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Review

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On the Origin of Free and Bound Staling Aldehydes in Beer

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ABSTRACT: The chemistry of beer flavor instability remains shrouded in mystery, despite decades of extensive research. It is, however, certain that aldehydes play a crucial role because their concentration increase coincides with the appearance and intensity of "aged flavors". Several pathways give rise to a variety of key flavor-active aldehydes during beer production, but it remains unclear as to what extent they develop after bottling. There are indications that aldehydes, formed during beer production, are bound to other compounds, obscuring them from instrumental and sensory detection. Because freshly bottled beer is not in chemical equilibrium, these bound aldehydes might be released over time, causing stale flavor. This review discusses beer aging and the role of aldehydes, focusing on both sensory and chemical aspects. Several aldehyde formation pathways are taken into account, as well as aldehyde binding in and release from imine and bisulfite adducts.

KEYWORDS: *beer aging, flavor stability, free aldehyde, bound aldehyde, imine, bisulfite*

1. SENSORY APPROACH TO BEER AGING

"Flavor" has been defined as "the sum of perceptions resulting from stimulation of the sense ends that are grouped together at the entrance of the alimentary and respiratory tracts".[1,2] In practice, "flavor" can be considered to comprise four different components: odor, aroma, taste, and mouthfeel. "Odor" is the perception of volatiles by the olfactory mucous membrane in the nasal cavity, after sniffing through the nose and entering the nasal passage. The experience of "aroma" is due to volatilization of compounds by body heat after taking the food product in the mouth. The volatiles reach the nasal cavity in a retronasal fashion, through the nasopharyngeal passage. "Taste" is the perception of soluble substances in the mouth by receptors located primarily on the surface of the tongue.[2−4] The amount of taste attributes is rather limited: sweet, sour, salty, bitter, umami, and fatty.[3] The term "mouthfeel" covers the haptic perception of the food product on the surface of the oral cavity, for example, the warming effect of alcohol, the sparkling of carbon dioxide, the oiliness of fats, and astringency.[3,5−7] Terminology for the description of beer flavor was visualized in the "Beer Flavor Wheel" by Meilgaard et al.[6] Since then, suggestions for adaptations[5,7] and variations[3] have been published. It must be kept in mind that the olfactory, gustatory, and haptic sensations are interconnected and that the perceived "flavor" is the result of very complex interactions between the senses. For example, higher levels of carbon dioxide in beer increase sourness and decrease astringency, whereas a higher ethanol concentration and higher beer pH increase the bitterness perception.[3] Furthermore, the presence of one substance can enhance or diminish the intensity of the perception of another substance. This way, the intensity of a mixture of components can be higher or lower than the sum of the individual intensities, called "synergy" and "suppression", respectively. For example, a mixture of ten aldehydes could be perceived even when they were present in a concentration of only one-tenth of their individual flavor threshold value,[8] but even certain combinations of, for example, two or three aldehydes at subthreshold levels have a perceivable effect on flavor.[2,4,9−17] The chemical similarity between these compounds seems of lesser importance for a synergistic effect, as it is rather a similar flavor sensation that matters.[9]

Flavor quality is, of course, very important in light of the general appreciation of consumers of a particular beer brand, but also important is the flavor stability of the brand they are accustomed to. Not all flavors associated with aging are necessarily regarded as off-flavors, and sometimes they are even preferred by the drinker. When a certain brand fails to meet the expectations of the consumer; for example, when the expected flavor is that of the fresh beer and the presented product shows aged flavors (or vice versa), it can lead to rejection of the brand.[18−24] Conversely, more flavor-stable beer allows greater flexibility in terms of the length of supply chain and temperature management in logistics.

A compound is generally detectable once its concentration becomes higher than its flavor threshold value. The lowest stimulus producing a sensation is called the absolute or detection threshold. If the recognition threshold is transgressed, which is generally higher than the detection threshold, the stimulus can be identified. The minimum concentration change to elicit a noticeable difference in a nonzero concentration matrix is the difference threshold.[2] Because both the concentrations and flavor thresholds of compounds can vary widely, the term "flavor unit" (FU) was introduced. This is the ratio of the concentration of a flavor-active compound and its threshold value. As a rule of thumb, a 0.5 FU increase or decrease is perceived by the taster but the cause may not be identified, whereas it can in the case of a 1 FU change.[9]

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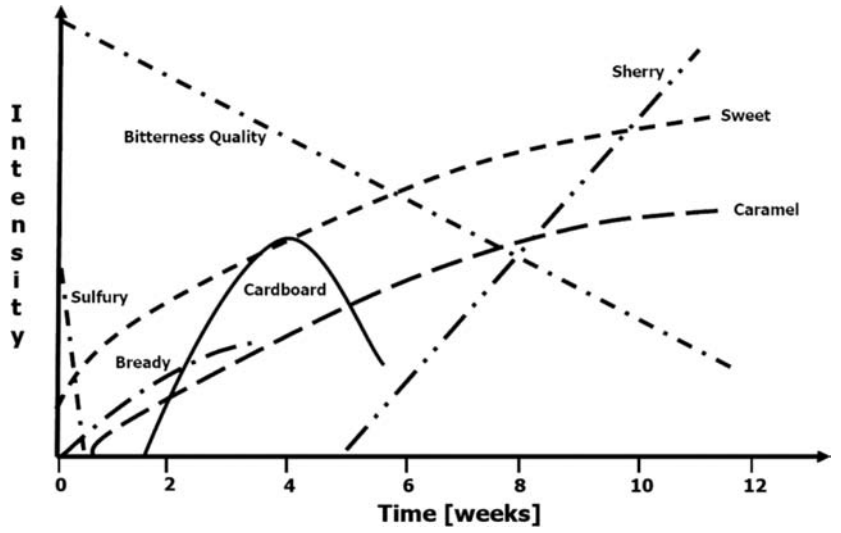

Figure 1. Graphical representation of the generalized flavor changes during aging of beer, as described by Zufall et al.[28] Reprinted with permission from ref 28. Copyright 2005 Fachverlag Hans Carl.

Normally, for sensory analysis of beer, trained experts are grouped into a sensory panel. Nevertheless, findings made by this panel are affected by the testing setup (e.g., number of samples to be evaluated), the testing environment (e.g., distracting odors in the testing room), personal factors (e.g., fatigue), and human bias, which make the results prone to unwanted variations. In the past, for the determination of flavor threshold values, myriad testing setups were used and reported. However, in 1979, the "ASTM Ascending Method of Limits" was proposed as standard procedure protocol.[25,26] In this method, the individual thresholds, as given by each panelist, are used to calculate the group threshold as the geometric mean of these individual values. Sensitive and/or trained individuals will, however, reward lower threshold values to certain components and, in some cases, large differences between individuals are observed.[2,27] Furthermore, it should be mentioned that the concentration of the compound already present in the fresh beer to which the compound is spiked is ignored in this procedure (difference threshold). Thus, flavor thresholds will vary depending on the beer type tested. Beer with a high endogenous concentration will likely result in a lower threshold value.[4,9,27] For this reason, flavor thresholds are also often reported in pure water (absolute thresholds). Due to possible influences of endogenous compound concentrations, synergistic and/or suppressive effects of a combination of compounds, masking flavors, and threshold variations between individuals, flavor threshold values as determined in beer are rather indicative than absolute, and it is therefore not surprising that data on flavor thresholds reported in the literature are often inconsistent.

An attempt was made by Dalgliesh[24] to generalize the sensory evolution of beer flavor during storage. Numerous papers make reference to the so-called Dalgliesh plot, and variations on this theme have been published as well, for example, by Zufall et al.[28] (Figure 1). As the aging pattern will differ between different beers, the depicted curves will vary in relative intensities and times.[24] In lager beers, for example, cardboard flavor is said to be the principal stale flavor. This negative attribute appears after a lag period and increases over time.[24] According to some, the cardboard flavor decreases again when aged even further.[28] This off-flavor may, however, not be perceived in aged specialty beers.[29] Because the largest part of the beer market comprises lager beers, most of the studies are focused on this market sector and, consequently, knowledge about the aging of specialty beers is relatively poor.[30]

Apart from cardboard flavor, aging beer may develop sweet, toffee-like, caramel, and burnt-sugar aromas, as well as a sweet taste. Also, a typical "ribes" flavor may appear very rapidly, but the intensity decreases upon further aging. This odor resembles the smell of crushed leaves and stems of black currant (*Ribes nigrum*) or flowering currant (*Ribes sanguineum*) and can also be referred to as "catty".[24] After very long aging, woody, wine- and whiskey-like notes can be detected as well. Also, sherry/madeira-like, solvent-like, metallic, earthy, straw, bread crust, and cheesy flavors can be detected in some cases.[18,21,24,28,30,31] Staling is not only characterized by an increase of undesired aging flavors, but the decrease of pleasant fresh flavors plays an important part as well. The loss of these positive flavor attributes, such as floral, fruity, and estery aromas, also comprises a loss in masking effect of negative flavor aspects.[4,18] Sulfury notes decline very rapidly. Bitterness becomes harsher, astringency develops, and mouthfulness decreases. The European Brewery Convention (EBC) Sensory Subgroup drafted a Flavor Stability Wheel, comprising descriptors relevant to flavor staling in beer (Figure 2). This tool was designed to facilitate the standardization of the used terminology when describing staling.[32]

2. CHEMICAL APPROACH TO BEER AGING

As generally recognized, many chemical reactions still take place during beer storage, indicating that freshly bottled beer is not in a state of chemical equilibrium. Moreover, bottled beer is not a perfectly closed system (e.g., oxygen ingress, light irradiation). It is stated, as a rule of thumb derived from the Arrhenius equation, that a temperature increase of 10 °C approximately doubles the rate of chemical reactions.[18,19,21,23,24] However, it was seen empirically that the degrees of flavor staling were comparable when beer was stored for 5 days at 37 °C, for 22 days at 30 °C, and for 42 days at 25 °C.[33] Therefore, to slow the chemical reactions in beer and prevent staling, it is advisible to maintain the lowest temperature possible for beer storage, while also taking into account other factors such as haze formation. However, at a fixed temperature, the rate of a particular

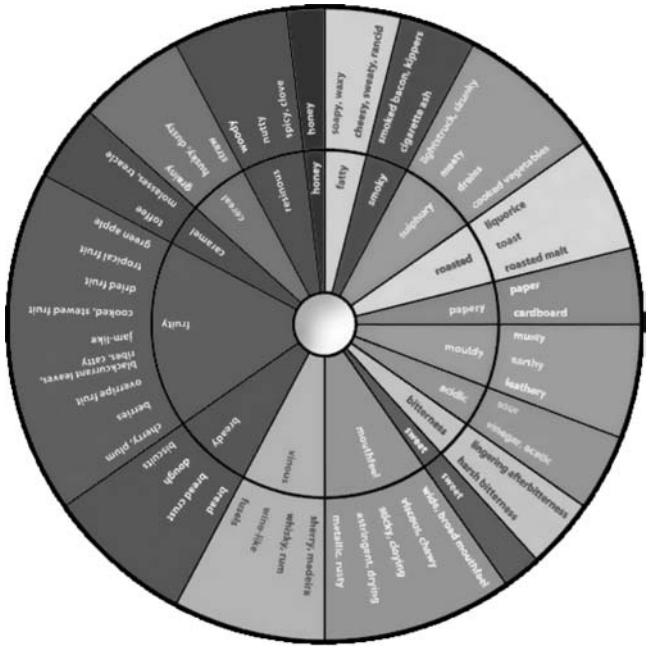

Figure 2. Flavor Stability Wheel, as proposed by the EBC Sensory Subgroup.[32] Reprinted with permission from ref 32. Copyright 2003 Fachverlag Hans Carl.

chemical reaction is determined by its characteristic activation energy. Hence, as different reactions have different activation energy values, reaction rates do not increase equally with increasing storage temperature.[21] This phenomenon has important consequences for conducting beer aging studies. Performing an aging experiment at a higher temperature (e.g., 60 °C) requires a shorter time period to develop staling, which makes it less time-consuming. Although the results might be indicative for staling principles, the obtained aging pattern may, however, not be representative for a more realistic and practical storage temperature (e.g., 7 or 20 °C). For example, beer aged at a higher temperature tends to develop more cardboard notes, whereas other notes, for example, caramel, might dominate in the case of a lower storage temperature.[33−38] Stated differently, one must exercise precaution in setting up forced beer aging experiments.

2.1. Aldehydes and Beer Aging. Hashimoto[39] was the first to observe that stale flavor formation in beer is accompanied by an increase of volatile carbonyls. Later, he found that the addition of hydroxylamine, a carbonyl scavenger, rapidly removes (part of) the stale flavor of aged beer.[40] Similar results were seen with the carbonyl scavengers 2,4-dinitrophenylhydrazine and semicarbazide.[18] Over the years, the role of volatile aldehydes in the sensory perception of staling in beer became indisputable, just like in several other alcoholic beverages. Fresh beer generally contains rather low concentrations of these aldehydes, below their respective flavor thresholds. Their concentration increase coincides with the appearance and intensity of "aged flavors".[41] Often, (*E*)-2-nonenal has been cited as the most important stale compound of lager beer, because its concentration was seen repeatedly to increase during aging to levels above the flavor threshold (approximately 0.03 μg L^{-1} according to Saison et al.[4]), causing cardboard/papery notes.[13,18,19,33,42−44] This attribute was first described by Burger et al.[45] in the 1950s. Over the years, it became clear, however, that (*E*)-2-nonenal is just a part of the bigger picture of staling and that the overall stale flavor is caused by a myriad of compounds. Multivariate data analysis identified a group of aldehydes, from different origins, as good staling markers.[37,46] These are listed in Table 1, together with aldehydes that are generally considered as important in beer staling.

Table 1. Boiling Point[170] of a Selection of Aldehydes, Their Respective Flavor Thresholds (Determined in Beer, Odor Thresholds Are Marked by an Asterisk)[4,13] and Flavor Descriptors[4]

aldehyde	boiling point	flavor threshold (ppb)	flavor description
acetaldehyde	21 °C	1114[4]	green apple, fruity
	at 760 mmHg	25000[13]	
Fatty Acid Oxidation Products			
hexanal	131 °C	88[4]	bitter, winey
	at 760 mmHg	350[13]	
(*E*)-2-nonenal	100−102 °C	0.03[4]	cardboard, papery, cucumber
	at 16 mmHg	0.11[13]	
Maillard Reaction Products			
furfural	161.8 °C	15157*[4]	caramel, bready, cooked meat
	at 760 mmHg	150,000[13]	
5-hydroxymethylfurfural	114−116 °C	35784*[4]	bready, caramel
	at 1 mmHg	1,000,000[13]	
Strecker Degradation Products			
2-methylpropanal	64 °C	86*[4]	grainy, varnish, fruity
	at 760 mmHg	1000[13]	
2-methylbutanal	90−92 °C	45[4]	almond, apple-like, malty
	at 760 mmHg	1250[13]	
3-methylbutanal	92−93 °C	56*[4]	malty, chocolate, cherry, almond
	at 760 mmHg	600[13]	
methional	165−166 °C	4.2[4]	cooked potatoes, worty
	at 11 mmHg	250[13]	
phenylacetaldehyde	195 °C	105[4]	hyacinth, flowery, roses
	at 760 mmHg	1600[13]	
benzaldehyde	179 °C	515[4]	almond, cherry stone
	at 760 mmHg	2000[13]	

2.2. Aldehyde Quantification. A wide range of aldehyde detection and quantification techniques are available (see Table 2). Although incomplete, this summary is indicative of the wide spectrum of methods available.

Our understanding of flavor and flavor stability rapidly increased with the introduction of gas chromatography in the 1960s, despite the method's limited potential at the time. About 30 years later, with the development of solid phase microextraction (SPME), another valuable milestone in aldehyde quantification was reached. This technique combines extraction and enrichment in one step, making extensive sample preparation and preconcentration superfluous. In addition, the required sample volume is rather low, and the use of solvents is strongly reduced, if not eliminated. Consequently, analyses became less expensive, less time-consuming, and easily automatable.[47−58] The solid phase is preferably exposed to the headspace, avoiding interference of nonvolatile matrix components, such as carbohydrates, proteins, lipids, and polyphenols, while strongly reducing its wear.[59,60] Other volatiles such as alcohols and esters, often with much higher abundances than aldehydes, can also interact with the SPME solid phase. To improve selectivity toward aldehydes from this volatile fraction, a derivatization of the carbonyl group can be introduced prior to

Table 2. Summary of Some Methodologies Used in Aldehyde Quantification in Beer and/or Other (Alcoholic) Beverages[a]

derivatization agent	extraction type	point of derivatization	extraction phase	separation technique	detection method
2,4-DNPH 44,143,148,234 cysteamine 131,235 MBTH 236 HH 237 PFBHA 10,23,47,48, 51–53,55,57–59, 107,145,238,239 PFPH 50 MHH 240	LLE 10,51,52,59,131,143, 148,235–237,239	in solution 10,23,34,44,47,51, 52,59,131,143,148, 234–237,239,240	liquid 10,34,44,47,51,52, 59,131,143,148, 234–237,239,240	TLC 44,143,234	densitometer 143,234
					MS 44
				HPLC 148	UV 148
				GC 10,23,34,47,48,50–53, 55,57–59,107,131,143, 145,207,225,235,236, 238–241	ECD 10,50–52,57,59,239
	SPE 34,240		headspace 23,47,48,50,51,53, 57–59,107,145, 207,225,238		NPD 235
	SPME 23,47,48,50–53, 57–59,107,145, 207,225,238	on fiber 23,48,50,51,53, 57–59,107,145,238			FID 34,48,238
	SBSE 55,241	in solution 55	liquid 55,241		MS 23,53,55,107,131,143, 145,207,225,236,237, 240–243
		on site 55,241			
-	-	-	headspace 242	SIFT 242	
D-cysteine 243 2,4-DNPH 244	-	in solution 243,244	-	LC 243,244	UV 244

[a]The table should be interpreted in a horizontal direction, where combinations of cells with common boundaries (except for the first column) have been encountered in the literature. 2,4-DNPH, 2,4-dinitrophenylhydrazine; ECD, electron capture detection; FID, flame ionization detector; GC, gas chromatography; HH, hydroxylamine hydrochloride; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LLE, liquid–liquid extraction; MBTH, 3-methylbenzothiazolin-2-one hydrazone; MHH, *o*-methylhydroxylamine hydrochloride; MS, mass spectrometry; NPD, nitrogen–phosphorous detector; PFBHA,*o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine; PFPH, pentafluorophenylhydrazine; SBSE, stir bar sorptive extraction; SIFT, selected ion flow tube; SPE, solid phase extraction; SPME, solid phase microextraction; TLC, thin layer chromatography; UV, ultraviolet spectrometry.

extraction. The derivatization agent *o*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine (PFBHA) has proven to be the most efficient and is therefore currently most commonly used.[10,23] PFBHA is preferably loaded onto the solid phase, where it can react with the aldehydes during extraction. The complex molecules formed are thermally desorbed from the solid phase, chromatographically separated, and subsequently detected, usually with a mass spectrometer.

For studying the sensory activity of individual compounds, a technique called gas chromatography–olfactometry (GC-O) is applied. After passage through the chromatographic column, the effluent is led through an olfactometric port, where the trained researcher is able to detect the potential odor of the separated compounds. From a wide range of compounds in a mixture, it is possible to identify the flavor-active ones among them, with their respective flavor descriptors and intensities. In parallel and simultaneously to this sensorial detection, a conventional analytical detector, such as a mass spectrometer, can be used by splitting the effluent.[11,23,61−65] The application of GC-O can be considered as a screening step in the search for potentially flavor-active components. Due to, among others, the lack of sensory interaction between flavor compounds, the results should be considered within the framework of more extensive sensory evaluation.

2.3. Mechanisms of Aldehyde Formation. The complex set of volatile constituents present in beer when it is consumed will determine its odor and aroma. Aldehydes play an important role in this matter, and some of their potential mechanisms of formation are discussed below.

2.3.1. Oxidation of Unsaturated Fatty Acids. For decades, extensive research has been performed to elucidate the formation pathways of (*E*)-2-nonenal, because this particular aldehyde was believed to be the main contributor to stale beer flavor. Rather soon, it became clear that this unsaturated aldehyde was derived from lipid oxidation.[14,66−69] This suggests that the fatty acids of most significance are linoleic acid (C18:2) and linolenic acid (C18:3), which contribute about 60 and 10%, respectively, of the total fatty acid content in malt.[70] Moreover, they contain a (*Z*,*Z*)-1,4-pentadiene entity. Linolenic acid is said to be oxidized approximately 3–4 times more rapidly than

linoleic acid, which in turn is oxidized about 30 times more rapidly than oleic acid (C18:1).[71] Linoleic acid shows the lowest recovery after wort separation, as only 81% of the amount present in malt is found in wort and spent grains, whereas for linolenic, oleic, and palmitic acid (C16:0), these percentages are 85, 85, and 99%, respectively.[72]

Fatty acids are released from the triacylglycerol structure by membrane-bound lipase present in the malt, via hydrolysis at the lipid−water interphase. Barley malt lipases have a pH optimum of 6.8 and are reasonably thermostable, as they may survive temperatures up to 67 °C. Therefore, they show the highest activity during mashing-in and remain active throughout most of the mashing process.[73] Subsequently, the free fatty acids are oxidized to hydroperoxy fatty acids, either via autoxidation or enzymatically through lipoxygenase activity. Another possible pathway that yields hydroperoxy fatty acids is the oxidation of the esterified fatty acids in the triacylglycerol structure, and the subsequent release of oxidized fatty acids by lipases (Figure 3).[70,74] The relative importance of autoxidation and enzymatic oxidation is still a matter of debate.[21,43,75,76] In addition to these two pathways, a third mechanism known as photo-oxidation may cause fatty acid oxidation.[71]

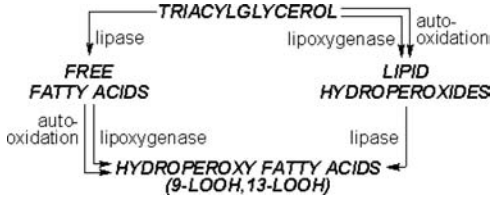

Figure 3. Formation of fatty acid hydroperoxydes by autoxidation and enzymatic activity of lipase and lipoxygenase during mashing, according to Kobayashi et al.[245]

2.3.1.1. Enzymatic Oxidation. Lipoxygenase enzymes (linoleate:oxygen oxidoreductases, EC 1.13.11.12) have an important role in the plant defense system of the viable kernel and living seedling, in response to wounding.[74,77] They recognize the (*Z*,*Z*)-1,4-pentadiene structure in linoleic and linolenic acid and oxidize these unsaturated fatty acids to hydroperoxy acids in the presence of oxygen. The latter are transformed by several enzymatic pathways to mono-, di-, and trihydroxy fatty acids (Figure 4), which can be further degraded nonenzymatically into a variety of carbonyls (e.g., (*E*)-2-nonenal, hexanal).[22,69,78−81] However, considerable amounts of hydroxy fatty acids are not degraded and remain present in the final beer.[71,82]

With respect to barley and malting, two different lipoxygenases are important: LOX-1 and LOX-2.[81,83] LOX-1 is present in barley, but its activity increases during germination. It oxidizes linoleic acid mainly to 9-hydroperoxyoctadeca-10,12-dienoic acid (9-LOOH). The optimum pH is 6.5, with 50% activity remaining at pH 5. LOX-2 is formed only during germination and is not present in raw barley. It mainly forms 13-hydroperoxyoctadeca-9,11-dienoic acid (13-LOOH) from linoleic acid. The optimum pH is also 6.5, but the pH range is narrower than that of LOX-1, the activity being practically zero at pH 5.[22,81,83−86] LOX-2 also shows a higher activity toward fatty acids esterified in triacylglycerol than LOX-1.[74]

During malting, especially kilning, most lipoxygenase activity is lost because inactivation of both enzymes takes place. The activity remaining after malting, mainly related to the slightly more heat-stable LOX-1, is partly transferred into the wort.[81] A rather high mashing-in temperature (e.g., 63 °C) and low mash pH (e.g., pH 5.2) result in a lower lipoxygenase activity.[18,19,21,22,71,75,87−89] Although this residual activity might appear minimal, the importance of this enzyme in beer staling was clearly indicated by laboratory-scale brewing trials with a null-LOX-1 barley line. The use of this barley resulted in a significant reduction of the (*E*)-2-nonenal concentration in beer, even after prolonged storage.[90] Some are convinced that the limiting factor of lipoxygenase activity is the oxygen and unsaturated fatty acid content, rather than the enzyme level.[76,91] In addition, some polyphenols, to date still unidentified but originating from the malt, seem to inhibit lipoxygenase activity.[86,92,93]

2.3.1.2. Autoxidation. The autoxidation of an unsaturated fatty acid (linoleic acid, linolenic acid) initiates by the abstraction of a weakly bonded hydrogen atom from the diallylic carbon atom in the (*Z*,*Z*)-1,4-pentadiene entity by a free radical (Figure 5). This results in a pentadienyl radical and comprises the rate-limiting step of the whole autoxidation process.[94] This initiation is most likely performed by relatively slow-reacting perhydroxy radicals ($HOO^{\bullet}$), or it can be propagated by peroxy radicals ($ROO^{\bullet}$) that are produced in this pathway (hence "autoxidation").[21,71,94,95] Other reactive oxygen species (ROS) with higher reactivity (e.g., hydroxyl radicals $HO^{\bullet}$, singlet oxygen) are not very likely to react with fatty acid, because they most likely react first with more abundant molecules, such as ethanol. For linoleic acid, the pentadienyl radical is stabilized by the formation of two different hydroperoxides with two conjugated double bonds each: 9-LOOH and 13-LOOH. The monoallylic carbon atoms present in linoleic acid can react as well, however, to a lesser extent than the diallylic site. The extraction of a hydrogen atom from these sites gives rise to four different hydroperoxy acids with two isolated double bonds each. The total proportion of these 8-, 10-, 12-, and 14-LOOHs is only about 4%.[21]

A wide variety of compounds can be formed from these hydroperoxy acid intermediates, by both enzymatic and nonenzymatic processes. The formation of (*E*)-2-nonenal and hexanal is initiated by protonation of the hydroperoxide group. A water molecule is eliminated, and the oxo-cation is inserted in the carbon−carbon bond next to the double bond. The formed carbenium ion is hydroxylated, and the molecule splits into an aldehyde and an oxoacid.[96] Higher temperatures, low pH values, and the presence of oxidants accelerate this mechanism. Transition metal ions, such as iron and copper, have a catalytic effect as they promote the formation of radicals from hydrogen peroxide.[21,71] The predominant step in the beer production process during which autoxidation takes place is said to be wort boiling.

2.3.1.3. Photo-oxidation. A variety of carbonyls (saturated, monounsaturated, diunsaturated) was seen to be produced by photo-oxidation of oleic and linolenic acid in beer.[69,71,97] Photosensitizers such as riboflavin (vitamin B_2) are activated by light irradiation. These activated species excite triplet oxygen to singlet oxygen, which in turn reacts with fatty acids to form hydroperoxides and aldehydes (Figure 6). The reaction is independent of the temperature. Singlet oxygen is much more reactive than triplet oxygen, and so without the influence of light, this pathway is of little significance. Therefore, beer packaging should aim for a minimal passage of light.[71]

2.3.2. Maillard Reactions. The reaction of an amine, amino acid, peptide, or protein with a reducing sugar and all possible reactions occurring thereafter are called "Maillard reactions" or "nonenzymatic browning reactions". As these reactions commence

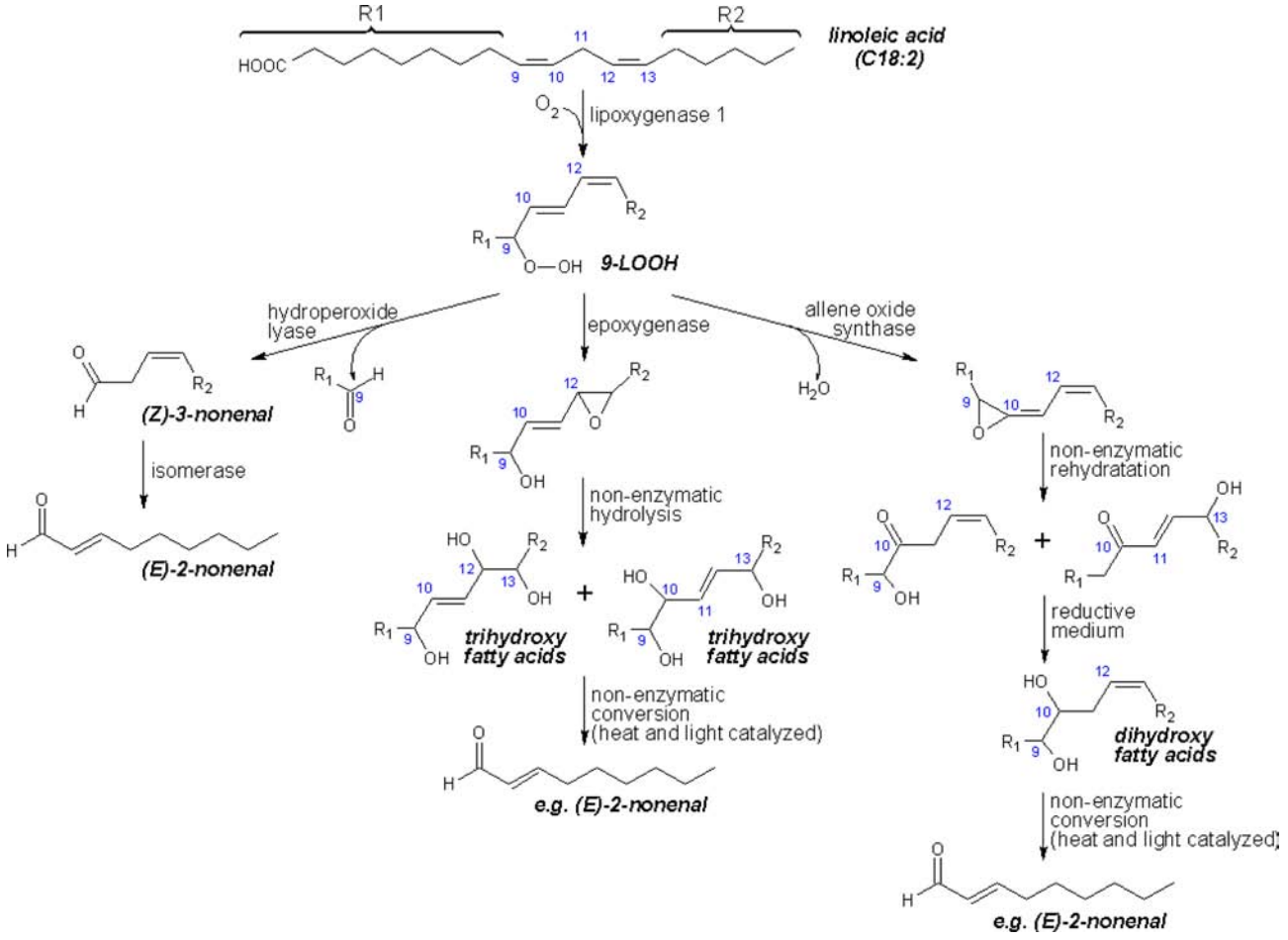

Figure 4. Schematic overview of some relevant published pathways[69,79,80,246] of the enzymatic breakdown of linoleic acid, starting with LOX-1 activity forming 9-LOOH. The epoxygenase and allene oxide synthase pathways result in a myriad of aldehydes and ketones, among others (*E*)-2-nonenal.

at 50 °C in the pH range of 4−7,[98] they are usually related to the application of heat and are responsible for an increase in color. The reaction of one type of amino acid with one type of sugar can already yield a myriad of products. Moreover, not only the primary and/or terminal amino groups interact but also, for example, the secondary amino group of proline and the ε-amino group of lysine in peptides of proteins might play an important role.[98] It is clear that the variety of Maillard products in beer is enormous and their chemical properties are very diverse.[69,98−109]

In general, the heterocyclic compounds furfural and 5-hydroxymethylfurfural (5-HMF) are quantitatively the most important Maillard products in beer. Their formation pathways are very similar (Figure 7). Both are important markers for the heat load placed on the mash, wort, and beer and for flavor staling in general.[17,46,110−117] Throughout the aging process, their concentrations increase at a linear rate.[110,112,113,117] According to several authors, furfural and 5-HMF concentrations do not exceed their respective flavor threshold values, and it is therefore said that they do not significantly affect beer flavor. This is however contradicted by more recent findings by De Clippeleer et al.,[118] in which spiking of furfural to fresh pale lager beer resulted in a sharper, harsher, more lingering bitterness and increased astringency. The effect on taste and mouthfeel is often discarded in flavor threshold determinations, which are usually based on odor and aroma, or only odor.

Furfural originates from a pentose, and 5-HMF is derived from a hexose. The carbonyl group of the sugar compound (in aldose form) reacts with an amine or with the amino group of an amino acid, peptide, or protein. This yields an imine (or Schiff base) and comprises the rate-limiting step of the early-stage mechanism.[119] This imine stabilizes by undergoing a so-called Amadori rearrangement, forming an Amadori compound (1-amino-1-deoxyketose). Higher temperatures are favorable for the rearrangement.[119] Due to instability at the beer pH, this Amadori compound can undergo 1,2-enolization. The subsequent release of an amine gives rise to 3-deoxyosone, an α-dicarbonyl (vicinal diketone). Cyclization yields the heterocyclic compound furfural, in the case of pentose, or 5-HMF, in the case of hexose.[21,98,113,120]

The Maillard cascade is initiated by the nucleophilic addition of the amino group to the reducing end of the open-chain sugar. At wort and beer pH, the sugar compounds are predominately in closed-chain form, and most of the amino acids present have lost their nucleophilic nature (pK_a values often around 9 or higher) and thus their reactive character. Therefore, the initiation of Maillard reaction, the formation of the Schiff base, is favored by a high pH.[23,119,121] After initiation, a high pH promotes 1- and 4-deoxyosone formation, which tempers the formation of 3-deoxyosone due to substrate limitations, rather than its being tempered by pH dependence. A lower pH indirectly promotes the formation of 3-deoxyosone for the same reason. In the case of beer production and storage, the

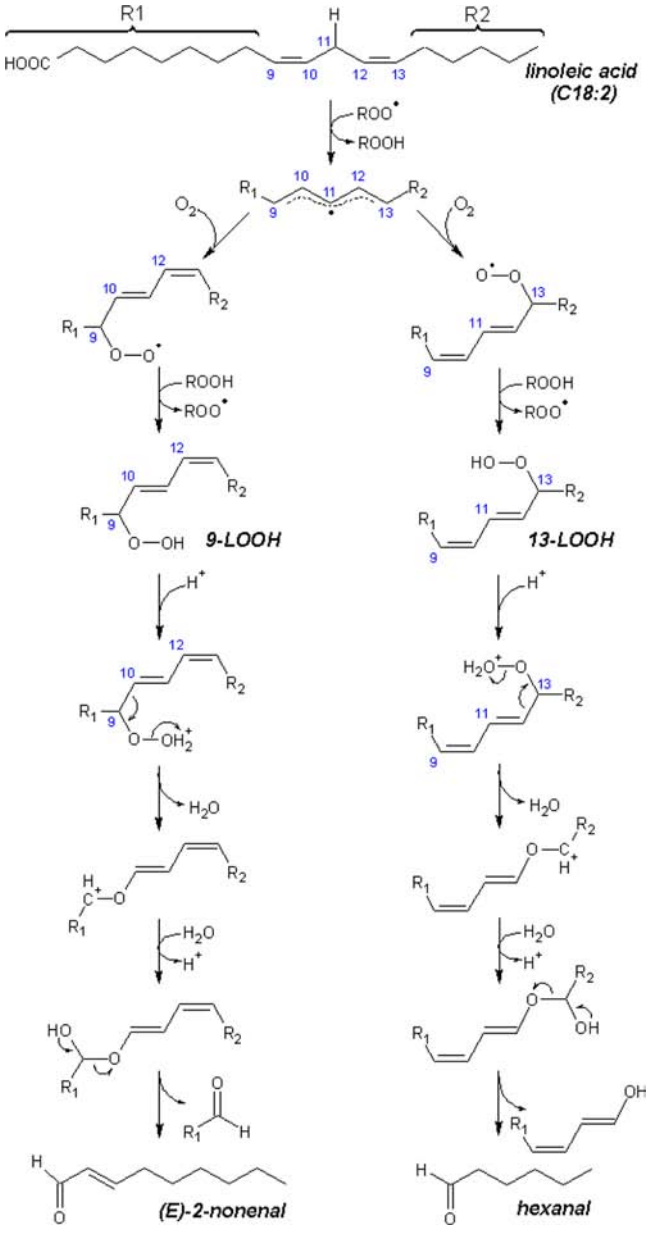

Figure 5. Formation of (*E*)-2-nonenal and hexanal through the autoxidation of linoleic acid, as described by Belitz et al.[94]

3-deoxyosones are predominant, which leads, among others, to furfural and 5-HMF.[104,113,121,122] Deoxyosones can also undergo cleavage by, among others, retro-aldol type reactions, leading to shorter chain α-dicarbonyls (C_2–C_4) such as glyoxal, 2-oxopropanal, and 2,3-butanedione.[98,102,123]

It is further thought that the Maillard reactions still take place at a slow rate during storage at a relevant temperature, as 9 months of beer storage at 20 °C is said to be comparable to 1 h of processing at 100 °C.[121] An accumulation in the individual concentrations of several α-dicarbonyls (in some cases up to 9-fold) during beer storage was seen in several studies.[106,107,124,125] On addition of the trapping agent aminoguanidine to the wort before boiling or to fresh beer, the formed α-dicarbonyls were bound and the flavor stability was enhanced.[106,107,121,124,125] Similar results were observed when 1,3-polydiamine resin was added to fresh beer for α-dicarbonyl scavenging during maturation.[126]

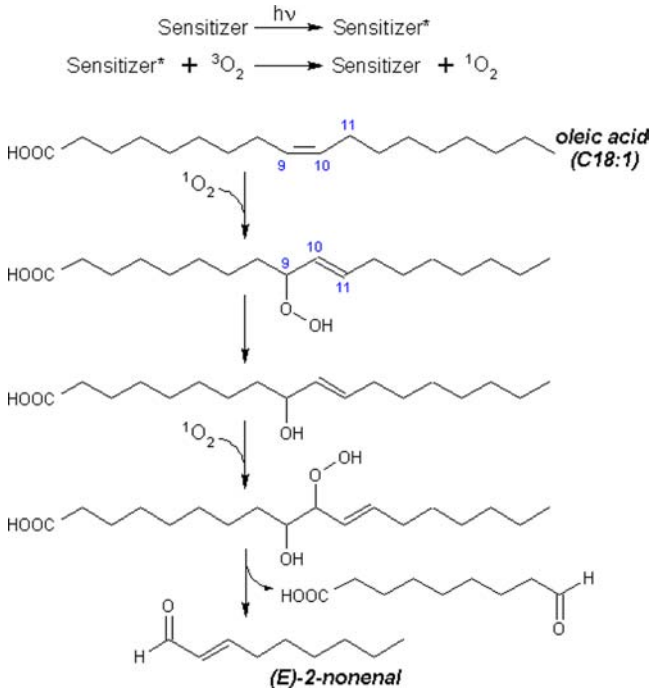

Figure 6. Photo-oxidation of oleic acid, according to Wackerbauer and Hardt.[71]

2.3.3. Strecker Degradation.

2.3.3.1. Strecker Degradation in a Strict Sense. Transamination can take place between an amino acid and an α-dicarbonyl in a reaction called "Strecker degradation" (Figure 8). The nucleophilic addition of the unprotonated amino group to the carbonyl group initiates the reaction, forming an unstable hemiaminal. This readily undergoes reversible loss of water, followed by irreversible decarboxylation, yielding an imine zwitterion. The addition of water results in an unstable amino alcohol, which decomposes into an α-ketoamine and a "Strecker aldehyde", containing one carbon atom less than the amino acid from which it is derived.[21,123,127]

In principle, the large number of different amino acids can give rise to different Strecker aldehydes. However, when the difference in concentration of individual amino acids is considered, in combination with the flavor threshold of the respective Strecker aldehydes, only a few Strecker degradation reactions are of interest in beer flavor: 2-methylpropanal from valine, 2-methylbutanal from isoleucine, 3-methylbutanal from leucine, methional from methionine, and phenylacetaldehyde from phenylalanine. Although benzaldehyde is thought to be formed indirectly from phenylalanine with phenylacetaldehyde as intermediate, it is still considered to be a Strecker aldehyde. Several pathways have been proposed, of which many involve the presence of oxygen.[128,129] An example is the free radical initiated oxidation, as described by Chu and Yaylayan[128] (Figure 9).

The Strecker degradation is often categorized under "Maillard reactions", because various α-dicarbonyls can be produced by Maillard reactions as shown before.[127] However, these compounds can originate from more diverse sources, such as oxidation of polyphenols or the transformation of 2,3-butanedione (diacetyl) and 2,3-pentanedione precursors, excreted by fermenting yeast.[130]

2.3.3.2. Strecker-like Reactions. The reaction of an amino acid with an α-unsaturated carbonyl compound, replacing the α-dicarbonyl in the Strecker degradation strictly speaking, is

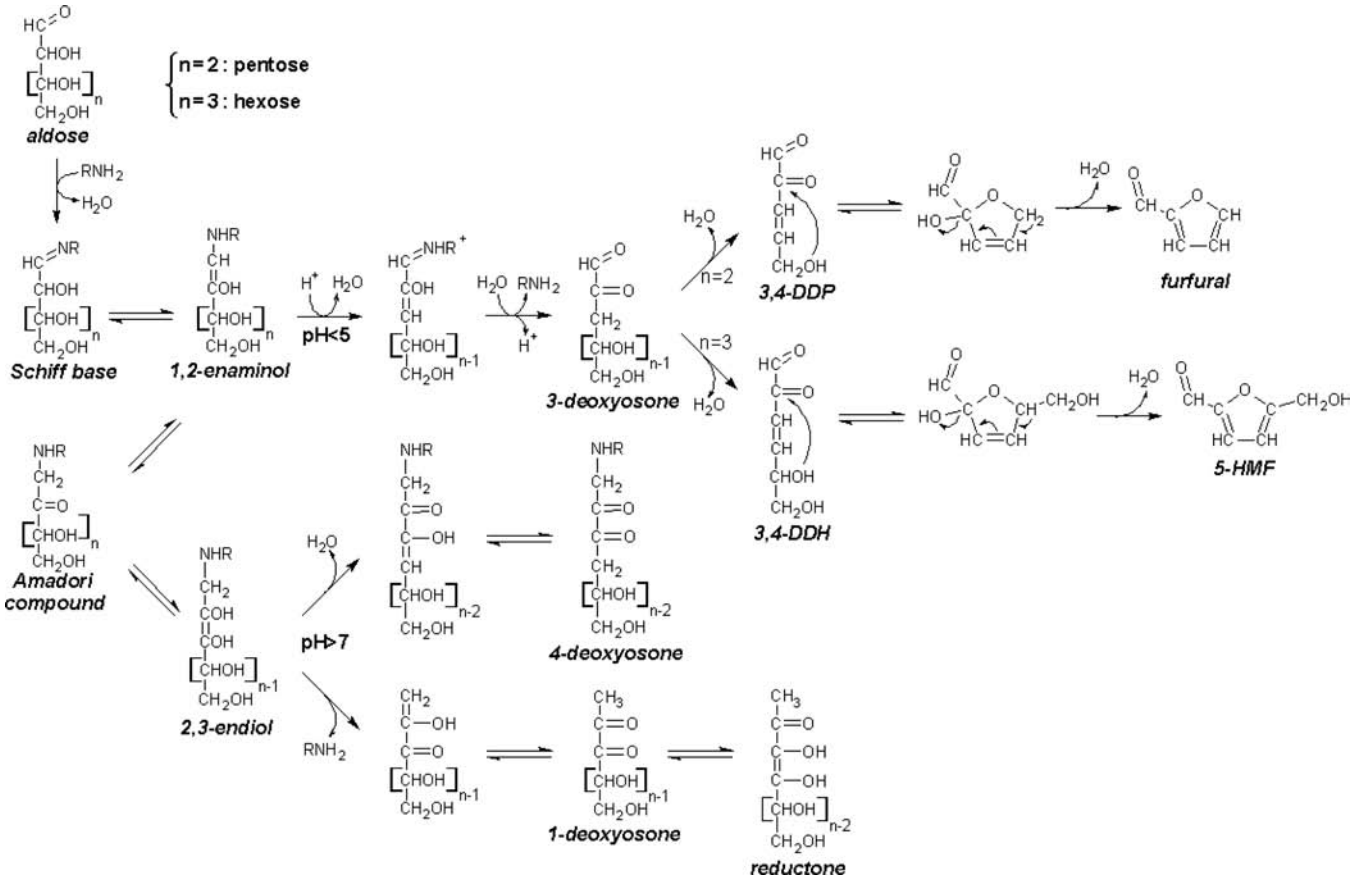

Figure 7. Overview of some Maillard reactions, starting from a pentose (n = 2) or a hexose (n = 3), yielding α-dicarbonyls (3-, 1-, and 4-deoxyosones) and some heterocyclic compounds (furfural and 5-HMF). Under acidic conditions, the formation of 3-deoxyosone is predominant over 1- and 4-deoxyosone formation.[21,98,120] (3,4-DDP, 3,4-dideoxypentosulose-3-ene; 3,4-DDH, 3,4-dideoxyhexosulose-3-ene; 5-HMF, 5-hydroxymethylfurfural).

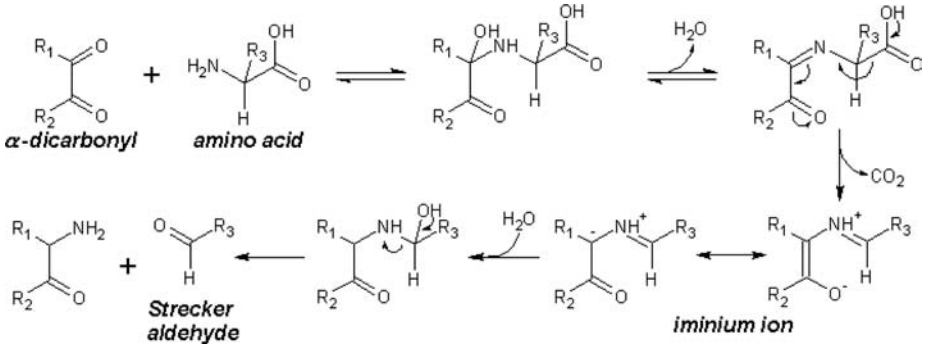

Figure 8. Strecker degradation reaction of an α-dicarbonyl and an amino acid, forming a Strecker aldehyde.

termed a "Strecker-like" reaction. An example of such an α-unsaturated carbonyl is (E)-2-nonenal, derived from lipid degradation. Furfural, derived from Maillard reactions, is an option as well,[123] as is benzaldehyde. The initiation of this Strecker-like reaction, the loss of water, and subsequent decarboxylation are similar to Strecker degradation, forming an imine zwitterion (Figure 10). Addition of water and degradation of the unstable amino alcohol can result in, among others, a Strecker aldehyde and, in some cases, a dihydro derivative of the initial unsaturated aldehyde (e.g., nonanal from (E)-2-nonenal) after release of ammonia. This pathway is, however, based on nonaqueous model systems and has not been confirmed in aqueous solutions, but it is likely that it comprises a Strecker

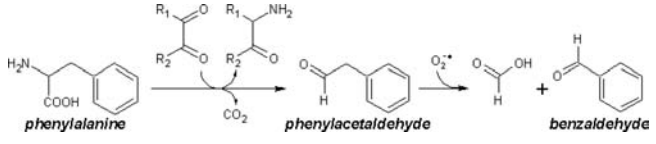

Figure 9. Strecker degradation of phenylalanine to phenylacetaldehyde, followed by the introduction of an oxygen atom at the benzylic carbon to form benzaldehyde.[128]

aldehyde source in food products.[123] Other similar Strecker-like reactions have been identified as well, involving α-cyclopropylcarbonyls, α-epoxycarbonyls, α-epoxyenals, α-epoxyenones, and 4-hydroxy-2-alkenals.[123] This fact illustrates an

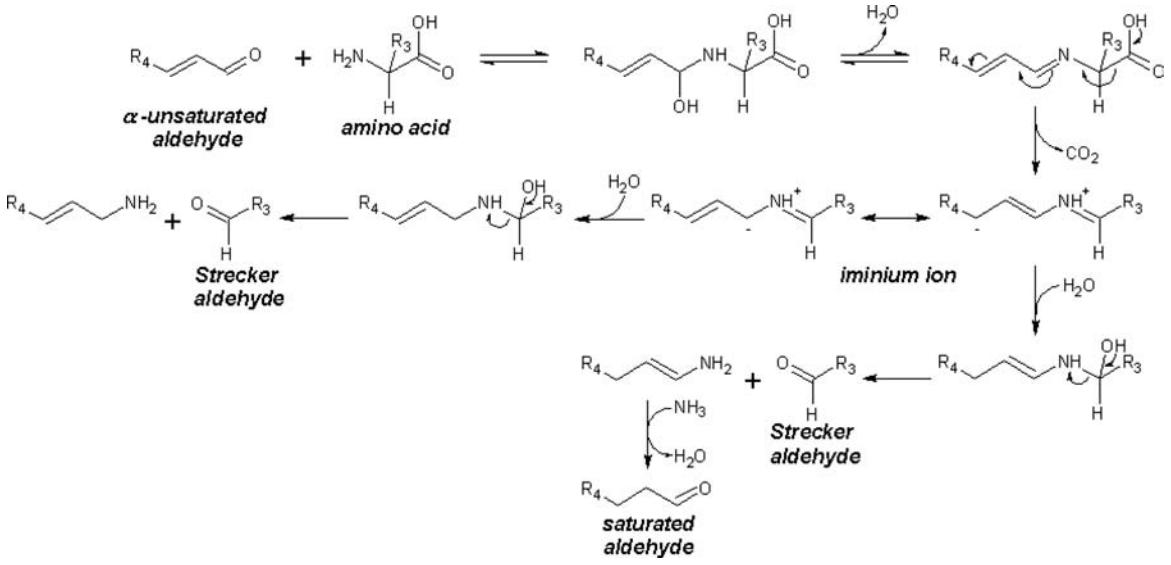

Figure 10. Strecker-like reaction of an α-unsaturated aldehyde and an amino acid, forming a Strecker aldehyde.[123]

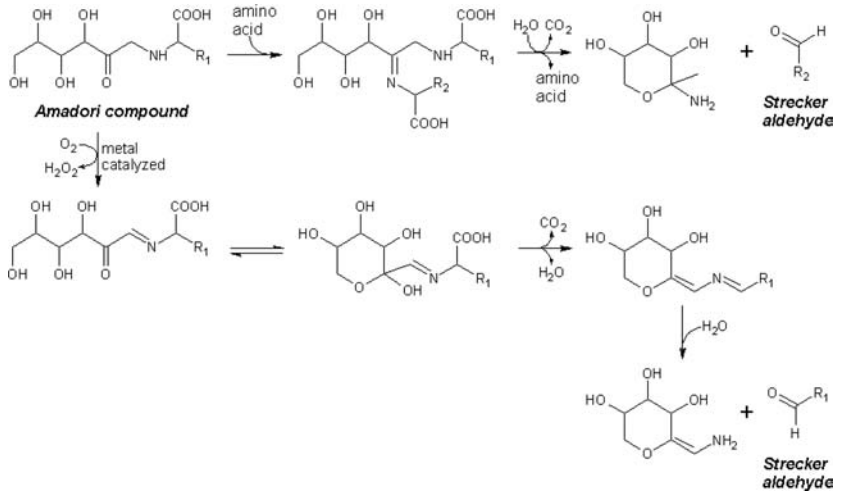

Figure 11. Proposed mechanisms for the formation of Strecker aldehydes starting from the Amadori compound.[127]

overlap in reaction mechanisms that were considered separately in the past, because some of these compounds can be found among lipid degradation products.[131]

2.3.3.3. Direct Strecker Aldehyde Formation from Amadori Compounds. Strecker aldehydes are also thought to be formed from Amadori compounds by direct reaction with amino acids[123,127,132] or via transition metal ion-catalyzed oxidation of the Amadori compound[123,127,133] (Figure 11). However, research on these reactions was performed in model systems and it is, therefore, not clear yet as to what extent these reactions are relevant in beer production processes. Nevertheless, the observation that more Strecker aldehydes are generated during beer aging in the presence of oxygen, and less in the absence, supports this hypothesis.[63,134]

2.3.4. Degradation of Bitter Acids. During wort boiling, the α-acids (six-carbon ring compounds, also called humulones) derived from hop products are heat-isomerized to the bitter tasting iso-α-acids (five-carbon ring compounds, also called isohumulones) (Figure 12). Previous studies demonstrated that, during beer aging, especially *trans*-iso-α-acids are prone to degradation, whereas *cis*-iso-α-acids remain largely unaltered, even after prolonged storage. Furthermore, the ratio of *trans*- over *cis*-iso-α-acids showed a good correlation with the observed decrease in bitterness intensity and quality over time.[31,135−142] In particular, a lower pH and a higher temperature appear to negatively affect *trans*-iso-α-acid stability.[138,141] Among myriad degradation products, a variety of volatile carbonyl products (e.g., 2-methylpropanal, 2-methylbutanal, 3-methylbutanal; Figure 12) was formed from these bitter acids in model solutions.[143] The exact aldehyde-producing degradation mechanism is, however, still unclear.

Hashimoto et al.[144] reported that beer brewed without hops hardly develops a characteristic stale flavor profile, not even after prolonged storage. This would indicate that hop product degradation might be an important stale flavor formation mechanism. This view is, however, contradicted by the results of more recent research by De Clippeleer et al.[145] They separated *cis*- and *trans*-iso-α-acids from a commercial isomerized hop extract on pilot scale and dosed these bittering principles to unhopped beer in milligrams per liter concentrations. After forced aging in the dark at 30 °C, results confirmed the higher instability of *trans*-iso-α-acids compared to *cis*-iso-α-acids. However, the formation of 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal could not be linked to

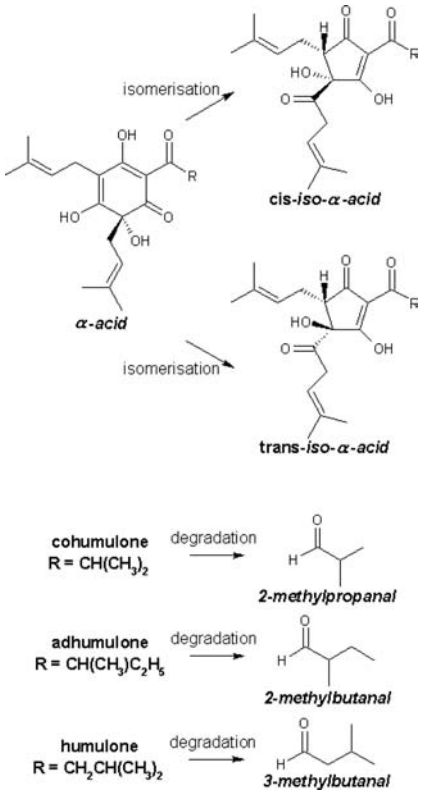

Figure 12. Isomerization of α-acids to *cis*- or *trans*-iso-α-acids. The relative configuration of these epimers differs only in the tertiary hydroxyl at C_4 and the prenyl chain at C_5.[136,145] Also shown are the hypothetical reaction products of the degradation of iso-α-acids to staling aldehydes through deacylation of the side chain at C_2. Adapted from De Clippeleer et al.[145]

hop product degradation, because the levels of these aldehydes increased to a similar extent, whether the beer was unhopped, hopped with commercial isomerized extract, hopped solely with *cis*-iso-α-acids, or hopped solely with *trans*-iso-α-acids. From these results, it can be concluded that stale aldehyde formation from iso-α-acid degradation must be of minor importance, if relevant at all, compared to other mechanisms.

2.3.5. Aldol Condensation. It was observed in model solutions that unsaturated aldehydes with a low flavor threshold can be formed by aldol condensation of saturated aldehydes with a higher flavor threshold, for example, (*E*)-2-nonenal from heptanal and acetaldehyde. Amino acids such as proline are thought to act as catalysts.[146] The general aldol condensation is shown in Figure 13, with heptanal and acetaldehyde as an example.

Besides the formation of (*E*)-2-nonenal, several aldol condensations have been reported, such as the reaction of two molecules of 3-methylbutanal giving 2-isopropyl-5-methyl-2-hexenal,[147] as well as the reaction of phenylacetaldehyde with acetaldehyde, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, and hexanal, yielding 2-phenyl-2-butenal, 4-methyl-2-phenyl-2-pentenal, 4-methyl-2-phenyl-2-hexenal, 5-methyl-2-phenyl-2-hexenal, and 2-phenyl-2-octenal, respectively.[121] Other combinations might occur as well, giving rise to a myriad of branched aldehydes with potentially totally different flavor attributes. However, the extent to which these reactions take place is unclear. For example, the yield of heptanal and acetaldehyde aldol condensation in model solutions was shown to be only about 0.2%. Combined with the generally low heptanal level in beer (order of magnitude 1 μg L^{-1}), the influence of this pathway on (*E*)-2-nonenal concentrations, and aldehyde concentrations in general, during beer aging under normal conditions is questionable.[18,44]

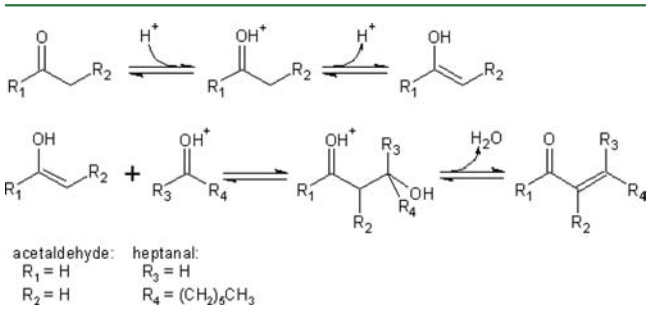

Figure 13. Aldol condensation of two carbonyls, based on Solomons and Fryhle,[166] with the reaction of acetaldehyde and heptanal, forming (*E*)-2-nonenal, as an example.

2.3.6. Melanoidin-Catalyzed Oxidation of Higher Alcohols. Next to ethanol, beer can contain significant amounts of higher alcohols (e.g., 2-methylpropanol, 2-methylbutanol, 3-methylbutanol, 2-phenylethanol). Oxidation of these compounds to their corresponding aldehydes can take place, although not directly by oxygen, but rather by the electron-accepting ability of melanoidins (high molecular weight polymers formed by Maillard reactions). The hydrogen atom of the hydroxyl group of the higher alcohol is transferred to a carbonyl group of the melanoidins. Oxygen facilitates this reaction, as does a lower pH.[146]

Supplementation of higher alcohols to beer results in higher amounts of the corresponding aldehydes.[40] However, their oxidation takes place only in the case of light irradiation, proceeds less readily with increasing molecular weight of the alcohol, and is inhibited by the presence of iso-α-acids and polyphenols.[71] Therefore, this pathway is believed to be of lesser importance.

As a footnote, melanoidins may also have positive side effects with regard to aldehyde formation, because they appear to inhibit the oxidation of fatty acids and the degradation of bitter acids.[146]

2.3.7. Secondary Autoxidation of Aldehydes. Unsaturated aldehydes, for example, (*E*)-2-nonenal, formed by one of the formerly described mechanisms can be further degraded to saturated shorter chain aldehydes (e.g., pentanal, hexanal, heptanal, octanal) by autoxidation.[146] This might be an explanation for the decline of the (*E*)-2-nonenal concentration (and of the related cardboard flavor) during prolonged beer storage.[71,97,148]

2.3.8. Aldehyde Secretion by Fermenting Yeast. Yeast is able to excrete Strecker aldehydes (e.g., 3-methylbutanal, methional) during fermentation via the Ehrlich pathway.[149−152] Oxoacids are formed anabolically from the main carbon source or they are derived from the catabolism of exogenous amino acids. Decarboxylation of these oxoacids yields Strecker aldehydes.[153,154] As an illustration, labeled 3-methylbutanal was produced and excreted by the yeast during cold contact fermentation in a medium containing leucine-d_{10}.[150] The contribution of this origin of aldehydes in the final beer is, however, most likely limited.

2.3.9. Acetaldehyde. Acetaldehyde is an aldehyde that is difficult to categorize under just one specific formation mechanism. It is sometimes called a "Strecker aldehyde", because it can be formed by Strecker degradation of alanine.[23,123] Furthermore, acetaldehyde is formed as a byproduct of glycolysis during fermentation, up to levels of 40 mg L^{-1}.[59,130,155]

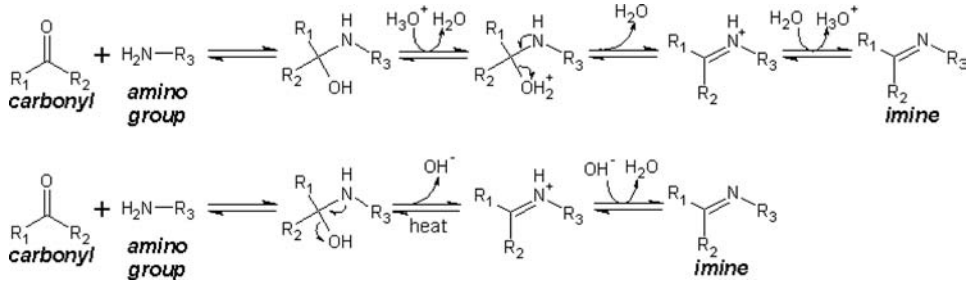

Figure 14. General mechanism for the imine formation reaction in organic (top) and in aqueous solution (bottom), as described by Solomons and Fryhle[166] and Pan et al.[167]

Moreover, ethanol can be oxidized to acetaldehyde in a free radical mechanism involving the Fenton reaction:[156]

initiation: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$ (Fenton reaction)

propagation: ${}^{\bullet}OH + CH_3CH_2OH \rightarrow CH_3{}^{\bullet}CHOH + H_2O$

$$CH_3{}^{\bullet}CHOH + Fe^{3+} \rightarrow CH_3CHO + Fe^{2+} + H^+$$

termination: $Fe^{2+} + {}^{\bullet}OH \rightarrow Fe^{3+} + OH^-$

Beer is predominantly a water−ethanol solution, with ethanol being the most abundant organic molecule present. Not surprisingly, the 1-hydroxyethyl radical is the most abundant free radical in beer, originating from the reaction of ethanol with a hydroxyl radical.[157] This 1-hydroxyethyl radical can bind oxygen, resulting in acetaldehyde and a hydroperoxyl radical, which propagates the radical chain reaction.

2.4. Free and Bound Aldehydes. The cause of increasing levels of (*E*)-2-nonenal during beer aging remains unclear. To estimate the relevance of (*E*)-2-nonenal release from a bound state, the concept of "nonenal potential" was introduced already more than two decades ago. According to Drost et al.,[75] the nonenal potential is a forcing test that determines the potential of a wort to form (*E*)-2-nonenal under beer conditions. Pitching wort is adjusted to pH 4.0 with phosphoric acid and heated for 2 h at 100 °C under an argon atmosphere. According to Liégeois et al.,[91] this procedure represents a way to determine the amount of (*E*)-2-nonenal formed during the production process and that is subsequently bound reversibly in an adduct. Adduct formation will reduce the volatility of (*E*)-2-nonenal and therefore will prevent it from evaporation during the wort production process. Additionally, as an adduct, (*E*)-2-nonenal will be insensitive to the reducing activity of yeast during fermentation (see further). Consequently, in its bound state, (*E*)-2-nonenal may remain present throughout the production process and end up in the final beer. Because analytical aldehyde detection methods are often based on volatilization of the compounds, this bound (*E*)-2-nonenal will be obscured and undetectable as such. The same accounts for the sensory perception of (*E*)-2-nonenal.[158] However, under the specific conditions during beer storage (beer pH, storage temperature), adducts may degrade, releasing (*E*)-2-nonenal, causing cardboard flavor and rendering the beer stale.[42,158−162]

Several studies support this hypothesis and point to the release of (*E*)-2-nonenal from a bound state during beer aging. For example, a close correlation was observed between the nonenal potential of clarified wort and the (*E*)-2-nonenal concentration in both naturally aged and forced-aged beer.[42,161,163,164] Moreover, Liégeois et al.[91] spiked deuterated (*E*)-2-nonenal during laboratory-scale mashing (when 63 °C was reached) to mimic its enzymatic formation by lipoxygenases. The beer produced from this mash was subsequently forced-aged, and estimates from the measurable aldehyde concentrations revealed that mashing may contribute around 30% of the (*E*)-2-nonenal in aged beer, whereas wort boiling contributes about 70% of (*E*)-2-nonenal. Furthermore, other studies excluded trihydroxy fatty acids as (*E*)-2-nonenal precursors in the bottled beer[67] and proved that lipid oxidation has no significant activity in bottled beer, because $^{18}O_2$ isotopes in the headspace were not incorporated into the carbonyl fraction.[161,164]

It is reasonable to assume that, besides the fatty acid oxidation-derived aldehyde (*E*)-2-nonenal, other staling aldehydes may form a similar "potential" during the beer production process and that (part of) these aldehydes are already present in a bound state in fresh beer. Indeed, based on several tests using the Strecker degradation inhibitor *o*-diaminobenzene, added to beer samples, and ^{13}C-labeled amino acids, spiked to filtered wort and beer samples, it has been reported that approximately 15% of total Strecker degradation aldehydes present in aged beer appear to be the result of de novo formation during storage, whereas about 85% seems to be derived from adducts, preformed during wort production.[165] The individual Strecker aldehydes showed, however, a different behavior; for example, 70% of phenylacetaldehyde was estimated to be derived from wort boiling and clarification, compared to practically 100% of 3-methylbutanal and methional.

The two adduct formation mechanisms considered to be most important, that is, imine formation and bisulfite adduct formation, are discussed below in more detail.

2.4.1. Imine Formation. When the carbonyl group of a compound interacts with the amino group of an amino acid, peptide, or protein, an imine (also called a Schiff base) can be formed. According to Solomons and Fryhle,[166] the general reaction mechanism (Figure 14, top) is acid-catalyzed with an optimum pH situated between 4.0 and 5.0. However, this reaction takes place in organic solution, and according to Pan et al.,[167] another reaction mechanism takes place in an aqueous environment (Figure 14, bottom). This was confirmed by Lermusieau et al.,[164] who noticed an increased imine formation with increasing pH, approximately up to a pH of 10. The higher availability of nonprotonated amino groups at a higher pH enhances their nucleophilic behavior, hence the increase in imine formation. A higher reaction temperature facilitates the leaving of the hydroxyl group after the attached carbon receives a pair of electrons from the nitrogen.[167] De Schutter[121] indeed noticed an increased imine formation at higher temperature. Destabilization of imines is said to take place by acidification of the medium (as is also performed in the "nonenal potential" forcing test).[159,162,164]

De Schutter[121] suspects the stabilization of imine adducts formed from 2-alkenals by resonance in the conjugate system.

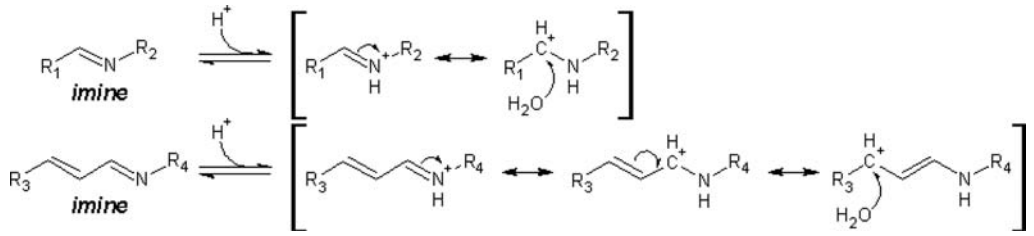

Figure 15. Suspected stabilizing effect by resonance in the structures of iminium adducts formed from 2-alkenals, compared to iminium ions from aliphatic aldehydes. Adapted from De Schutter.[121]

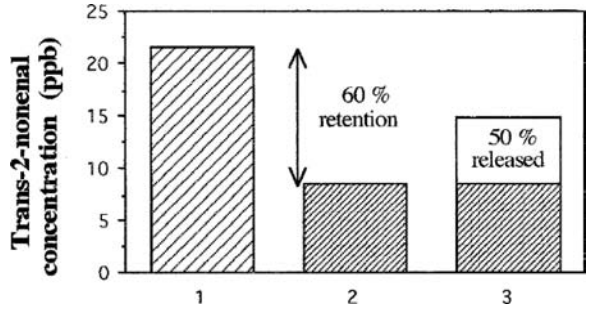

Figure 16. Model solution of (*E*)-2-nonenal (21.4 ppb) and malt albumins (886 ppm of bovine serum albumin equivalent): (1) initial concentration of (*E*)-2-nonenal before interaction with albumins; (2) after 25 min at 50 °C and pH 5.4; (3) after 2 h at 100 °C and pH 4 under argon atmosphere.[159,164] Reprinted with permission from ref 164. Copyright 1999 American Society of Brewing Chemists.

The positive charge may be distributed, making these iminium ions less susceptible for a nucleophilic attack of water molecules than iminium ions formed from aliphatic aldehydes (Figure 15).

Lermusieau et al.[164] used a malt albumin–alkenal model mixture to confirm the binding of free (*E*)-2-nonenal (Figure 16). The initial (*E*)-2-nonenal concentration was compared with the residual concentration after reaction with albumin proteins. After 25 min at pH 5.4 and 50 °C, the measurable concentration of (*E*)-2-nonenal dropped by approximately 60%. However, when the "nonenal potential" method, as described by Drost et al.,[75] was applied, around half of this 60% was released again.[159,164] In other words, although part of the protein-bound (*E*)-2-nonenal remains obscured, determination of the nonenal potential provides a good indication of the presence of bound (*E*)-2-nonenal and thus the potential of (*E*)-2-nonenal release.

Nikolov and Yaylayan[168] investigated the chemical reactivity of 5-HMF with, among others, lysine, glycine, and proline in model systems using isotopic labeling. The interaction of 5-HMF with a primary amino acid such as lysine or glycine yields an imine, which can subsequently be decarboxylated (Figure 17). The compound formed by interaction with a secondary amino acid such as proline can also be decarboxylated, creating two isomeric iminium ions. One isomer, which contains a conjugated structure, is stabilized by vinylogous Amadori rearrangement, whereas the other, nonconjugated isomer, can undergo dehydration.

Aldehydes can also bind proteins by hydrophobic interaction. The binding of aldehydes such as benzaldehyde, hexanal, and (*E*)-2-nonenal to bovine serum albumins was modeled as a function of the number of hydrogen atoms and boiling point of the aldehydes. A higher number of hydrogen atoms and higher aldehyde boiling point correlate with a higher fraction bound to the albumins.[169] In practice, this aldehyde scavenging potential

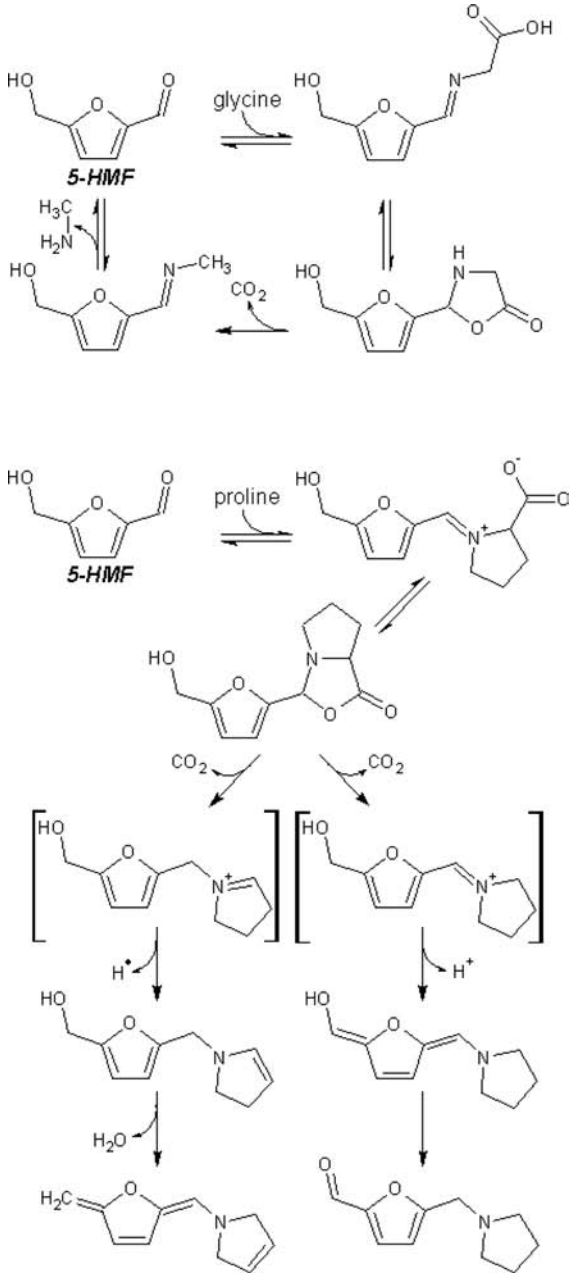

Figure 17. Reaction of 5-hydroxymethylfurfural with glycine (top) and proline (bottom), as described by Nikolov and Yaylayan.[168]

of proteins might be important in the removal of aldehydes from the medium during the brewing process, for example, with the trub.

During Strecker degradation, an imine zwitterion is formed as well (Figures 8 and 10). Protonation of this intermediate

leads to a more stable imine, which has been isolated.[123] Therefore, the (incomplete) Strecker degradation pathway can also be considered a source of imine adducts.

2.4.2. Bisulfite Adduct Formation. Sulfur dioxide (SO_2) is a gas that is 85 g L^{-1} soluble in water at 25 °C and has a boiling point of −10 °C.[170] In solution, it undergoes equilibrium reactions with $SO_2.nH_2O$, the bisulfite ion (HSO_3^-), and the sulfite ion (SO_3^-). At beer pH, which is generally 3.8−4.4, the predominant form is the bisulfite ion.[158,171] Because all of these species can be converted to, measured as, and reported in terms of SO_2, they are often generalized under "SO_2" or "sulfites".[158]

The human body is able to metabolize these sulfites by enzymatic conversion to sulfate and subsequent excretion in urine, although high levels can lead to, among others, gastric problems. Some individuals exhibit higher sensitivity than others, leading to adverse reactions such as anaphylactic shock, headache, abdominal pain, nausea, dizziness, hives, and asthma.[158,171] In 1994, the Scientific Committee on Food (SCF) of the European Commission set an acceptable daily intake (ADI) of 0.7 mg (kg body weight)$^{-1}$ day^{-1}.[172] The usage and labeling of sulfites in beer and other beverages are strictly regulated in many countries. In the European Union (EU) and the United States, their presence must be declared on the label when exceeding 10 mg total SO_2 L^{-1}. For EU legislation, the total SO_2 content cannot exceed 20 mg L^{-1} in low-alcohol and alcohol-free beer and 50 mg L^{-1} in beer with a second fermentation in cask.[173] The flavor threshold of SO_2 in beer is approximately 20 mg L^{-1}. At higher concentrations, for example, >30 mg L^{-1}, it can negatively affect the flavor quality, yielding a struck match flavor.[171,174]

Management of SO_2 and derived species is common practice in the brewing industry, because they have antimicrobial and flavor stabilization activity. Sulfite can be introduced when present in the ingredients (e.g., as preservative in syrups and fining agents), but the major source of sulfite in beer is the reduction of sulfate in water and grist by the yeast metabolism (endogenous SO_2). The SO_2 content is also increased by the addition of sulfiting agents (exogenous SO_2) such as SO_2 (E220), Na_2SO_3 (E221), $NaHSO_3$ (E222), $Na_2S_2O_5$ (E223), $K_2S_2O_5$ (E224), $CaSO_3$ (E226), $Ca(HSO_3)_2$, (E227), and $KHSO_3$ (E228) before beer packaging.[115,158,171−173] According to Johannesen et al.,[175] no difference could be noticed between the (*E*)-2-nonenal concentrations of forced-aged beer with sulfite derived from endogenous or exogenous origin.[175]

It is generally accepted that sulfites protect beer from staling in two different ways.[23,43,158,171,176−178] First, they can act as antioxidants, improving beer flavor stability by inhibiting oxidative chain reactions through radical scavenging of both ROS and other radicals. Sulfite seems to interact with peroxides in a two-electron nonradical producing reaction, preventing the formation of staling aldehydes and many other undesired products.[21,179] Second, they have a role as carbonyl-binding agents through the formation of aldehyde−bisulfite adducts, the so-called hydroxysulfonates (Figure 18). As an illustration, the addition of sulfite to fresh beers strongly delayed the

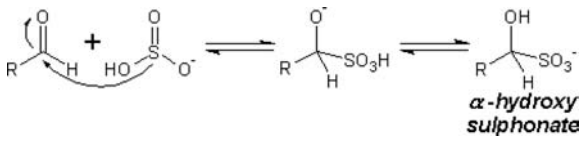

Figure 18. Formation mechanism of an α-hydroxysulfonate by the addition of sulfite to the carbonyl group, based on Guido.[158]

appearance of cardboard flavor during beer aging, and the level of free flavor-active (*E*)-2-nonenal lowered upon addition.[38,40,44] To date, it remains unclear what stabilizing mechanism is the most effective in practice and, although some are convinced of the first one,[180] in this paper, the focus will be on the adduct formation.

The formation of hydroxysulfonate has been confirmed indirectly by ^{1}H NMR spectroscopy and directly by LC-MS in aqueous solution at beer pH.[178] In the pH range of 1−8, the flavor-inactive aldehyde−bisulfite adduct form predominates, whereas at a higher pH dissociation occurs, resulting in free carbonyls. In the pH range 2−6, the equilibrium constants remain more or less constant.[158,181,182] According to ^{1}H NMR research performed with (*E*)-2-butenal as a model component for (*E*)-2-nonenal, a disulfonate is the product of the interaction with this unsaturated aldehyde (Figure 19).[160] Sulfite can be

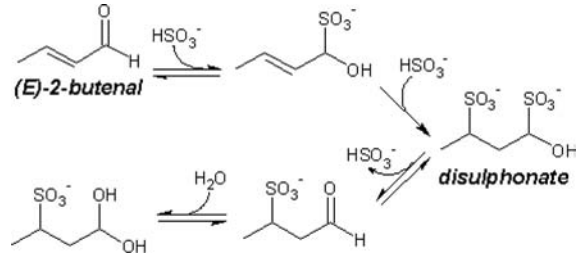

Figure 19. Reaction equilibria of (*E*)-2-butenal with sulfite, according to Dufour et al.[160]

added to the carbonyl functional group, which proceeds rather quickly and yields a reversible bond, but irreversible addition to the unsaturated double bond can also take place, yet more slowly.[160] This would imply that unsaturated aldehydes could not be fully recovered from bisulfite adducts.

Free SO_2 disappears from beer over time, with a very low, but nonzero rate, at 0 °C, and faster with increasing temperature, following first-order kinetics. These rates are barely affected by the initial SO_2 content.[183] Free SO_2 is most likely lost as an antioxidant pool, but likely also as a pool for binding de novo formed aldehydes or aldehydes released from, for example, imine adducts,[38,176,183] as well as reversible or irreversible interaction with a whole range of other components such as reducing sugars, Maillard intermediates (thus inhibiting the Maillard cascade), cysteine residues, thiamins, quinones, and polyphenols.[44,158,171,177,180] According to Barker et al.,[44] short-chain aldehydes bind bisulfite more strongly than long-chain aldehydes, and the addition of acetaldehyde to a model solution gradually removed bisulfite from other aldehyde bisulfite adducts. As acetaldehyde represents >95% of all aldehydes in beer, the majority of carbonyl-reacted SO_2 will be associated with this compound.[176,181,184] Based on dissociation constants of aldehyde−bisulfite adducts found in the literature,[185−187] Bradshaw et al.[156] calculated that, in the presence of 25 mg L^{-1} free sulfur dioxide, only 0.5% of acetaldehyde is unbound at pH 3.0, whereas for furfural, 48 and 73% are unbound at pH 3.6 and 7.0, respectively. It has been suggested that most of (*E*)-2-nonenal is bound as a sulfite adduct as long as the total amount of SO_2 in aging beer exceeds 2 mg L^{-1}.[38] For the total carbonyl content, a maximum of 40% appears to be bound when 5−10 mg L^{-1} sulfite is added, which has been mentioned by Bushnell et al.[180] as the optimal sulfite concentration in beer. Kaneda et al.[177] found a similar optimal sulfite content in packaged beer, being 8−9 mg L^{-1}.

From the above, it is clear that the precise role of SO_2 in beer flavor stability is complex and that additional research is required. For instance, it has been mentioned that acetaldehyde−bisulfite adducts still show antioxidant activity in aging beer, protecting other compounds from oxidation,[177,188] and it has even been proposed by Kaneda et al.[188] that this activity may be more important than the actual carbonyl scavenging ability of sulfite.

2.5. Yeast Metabolism toward Aldehydes.

2.5.1. Sulfite Secretion. Sulfite comprises an intermediate product of cysteine and methionine biosynthesis, and its excretion by yeast proceeds in four stages[22,171,175,188,189] (Figure 20). In stage 1, methionine and threonine present in

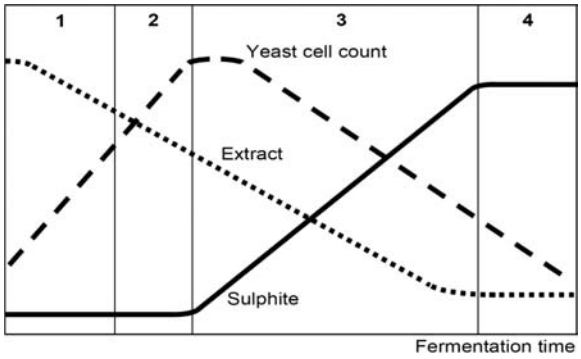

Figure 20. Four stages of the sulfite secretion by yeast during fermentation. Available extract and yeast cell count are also indicated.[189] Reprinted with permission from ref 189. Copyright 1991 Oxford University Press.

wort inhibit and repress certain enzymes, preventing sulfite excretion. During the second stage, the pathway is switched on, but sulfite excretion remains low due to a high demand for sulfur-containing amino acids. In stage 3, yeast growth ceases, which lowers this amino acid demand. However, extract, and thus energy, is still available, which favors sulfite production. Sulfite excretion commences due to an oversupply in the metabolism. The alcohol level at this moment is about 1.5% w/w.[75,190] In the fourth stage, the extract is depleted, sulfate reduction stops, and sulfite excretion stops accordingly.[189] The extent of sulfite excretion depends on the yeast strain used; lager strains often produce more SO_2 than ale strains, for example.[191] It has been found that beer produced with a yeast strain with augmented sulfite secretion shows better flavor stability.[192] Furthermore, higher sulfate supply to the yeast, higher original wort gravity, higher wort clarity, higher fermentation temperature, lower pitching rate, and lower wort oxygenation all result in higher SO_2 contents.[158,171,189,190] In general, sulfite secretion is inversely proportional to yeast growth, independent of the applied parameters.[189]

2.5.2. Reducing Activity of Yeast. It is generally accepted that yeast metabolism can reduce aldehydes in the wort to their corresponding alcohols. The system responsible for this reduction has been found to be very complex and heterogeneous.[149,154] Some aldehyde reductases regenerate $NAD(P)^+$ from NAD(P)H and, therefore, maintain a suitable redox balance within the cell.[154,193] Spiking of aldehydes to wort with subsequent laboratory-scale fermentation results in a lack of measurable aldehyde levels directly after fermentation and yeast removal. Moreover, the malt-like aroma disappears completely by this fermentation step. On the other hand, the corresponding alcohols and acetate esters showed to be present.[153,165,193] Collin et al.[155] suggested that the limiting step of carbonyl reduction is the uptake rate by the yeast, but this was countered by the findings of Debourg et al.,[149] who worked with permeabilized yeast cells.

Linear saturated aldehydes appear to be reduced more rapidly with increasing carbon number, and their reduction rate is higher than their corresponding branched or unsaturated aldehydes.[149] Furfural and (*E*)-2-nonenal are reduced early in the fermentation process.[63,115,194] Vesely et al.[195] observed a clear decrease in, among others, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, furfural, and methional concentrations, at both 10 and 15 °C fermentations. Although the reduction rates were slightly higher at 15 °C, the resulting aldehyde concentrations were lower at 10 °C. Perpète et al.[182,193] reported an initially fast reduction of Strecker aldehydes in cold contact fermentation, which slowed and resulted after a few hours in a constant concentration. This end concentration is aldehyde-dependent, but can reach up to 40% of the initial concentration. Higher fermentation temperatures led to lower, but nonzero, end concentrations. Neither higher pitching rates nor different yeast strains or even a second pitching with fresh yeast affected the concentration of aldehydes at the end of fermentation. Similar results were obtained with laboratory-scale and industrial fermentation trials. This points to the interactions of the aldehydes with wort components rendering them nonreducible by the yeast, for example, imine formation and bisulfite adduct formation, but also, for example, weak binding to flavonoids at fermentation temperatures.[153,182,193] As the free aldehydes are reduced by the yeast, the equilibrium between free and bound aldehydes restores the free form, yet this seems insufficient for complete aldehyde reduction.[149]

Aldehyde reduction by the yeast starts very early in the fermentation process, whereas sulfite production occurs at a later time.[75] The protective effect of sulfite binding is, therefore, thought to be of rather limited importance.[182]

Yeast also reduces α-dicarbonyls, the intermediates of the Maillard reaction pathway and part of the Strecker degradation pathway. Addition of an isolated yeast reductase to beer with subsequent forced aging resulted in a lower concentration of dicarbonyl compounds.[196] Overexpression of an involved reductase resulted in beers with 30−40% lower concentrations of Strecker aldehydes.[197]

3. PRACTICAL MEASURES TO REDUCE ALDEHYDE STALING IN BEER

The process that converts raw materials into the final product, beer, consists of several consecutive but inseparable steps that all have the potential to influence the flavor stability of the end product. In Figure 21, a scheme is given that includes the mechanisms of formation and removal of free aldehydes, as mentioned in the previous sections, and the potential dynamics between free and bound aldehydes. In what follows, some general principles to improve flavor stability throughout the entire brewing process are given. Furthermore, specific suggestions from a practical point of view are made as a function of the production steps. Often, these suggestions should be weighted in relation to other important beer properties, such as colloidal stability, foam stability, and overall flavor quality. In addition, some of these recommendations are still in a rather "philosophic stage", whereas others are already widely applied through the use of innovative technology.

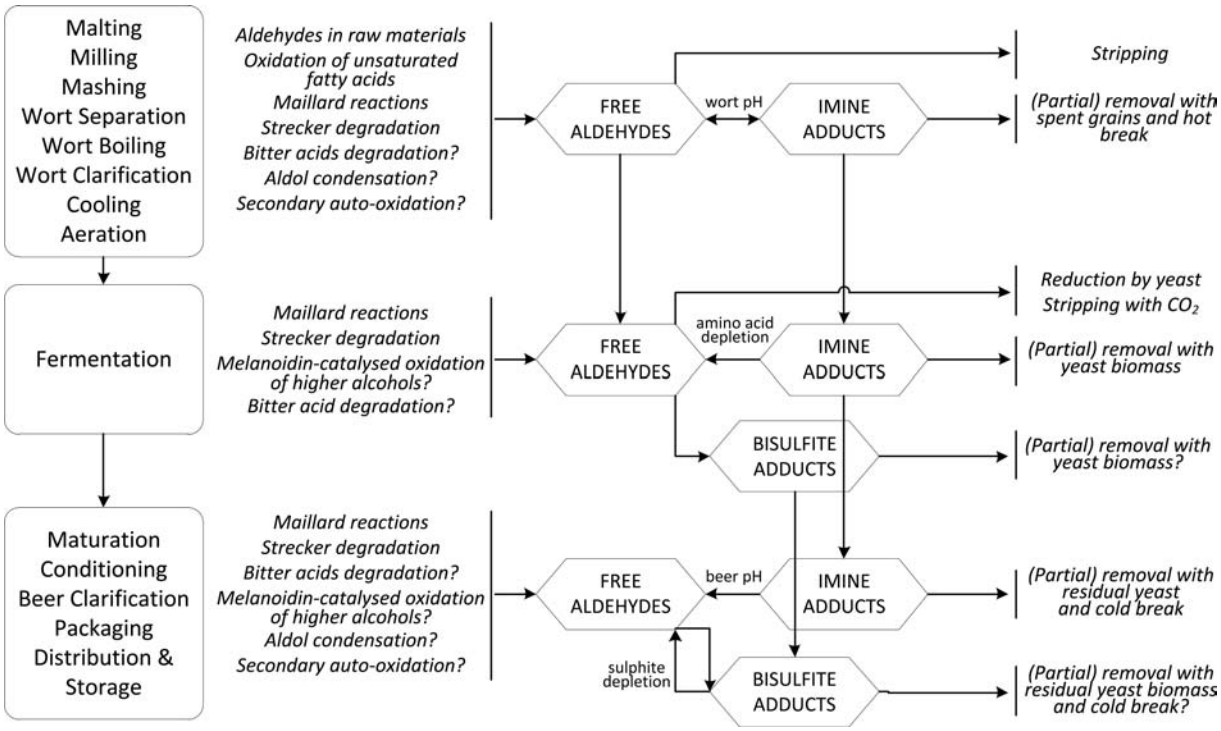

Figure 21. Overview of the potential mechanisms of formation and removal of aldehydes throughout the beer production process and the dynamics of free aldehydes with imine and bisulfite adducts.

General principles to improve beer flavor stability and diminish aldehyde staling:

- Oxygen uptake should be avoided at all times (except during aeration, of course, when yeast works as an oxygen scavenger requiring oxygen for its metabolism). The construction of the brewing installation should be designed accordingly. All pipes and tanks should be flushed with CO_2 or N_2 of high purity, air pockets should be avoided, and bottom filling of the tanks should be applied when possible. When all containers are emptied, pulling in air should be avoided. Oxygen-free water should be used as much as possible.[18,22,24,76,77,198]
- Heat load should be minimized as much as possible throughout the malting and brewing, because this favors several unwanted processes in regard to flavor stability (e.g., autoxidation of unsaturated fatty acids, Maillard reactions, Strecker degradation). For example, all hot transfers between vessels should be as short as possible.[121]
- The presence of iron and copper should be minimized, because they can initiate free radical reactions. The transition metal ions that do end up in the medium, for example, originating from the brewing installation, can be chelated by, for example, amino acids, melanoidins, and phytic acid.[24,95,198]
- All adjuncts used throughout the process should contain as few aldehydes and aldehyde precursors as possible. In some cases, the substitution of malt, for example, by maltose syrups, was shown to have a neutral to positive effect on flavor stability [77] Sadly, most adjuncts do not contribute to antioxidant activity, nor do hop extracts.[22]
- Antioxidant activity in beer is supplied by different components, the most important ones being polyphenols. Generally around 80% of the polyphenols in beer originate from malt, whereas hops contribute about 20%.[199] The majority of oxygen that enters beer and interacts with beer components has been shown to be incorporated in polyphenols (approximately 65%, whereas about 30% was found in the volatile carbonyl fraction and about 5% was associated with bitter acids). Moreover, polyphenols chelate transition metal ions.[22,95,200] However, not all polyphenols are antioxidant, such as catechin (3′- and 4′-hydroxyl groups on the flavan ring); some are pro-oxidant, such as delphinidin (3′-, 4′-, and 5′-hydroxyl groups on the flavan ring) due to their ability to transfer electrons to transition metal ions.[18,21,95,201] Besides polyphenols, a wide spectrum of valuable antioxidants is present in beer, such as reductones, melanoidins, and vitamins. The upstream production process should aim at promoting and protecting the endogenous presence of antioxidants.[43,92,163,199,202−205]

Potential measures in malting:

- The variations in levels of aldehydes, aldehyde precursors, and, for example, antioxidant activity and copper content in barley should be monitored, as these concentrations in the raw material vary with barley variety and growth conditions.[18,22,75,77,145,199,206−209]
- Barley batches with low levels of soluble nitrogen and low Kolbach indices should be selected, because a correlation was seen with the appearance of Strecker aldehydes in aging beer.[210,211]
- Barley varieties with low lipoxygenase potential should be selected.[21,75,78,81,91,212]
- Embryo development should be suppressed by, for example, rootlet inhibitors to reduce formation of aldehydes and aldehyde precursors.[21,198]
- "Good malting practice" should be performed in regard to the type of malt: temperature and moisture profiles should be chosen and monitored carefully. For example,

malt kilning at high (end) temperature inactivates LOX enzymes, which reduces enzymatic oxidation of unsaturated fatty acids, but promotes, among others, Maillard reactions, Strecker degradation, and imine adduct formation.[18,21,22,43,71,75,78,145,163,200,210]

- The different temperature and moisture profiles between top, middle, and bottom layers of the kiln should be monitored. For instance, malt from the bottom layer shows lower LOX activity, but a higher nonenal potential.[43]
- Intelligent management of the endogenous microflora and/or inoculation with beneficial micro-organisms will produce, for example, cell-wall degrading enzymes, for more efficient wort production.[213]
- Storage of barley before malting and storage of malt before further processing should be limited in time, because an increase in free and triglyceride-bound trihydroxy fatty acids is observed during this storage.[74]

Potential measures in milling:

- The malt and the milling installation with CO_2 or N_2 should be sparged to reduce oxidation.[18,22,198] Some studies indicate that enzymatic oxidation of unsaturated fatty acids occurs especially during wet milling, although others contradict this statement.[74]
- Milling regimens should be applied that minimize damage to the embryo, activation of lipoxygenases, and production of aldehydes and their precursors.[19,42,198,206,212]

Potential measures in mashing and wort separation:

- Mashing-in at higher temperatures, for example, 63 °C, and lower pH, for example, 5.2, should be used to quickly denature lipoxygenases that were not inactivated during malting.[18,19,21,22,71,75,87−89,214,215]
- Gallotannins should be added at mashing-in, working as antioxidants, metal chelators, radical scavengers, lipoxygenase inhibitors, and aldehyde binders.[77,87,200,216]
- Mashing should be performed with low oxygen levels to prevent enzymatic and autoxidation of unsaturated fatty acids and other oxidation processes.[74,76,82,146,215,217]
- The use of an oversized chimney with condensate trap promotes removal and prevents re-entrance of unwanted volatiles, including aldehydes.[218]
- The time of wort separation, certainly when performed at high temperature, must be limited. However, a good wort separation is essential to remove aldehyde precursors, for example, lipids, and aldehydes bound to insoluble proteins from the mash together with the spent grains.[19,22,75,91,161,215]
- The use of acidified sparging water releases aldehydes from imine adducts, which can be stripped in later stages.[42]
- Excessive amino acid concentrations must be avoided, because these can lead to Strecker degradation and imine adduct formation throughout the brewing process and even in the packaged beer.[146,219,220]

Potential measures in wort boiling and wort clarification:

- The use of an oversized chimney with condensate trap promotes removal and prevent re-entrance of unwanted volatiles, including aldehydes. Other wort stripping techniques that promote removal of volatiles (e.g., depressurization) are recommended as alternatives.[18,22,75,77,121,165,218]
- The oxygen content during wort boiling should be limited, as this process step has been shown to be the main step of autoxidation of unsaturated fatty acids throughout the brewing process.[71,161]
- Deintensified boiling, a shorter boiling time, and effective convection in the vessel must be sought, as wort boiling is the main step for Maillard reactions and Strecker degradation, and these are promoted by a high heat load.[71,77,121,146,165,198] Furfural and 5-HMF formation rates increase with increasing boiling time, and Strecker aldehyde formation proceeds at a pseudo-zero-order rate, whereas lipid oxidation hardly proceeds.[121] Heat should be added via the smallest temperature difference and through the biggest exchange surface area.[121]
- Boiling should be performed at a lower pH, which promotes aldehyde production from precursors in this step, but subsequently removes them from the wort by stripping. This approach limits carry-over of precursor compounds further downstream, where removal is more difficult. Moreover, Maillard reaction initiation is reduced at a lower pH.[121]
- Instead of wort boiling (e.g., during 1 h), mashing-off at 95 °C, membrane-assisted thin bed filtration of the wort derived from fine-milled malt, injection of clean steam (in-line and in-kettle), stripping of the wort, and decantation via a combination vessel should be performed. This speeds the wort production process (fast wort filtration and no wort boiling) and lowers the heat load on the wort.[218]
- Fresh hops, rather than aged hops, should be used, because the latter contain more aldehydes and aldehyde precursors.[71]
- The use of high-tech hop products (e.g., tetrahydro-iso-α-acids) has been shown to be at least neutral to flavor stability and positive in terms of other attributes such as iso-α-acids utilization, bitterness quality, and bitterness stability.[139,144,146,221,222]
- Addition of sulfites to the filtered wort showed a suppression of lipid oxidation and imine formation.[19,42,159]
- Clarification time should be limited, but a good wort clarification is essential to limit carry-over of aldehyde precursors (such as lipids) to the pitching wort and to maximize the removal of aldehydes bound to insoluble trub particles.[22,71,75,198,205,215] However, a complete removal of lipids will negatively influence the yeast fermentation process.[22,82]

Potential measures in cooling, aerating, and fermentation:

- The time between the end of boiling and cooling should be limited.[215]
- Swift cooling of the wort slows all aldehyde formation processes.[77]
- Excessive aeration must be prevented, as it suppresses SO_2 secretion by the yeast.[146] Moreover, introduced molecular oxygen is depleted rapidly, but excesses might initiate oxidation processes before uptake by the yeast.[22]
- A yeast strain with a high aldehyde reducing activity should be selected.[193]
- A yeast strain with a larger cellular volume should be used, which appears to promote a higher pH further downstream.[223] A higher beer pH generally leads to prolonged flavor stability, because it increases iso-α-acid stability, reduces oxidation of higher alcohols, and reduces protonation of the superoxide radical to the much more reactive perhydroxyl radical.[138,214,224−227]

Moreover, the binding of aldehydes in imine adducts is enhanced at a higher pH.[224] Furthermore, improved flavor stability might also be related to the higher ploidy of the larger yeast cells.[223]

- A yeast strain with a high SO_2 secretion should be combined with the application of a relatively high fermentation temperature, which also promotes SO_2 secretion.[146,215]
- Alternatively, an attempt to minimize SO_2 secretion should be made to reduce the formation of aldehyde−sulfite adducts and allow the yeast to reduce the free aldehydes. Addition of exogenous SO_2 before packaging provides antioxidant activity and aldehyde masking.[198]

Potential measures in packaging:

- The lowest O_2 concentration possible in the packaged beer (no more than 50 ppb) must be achieved, for example, by purging the beer containers with CO_2, fobbing the beer prior to closing the container, and limiting headspace air.[18,22,75,76,228]
- Antioxidants, for example, sulfite, ascorbic acid (E300), ferulic acid, catechin, and/or the enzyme glucose oxidase-catalase, should be added, although their capabilities are often contradicted.[18,22,146,159,171,201,229,230]
- Arginine should be added, which can (theoretically) perform nucleophilic attacks on α-dicarbonyls and/or aldehydes by its two adjacent end-standing amino groups, thereby acting as a Maillard reaction inhibitor and/or an aldehyde scavenger. Lower aldehyde and Maillard intermediate contents were observed upon the addition of arginine, but this effect may (in practice) be caused by the pH increase associated with the excessive amounts added to the beer in this research.[121]
- Enzymes that are able to reduce α-dicarbonyls, either directly or throughout the aging process, should be added, and/or so-called Amadoriase enzymes that degrade Amadori compounds should be added.[121] The feasibility of this measure still needs to be proven, however, as research on this topic still needs to be performed.
- Yeast should be added to the bottle for refermentation ("bottle conditioning" or "bottle krausening"). Its reducing activity significantly improves the flavor stability, even without the addition of fermentable sugar and at low cell counts (10000 cells mL^{-1}). Aged lager beer has been shown to be difficult to separate from fresh beer, and haze formation is only limited.[152,153,231] An additional advantage is the oxygen scavenging activity of the yeast, thereby protecting beer components from oxidation.[232]
- When pasteurization is performed, limit the pasteurization temperature.[36]
- A crown cork liner for beer bottles, which efficiently excludes oxygen ingress, preferably with oxygen scavenging ability, should be used.[21,22,75,228,233] The undesirable effects of light are reduced significantly (but not eliminated) by the use of brown glass, which is, therefore, favored over, for example, green glass bottles.[205]
- Beer packaged in cans showed a lower Strecker aldehyde increase, compared to glass and PET bottles, which can be kept in mind in the selection of the beer container type.[228]

Potential measures in transportation and storage:

- Refrigerated temperature (e.g., ≤7 °C) should be maintained during transportation and storage to slow all chemical reactions causing staling.[18,22,36,75,110,146,198,229,230]
- Exposure to sunlight and intense shaking should be prevented.[22]
- Stock turnover must be made in a timely manner.[24]

4. CONCLUSIONS

Over the years, knowledge and understanding of beer flavor stability has improved substantially, and the role of aldehydes has been demonstrated indisputably. From a chemical point of view, the potential formation mechanisms of staling aldehydes have been unraveled, either in detail or up to a level where a reasonable understanding has been reached. However, controversy still exists about the relative importance of the different mechanisms in brewing practice. In particular, it remains unclear to what extent staling aldehydes are formed de novo during beer storage. Increasing evidence suggests that they find their origins further upstream, throughout the beer and even the malt production process. Obscured in a bound state, these aldehydes may be transferred into the fresh beer, where they may subsequently be released over time due to the chemical disequilibrium. Yielding stale flavor, this transfer should be minimized to obtain and maintain the consumers' appreciation.

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