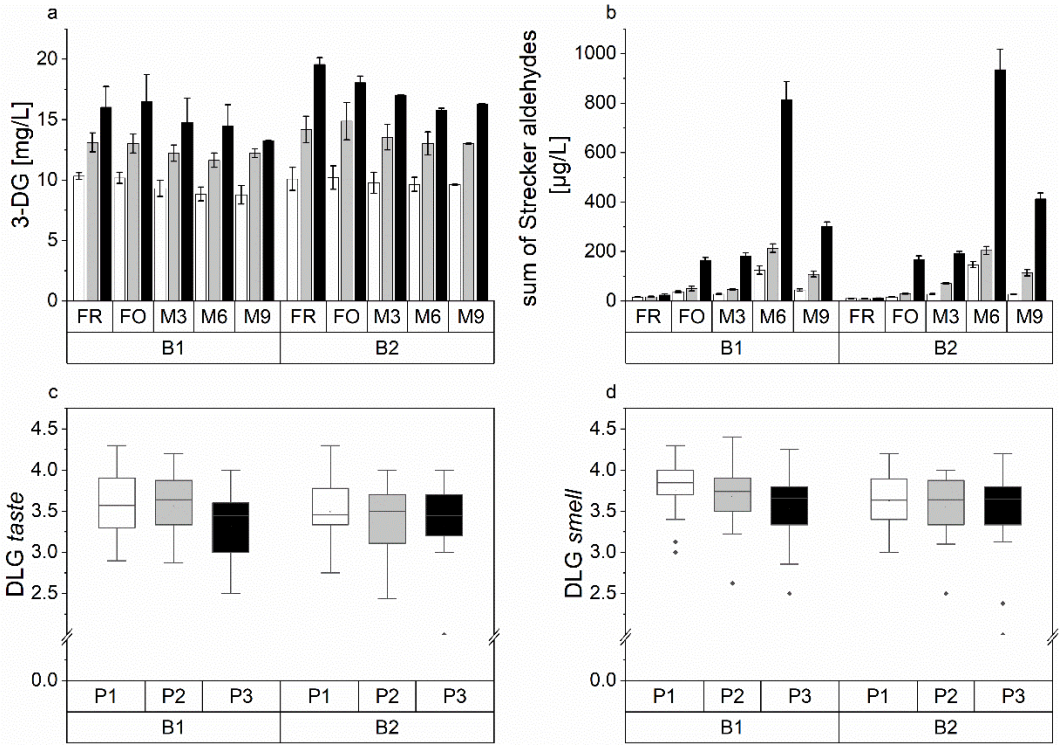

Figure 9 Changes in the concentration of (a) 3-DG and (b) sum of Strecker aldehydes in fresh (FR) samples and after 3 (M3), 6 (M6), and 9 (M9) months of natural aging, and after forced (FO) (n = 3) and sensory attributes (c) DLG smell and (d) DLG taste in subject to malt modification level (none-colored: P1, grey: P2, black: P3)

The 3-DG content in fresh beer was successfully varied by the varying malt modification level. As already observed during malting (105) and wort boiling (109, 110) the increased concentration of reactants (amino acids and sugars) as a consequence of an enhanced malt modification level leads to a stronger reaction to 3-DG during malt and beer production further elevating the 3-DG content in fresh beer.

It could be shown that an increased initial technologically varied content of 3-DG in fresh beer causes a higher absolute degradation after 9 months of natural aging (B1P1: 1.5 mg/L; B1P2: 0.9 mg/L; B1P3: 2.8 mg/L; B2P1: 0.5 mg/L; B2P2: 1.2 mg/L; B2P3: 3.3 mg/L). The exception at B1P2 may be caused by a slightly increased 3-DG content from 6 months to 9 months of natural aging. An increase of 3-DG content during beer aging can be explained by degradation of Amadori products or re-interconversion of 3-DGal (18). In comparism to part IV, the monthly reduction rates are lower in part VI. Here, the 3-DG reduction rate is 0.1–0.4 mg/L per months, in part IV the reduction rate ranged from 0.6–1.1 mg/L per month. Therefore, it could be assumed that the artificially increased 3-DG content is degraded to a higher degree compared to a technologically varied 3-DG content. In particular, the 3-DG interconversion to 3-DGal is advantaged for artificially spiked samples (111), because the initial 3-DGal is also elevated at the technologically modified variations by an increased initial 3-DG-content (113). Therefore, the interconversion reaction of 3-DG is less advantaged in these aging experiments.

Regarding important degradation reactions, analytical changes (Strecker aldehydes) and sensory changes were observed in part V and part VI. Free Strecker aldehydes are significantly increased by an increased malt modification level. This effect can be explained by the increased initial amino acid and dicarbonyl content in fresh beer. The *de novo* formation of Strecker aldehydes obviously becomes important mainly after 4 months of natural aging (112, 113). This confirms the outcome of part IV, where a *de novo* formation of Strecker aldehydes through 3-DG degradation also occurs mostly after prolonged storage (> 6 months of natural aging) (111). Based on the conversion rate of 14% (degraded 3-DG to Strecker aldehydes) in part IV presented in Figure 8 after 12 months of natural aging (111), about 60% of the formed sum of Strecker aldehydes are explainable after 9 months of natural aging at medium (P2) and high (P3) malt modification levels by the observed 3-DG degradation (Figure 9) in part V. This indicates that the Strecker aldehydes are mainly formed by 3-DG degradation. However, at low malt modification levels (P1) the degraded 3-DG covers more than 100% (B1: 375% and B2: 189%) of the formed Strecker aldehydes. Here, it can be assumed that 3-DG undergoes alternative degradation reactions such as fragmentation or HMF formation to a higher percentage compared to medium or high malt modification levels and that 3-DG is not converted by 14% within Strecker

degradation at theses stages. Therefore, Strecker degradation of 3-DG is decreased at lower malt modification levels.

Although the Strecker aldehydes concentrations show significant absolute differences employing forced aging and natural aging, a significant effect in sensory characteristics such as DLG *smell* or DLG *taste* by the malt modification level (Figure 9) could not be detected. However, a higher malt modification level tends to decreased averages for DLG quality parameters (112). Therefore, in determining differences between beers with different malt modifications, a discriminative triangle test was carried out after 9 months of natural aging when the differences between the Strecker aldehydes concentrations are at maximum. With the exception of B1P1 and B1P2, all pairs reveal a significant difference. Therefore, the variations (different malt modification levels) are clearly sensory distinguishable after 9 months of natural aging (112).

In conclusion, a technologically increased 3-DG content in fresh beer by an enhanced 3-DG formation potential during beer production accelerates analytical and sensory natural aging. Thus, the 3-DG content in fresh beer serves as a suitable parameter to predict flavor instability due to its reactive precursor activity during natural aging.

4.4 Course of 3-DG during the malt and beer production

Summing up all presented results, the survey of 3-DG during malt and beer production is to be considered. Figure 10 shows a qualitative overview of the formation and degradation of 3-DG during the malt and beer production.

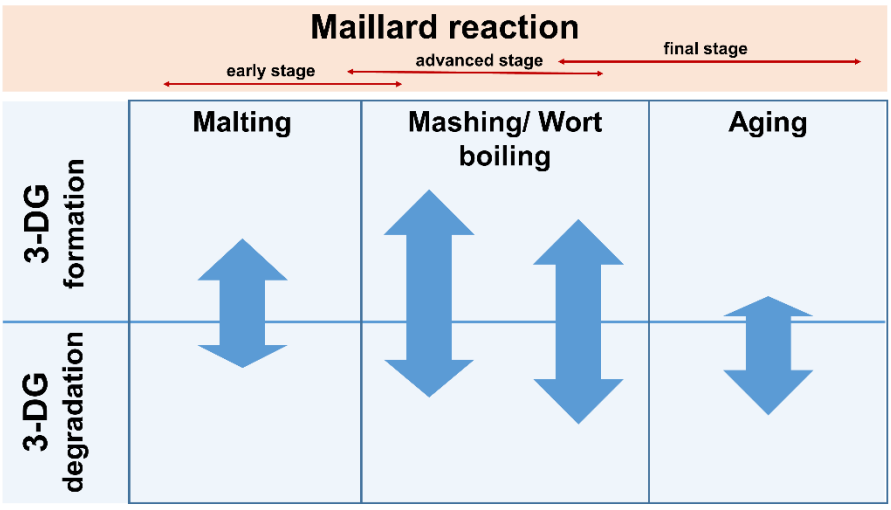

Figure 10 Qualitative overview about the 3-DG formation and degradation during the malt and beer production

During malting (kilning step), Amadori products such as FL and 3-DG were simultaneously formed, which indicates that the Maillard reaction stays in its early stage (19, 105). Nevertheless, 3-DG degradation can already occur at minor reaction rates due to the high reactivity of the dicarbonyl and the high temperatures during kilning. Stepping further through beer production, 3-DG is mainly formed during mashing and at the beginning of wort boiling where FL as its direct precursor is degraded simultaneously (109). This behavior indicates the progress of the Maillard reaction to the advanced stage (19). At the end of boiling, 3-DG formation stagnates because 3-DG degradation starts to dominate. This effect is confirmed by enhanced HMF (109) and Strecker aldehyde formation (110) during the boiling process. Proceeding to beer aging, the degradation of 3-DG completely dominates over formation. With simultaneously occurring constant contents of AGEs and rising Strecker aldehydes and HMF contents, it can be stated that the Maillard reaction reaches its final stage mainly characterized by dicarbonyl degradation in the brewing process (19).

This thesis confirms the progress of the Maillard reaction based on the formation and degradation reactions of 3-DG, its precursors, and the respective follow up products. Starting from malting the Maillard reaction develops to the final stage ending up at beer aging while the transitions of the three stages between the malting and beer production steps are not discrete but shows some overlapping.

4.5 Thesis Outcome: Discussion of the role of 3-DG regarding flavor instability

Flavor instability is caused by rising aldehydes during beer aging. As already shown, they can be formed in two ways: Releasing from their bound form or *de novo* formation by chemical reactions such as the Maillard reaction (112). 3-DG has influence on the *de novo* formation pathway due to its reactivity within the Strecker degradation according to model studies (5). In this thesis, the formation of Strecker aldehydes from 3-DG degradation is confirmed after prolonged natural aging (> 6 months) by spiking experiments (111). About 60% of the formed Strecker aldehydes during natural aging are covered by 3-DG degradation by a technologically varied 3-DG content in fresh beer after 9 months of aging at medium (P2) and high (P3) malt modification levels. However, the role of dicarbonyls, in particular 3-DG, regarding flavor instability needs to be considered in an enlarged view comprising free and bound aldehydes as well as

their precursor compounds (dicarbonyls, Amadori products, and amino acids). Figure 11 shows the molar distributions of the sum of free and bound aldehydes, Amadori products (FL and ML), Strecker-active amino acids (valine, isoleucine, leucine, methionine, and phenylalanine), and dicarbonyls (3-DG, 3-DGal, and 3-deoxymaltosone) according to part V and part VI during natural aging.

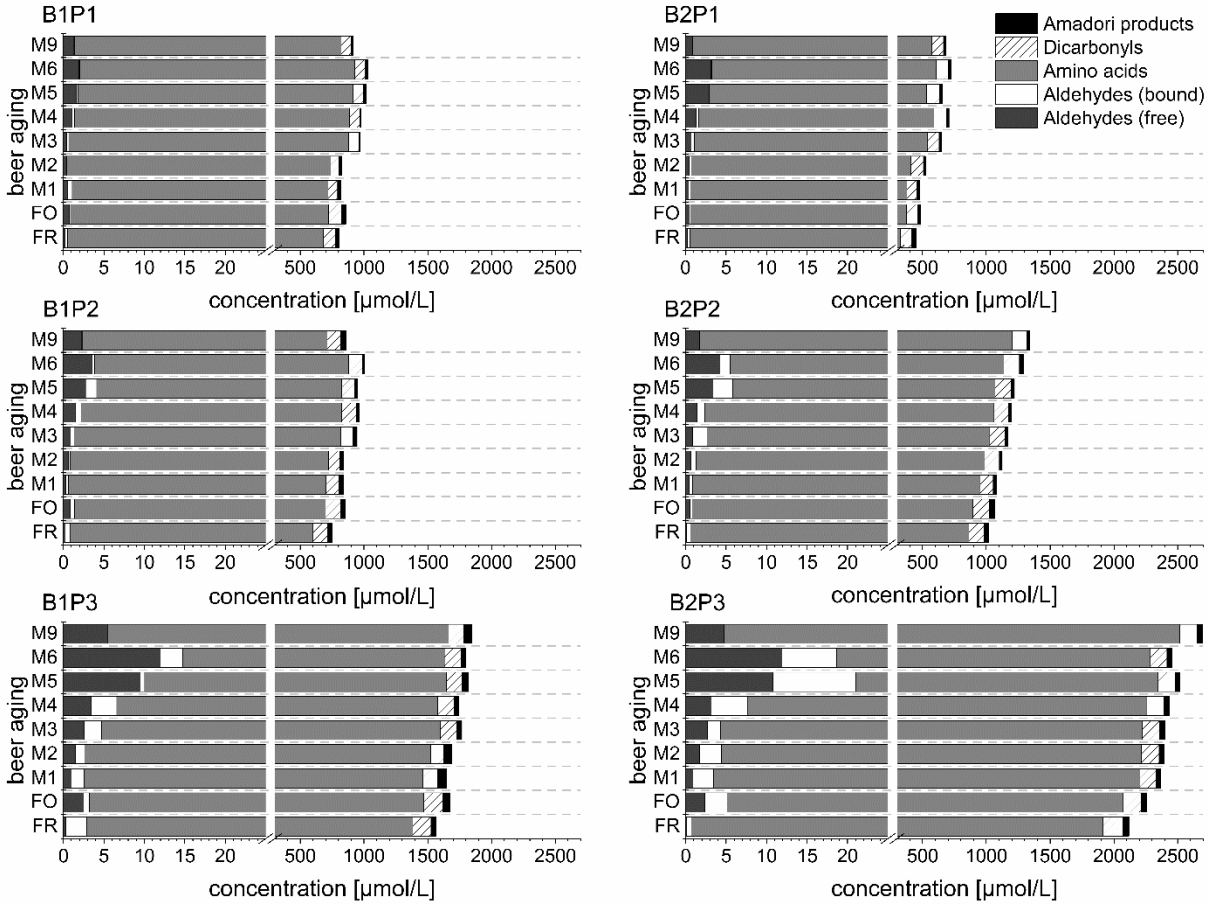

Figure 11 Molar distribution of free and bound aldehydes and precursor compounds (amino acids, dicarbonyls, Amadori products) during aging in beer at three different malt modification levels (P1, P2, and P3) and two barley varieties (B1 and B2) (113)

Generally, it could be shown that the precursors strongly predominate the total sum of all compounds. Free and bound aldehydes contribute to the molar distribution in sum only up to 0.7% in total. Therefore, the degradation of precursors is covering the formation of the free aging aldehydes completely. However, it could be assumed that release and *de novo* formation of aging aldehydes take place simultaneously. The aging period where the concentration of free aldehydes surpasses the total of free and bound aldehyde content in fresh beer is determined as release dominated aging stage. Here, free aldehydes are mainly formed by release of their bound forms. This time period ranges from 1–4 months of natural aging depending on the malt modification level. The result indicates that the *de novo* formation of aging aldehydes becomes

dominant after 1–4 months of natural aging. Significant sensory changes were observed after 3–5 months of natural aging (112). Thus, the release of aldehydes may basically provide the potential for sensory changes. Furthermore, the *de novo* formation, e.g. from 3-DG degradation, mainly reinforces and accelerates sensory changes during natural beer aging after 3–5 months. These findings further confirm the results of part IV, where an artificially induced increase of the initial 3-DG content shows a significant analytical effect after prolonged natural beer aging (> 6 months).

Focusing on the role of 3-DG on the *de novo* formation pathways, Figure 11 shows that the Strecker relevant amino acids are the predominant substance class out of the precursors. They are covering 73–93% of the total molar distribution. The dicarbonyls represent a minor substance class of precursors (5–20% of the total sum). Nevertheless, precisely because of this fact, dicarbonyls are playing the critical role for the *de novo* formation of Strecker aldehydes. The less concentrated precursor is known to be decisive for the kinetics (reaction speed) of the Strecker degradation. Furthermore, dicarbonyls, in particular 3-DG, are the only precursor class showing a constant reduction with all experimental variations during natural aging. This degradation further indicates the importance of the dicarbonyls for Strecker degradation. 3-DG covered 61–79% of the total molar sum of the observed dicarbonyls (Figure 11). Therefore, 3-DG is the predominant dicarbonyl compound during natural beer aging according to this study. In conclusion, the original hypothesis stating that the concentration of 3-DG in fresh beer can be used as an analytical indicator for flavor instability of pale lager beers is confirmed.

4.6 Outlook

This thesis points out the importance of dicarbonyl 3-DG for understanding the flavor instability of pale lager beer. The investigated results offer themselves to be further used to established a rapid-response analytical method. After derivatization with o-PD 3-DG forms a stable quinoxaline and can be monitored at 312 nm by UV absorption (23). Using 2,3-diamonnaphtalene as a derivatization reagent forms a benzo[*g*]quinoxaline which can also be monitored by fluorescence detection at an excitation wavelength of 271 nm and an emission wavelength of 503 nm (118). This could be used for a photometric or a fluorometric fast response method where the dicarbonyls could be measured directly from fresh beer. The establishment of such a

method certainly requires further validation and investigation of a strongly enlarged sample set of pale lager beers.

Flavor instability is a complex phenomenon. Beside important precursors, antioxidants play a role to determine the flavor instability of beer. A key antioxidant is SO_2 in beer. Therefore, studies should be applied with a constant 3-DG content and varied SO_2 concentrations to investigate the role of 3-DG by varied antioxidant concentration. The SO2 variation could be performed by varied yeast strains or varied fermentation conditions.

Furthermore, dicarbonyl compounds are potentially suitable as predictors for the flavor stability of other beer types such as dark beers or wheat beers. In comparable experiments, the functionality of 3-DG in these types could be observed. Finally, the potential 3-DG metabolism during yeast fermentation could be investigated. Follow-up products such as 3-deoxyfructose may also be promising analytical indicators for flavor instability.

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Appendix

None peer-reviewed publications

- „Frage des Quartals: Wie altert Bier?“ (Arndt Nobis, Julia Wannenmacher); Bier und Brauhaus (2017)

- „Frage des Quartals: Wie funktioniert die Maillard Reaktion?“ (Raphael C. Prado, Arndt Nobis); Bier und Brauhaus (2019)
- „Vokabular der Bieralterung – Sensorische Analyse der Bieralterung im Rahmen der Qualitätssicherung“ (Florian Lehnhardt, Arndt Nobis, Martina Gastl, Thomas Becker)
- „Es braut sich was zusammen“ (Arndt Nobis, Florian Lehnhardt), Nachrichten aus der Chemie (2020)
- „Wie entsteht verborgenes Alterungspotential im Malz?“ (Arndt Nobis, Melanie Kathke, Michael Hellwig, Thomas Henle, Martina Gastl, Thomas Becker); Brauwelt (2021)

Peer-reviewed publications

- 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. Journal of Agriculture and Food Chemistry. 64. (2016): 2746-2753
- Alpers, T., Kerpes, R., Frioli, M., Nobis, A., Hoi, K., Bach, A., Jekle, M., Becker, T.: Impact of Storing Condition on Staling and Microbial Spoilage Behavior of Bread and Their Contribution to Prevent Food Waste. Foods. 10. (2020): 76
- Ritter, S., Nobis, A., Gastl, M., Becker, T.: Evaluating raffinose family oligosaccharides and their decomposition products by ion chromatography – a method development and advanced repeatability study. Talanta Open. 5. (2022): 100086

Oral presentations

- „Dextrin-Profiling im Darrprozess mittels MALDI-TOF-MS-Imaging“, 51. Technologisches Seminar 2018
- „Bioverfügbarkeit von Zink – Was steckt hinter dem Gesamtpotential?“, 52. Technologisches Seminar 2019
- „Campus Connect: Best practices in HPLC“, Fortbildung Graduiertenzentrum Weihenstephan 2019

- „Bier bewusst genießen – Die Chemie der Bieralterung“, Fachvortrag JCF Rostock 2021
- „Precursor from the Maillard Reaction – a New Early Possibility to Predict Beer Aging Stability?”, Virtual ASBC Meeting 2021
- „Einfluss der Malzmodifikation auf die Alterungsstabilität des Bieres“, 19. Rohstoffseminar 2022
- „3-Desoxyglucoson – Potential der Dicarbonylverbindungen aus dem nicht-enzymatischen Polysaccharidabbau“, 54. Technologisches Seminar 2022