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

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A review

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1. Abstract

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

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

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

2. Introduction

a. Flavor of fresh and aged beer

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

More than 40 years ago, Meilgaard et al. investigated single volatile compounds in beer with respect to their flavor activities for the first time. The odor activity values (OAVs) of 239 compounds in beer

were determined, revealing that ethanol, carbon dioxide, several esters (e.g., 3-methylbutyl acetate and ethyl hexanoate), higher alcohols (e.g., 3-methylbutanol), dialkyl sulfides (e.g., dimethyl sulfide (DMS)), and short-chain fatty acids (e.g., butanoic acid) were key aroma compounds in American lager beers (Meilgaard 1975). In sensory omission tests, ethanol, (R)-linalool, ethyl butanoate, DMS, 3-methylbutanol, ethyl hexanoate, 2-phenylethanol, and furaneol were found to be significant for the overall flavor in a synthetic aroma recombination study of pilsner beer (Fritsch et al. 2005).

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

The course of aging of lager beer is well documented since it is especially susceptible (Baxter and Hughes 2001). Typically, staling flavors develop after 3–6 months of storage at 18 °C (Ilett and Simpson 1995). Dalgliesh et al. (Dalgliesh 1977) reported in 1977 the changes in aroma during the aging of lager beer in detail, and in 2005 Zufall et al. (Zufall et al. 2005) extended Dagliesh's model using forced-aging at 28 °C. During aging, bitterness is reduced, whereas sweetish and caramel notes are amplified. A cardboard flavor as well as ribes-like flavor can develop, but also disappears after time, and even bread, wood, or sherry flavors can be presented after longer storage times. Additionally, fresh aromas and masking effects are diminished (Saison et al. 2009). However, owing to the reasons stated above, the development of a certain flavor cannot be correlated to a single compound.

a. Analytically detectable indicators of beer aging

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

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

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

Furthermore, some indicators have been criticized and should be used carefully. For instance, since its discovery by Palamand and Hardwick in 1969 (Palamand and Hardwick 1969), (E)-2-nonenal was considered to be a key aging compound for a long time. Due to its extremely low flavor threshold and its characteristic cardboard flavor, the complex phenomenon of beer aging was reduced to the presence

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

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

high flavor thresholds, they seem to have little impact on total flavor. 2-Furfurylethylether is particularly well known to increase in all beers during aging and has been suggested as a potent analytical storage indicator (Eichhorn 1991; Vanderhaegen et al. 2004).

b. Flavor-inactive bound-state aldehydes

Evans et al. found that relatively few flavor-active compounds are either newly formed or entirely diminished during aging, while most are already present in fresh beer and somehow masked from sensory and analytical perception (Evans et al. 1999). It has been suggested that, after packaging, non-extreme storage conditions allow the ongoing formation of staling compounds through oxidation and reduction reactions. Furthermore, the contribution of interactive effects between aroma compounds has been discussed (Narziss 1986). It has been found that staling aldehydes exist in two forms. In their **free form**, they directly contribute to flavor, but they can also occur in a trapped or **bound state**, both reversibly and irreversibly. In this way, aldehydes are not evaporated during wort production nor reduced to their corresponding alcohols by yeast. Thus, they are obscured from sensory and analytical perception. Then, in the course of aging, these aldehydes will be released from their adducts depending on factors like temperature, pH value, binding strength, and thermodynamic stability, and cause off-flavors (Lermusieau, Noël, and Collin 1999; Liégeois et al. 2002; Noël et al. 1999a). Thus, the determination of these precursors can provide an indication for the endogenous aging potential of a particular beer.

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

Other authors have assumed that oxygen is a release-promoting agent since it is known that oxidative conditions promote staling, and investigations into beer-flavor instability in terms of solely *de novo* formation of compounds were inconclusive and showed contradictions (Wietstock, Kunz, and Methner 2016a; Noël et al. 1999b). This indicates that the chemistry of beer aging has become more nuanced, and that bound-state precursors have attracted increasing research interest. For instance, Suda et al.

showed in experiments with ^{13}C-labeled amino acids that 85% of Strecker aldehydes are derived from wort boiling and clarification processes, whereas only 15% are formed *de novo* from the remaining amino acids in bottled beer. They suggested that Strecker aldehydes may be trapped by either amino acids, proteins, or sulfites (Suda et al. 2007). A reactive-oxygen-species (ROS)-induced oxidative degradation pathway for the development of Strecker aldehydes has been previously proposed (Wietstock, Kunz, and Methner 2016b). Noël et al. found that after spiking $^{18}O_2$ to bottled beer and subsequent aging, ^{18}O was incorporated in sulfites, polyphenols, and isohumulones, but not in the carbonyl fraction. Thus, they concluded that lipid peroxidation might not occur in bottled beer (Noël et al. 1999a; Noël et al. 1999b).

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

i. Bisulfites

Sulfur dioxide can delay the staling of beer by either scavenging ROSs and acting as an antioxidant or forming hydroxysulfonates and stepwise disulfonates with carbonyls through bisulfite ions (HSO_3^-) (Guido 2016). In special cases, e.g., with α-unsaturated aldehydes, irreversibly trapped adducts can be formed where the second addition to the double bond is the rate-limiting step (Dufour et al. 1999). Kaneda et al. (Kaneda et al. 1994) regarded the optimal SO_2 content of beer to be 8–9 mg L^{-1}. In the pH range 3–6, nearly all the sulfites are present as SO_3^- and most carbonyls are bound. However, since

acetaldehyde is the most abundant aldehyde in beer (> 95%) (Hashimoto and Eshima 1977; Kaneda, Takashio, and Tamaki 1997) and its concentration rises during aging, other aldehydes may gradually be released from their adducts and thus contribute directly to flavor deterioration. The formation of adducts with acetaldehyde is favored thermodynamically due to its carbon chain length (Barker et al. 1983). For commercial beers, acetaldehyde-bisulfite adduct concentrations of up to 54 μM have been reported (Kaneda et al. 1996). Using results from 10 real wine samples, de Azevedo et al. showed that up to 90% of the acetaldehyde (19 mg L^{-1}) and 50% of the 2-furfural (12 mg L^{-1}), butanal, hexanal, and benzaldehyde are bound as bisulfites (Azevedo et al. 2007). Bueno et al. reported that, due to the depletion of SO_2, bisulfite adducts are cleaved during wine oxidation and thus aldehydes are released (Bueno, Carrascón, and Ferreira 2016).

ii.Imines

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

under different conditions by Baert et al. failed to demonstrate the formation of imines between several different amino acids and marker aldehydes (Baert 2015).

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

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

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

iv. Glycosides

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

partly due to acidic hydrolysis of glycosides (Chevance et al. 2002). Thus, it is conceivable that these compounds are formed during beer production. Later, Gijs confirmed that lower pH elevates the levels of β-damascenone during the artificial aging of beer (Gijs et al. 2002).

<p style="text-align:center">v. Acetals</p>

Acetals are the product of a reaction aldehydes with alcohols. These can either be monohydric or polyhydric. The best known example of the former type is diethylacetal from acetaldehyde and ethanol, while the latter one yields cyclic acetals, such as 2,4,5-trimethyl-1,3-dioxolane from acetaldehyde and 2,3-butanediol (Peppard and Halsey 1982). Vanderhaegen et al. found that 2,4,5-trimethyl-1,3-dioxolane increases during aging similarly to the concentration of acetaldehyde while oxygen seemed to have the biggest impact. This suggests that there is an equilibrium between both compounds (Vanderhaegen et al. 2003). Recently, Liu et al. monitored the course of acetaldehyde and diethylacetal during the brewing process and storage. In contrast to the previous study they observed the gradual decline of diethylacetal, while acetaldehyde increased continuously (C. Liu et al. 2018).In the literature, there is a clear lack of data on adducts and precursors, despite several authors being aware of the necessity of their investigation (Kaneda et al. 1995). In addition to the above-mentioned forms, other types of adducts (e.g., with β-glucans) may also occur in beer. The fact that different types of adducts react differently under the influence of factors such as pH and temperature makes it difficult to determine the degree of release during storage. For instance, while imines are more stable at higher pH, bisulfites tend to be cleaved (Guido 2005). Thus, bound-state aldehydes seem promising for estimating aging potentials in a non-discriminative way.

3. Forced-aging

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

Table 2 gives an overview of different regimes, the time equivalent for natural aging, the used analytics and sensorics, and the investigated substances in lager/pilsner beers.

a. Influence of temperature on forced-aging

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

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

b. Influence of pH on forced-aging

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

reported decreased aged flavor with increasing pH. It has also been shown that, at lower pH, the appearance of cardboard notes is favored (Saison et al. 2010b).

c. Application of forced-aging parameters

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

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

Taken together, the results of forced-aging tests present a rather unrealistic picture of staleness. Elevated temperatures cause differences in reaction rates (e.g., Strecker aldehydes like methional and phenylacetaldehyde are temperature-dependent and favored at higher temperatures) (Soares da Costa et al. 2004) and also lead to reactions that might not occur at lower temperatures owing to their high activation energies (e.g., release of certain aldehydes from adducts). The same is true for acidification

due to the cleavage of adducts and catalysis of reactions. It seems that some authors are well aware of these disadvantages, while others are not, since they do not report on the degree of staling or the flavors developed (Vesely et al. 2003). After taking into account bound-state aldehydes for a wider picture of beer flavor instability, it seems that, in previous tests, adducts were cleaved by chance rather than intention. Thus, staleness engendered in that way should not be compared to that of naturally aged beers. Other non-discriminative approaches to predicting the shelf-lives of beers seem more promising and should be explored further, since they yield a more realistic representation of beer aging.

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

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

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

a. **Selection of extraction technique**

Due to each method´s particular advantages and disadvantages, there is no absolute way to determine the compounds responsible for beer aging. The choice of extraction method greatly depends on the matrix and analytes (Werkhoff et al. 2002). In general, the most serious analytical errors occur during

sample extraction, and numerous studies have stated that the outcome of an analysis is highly dependent on the extraction and analysis techniques used. Thus, a suitable method for extracting volatile compounds has to cover all key aroma compounds without discriminating, decomposing, or modifying them. Additionally, no new aroma compounds should arise, and non-volatiles should be removed, which can interfere with chromatographic separation (Engel, Bahr, and Schieberle 1999). Thus, to detect the compounds of interest in the best way, a suitable method needs to be selected.

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

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

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

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

b. Derivatization

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

For carbonyls, derivatization with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA) is a well-established method. In the reaction, two oximes arise, leading to two peaks in gas chromatography (cf. Figure 2). When performing SPME it is easily applied using on-fiber derivatization, which makes it a simple and rapid technique to lower detection limits to the high-ppt range. Saison et al. optimized the method for 32 beer aging compounds (Saison et al. 2008).

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

c. Selection of separation and detection techniques

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

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

d. Possible strategies for determination of bound-state compounds

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

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

Kim et al. reported an LC-MS/MS method for the determination of linear aldehydes after derivatization with D-cysteine in the form of 2-substituted 1,3-thiazolidine-4-carboxylic acids (Kim and Shin 2011). By determining the specific bound-state aldehydes in the form of 2-substituted 1,3-

thiazolidine-4-carboxylic acids in beer, more insight on the parameters of formation, stabilities and behavior during brewing process can be gained.

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

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

5. Conclusion

Due to years of research, as well as technological and raw-material-related improvements, more flavor-stable beer is produced today. The most common way to estimate flavor stability is forced-aging. Due to the high temperatures commonly employed in force-aging, force-aged beers are not particularly comparable to naturally aged beers since different aroma profiles are obtained. In most samples, no correlation can be drawn for products aged at 20 °C to 60 °C. Therefore, both industry and science require non-discriminative methods to study flavor stability.

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

To gain a holistic understanding of beer aging, more focus on bound-state aldehydes is required. Alternatively to forced-aging, a method to assess the pool of bound-state aldehydes involving the use

of 4-vinyl pyridine has been developed. Other promising analytical techniques may be LC-MS, UPLC-ToF-MS, MS[2], or assays involving (quantitative) NMR. From the obtained data, an easily manageable number of meaningful indicators (volatile carbonyls and non-volatile precursors) must be defined. This will, in combination with sophisticated data analysis, lead to more reliable predictions of flavor stability that can be tailored to different breweries and beer styles.

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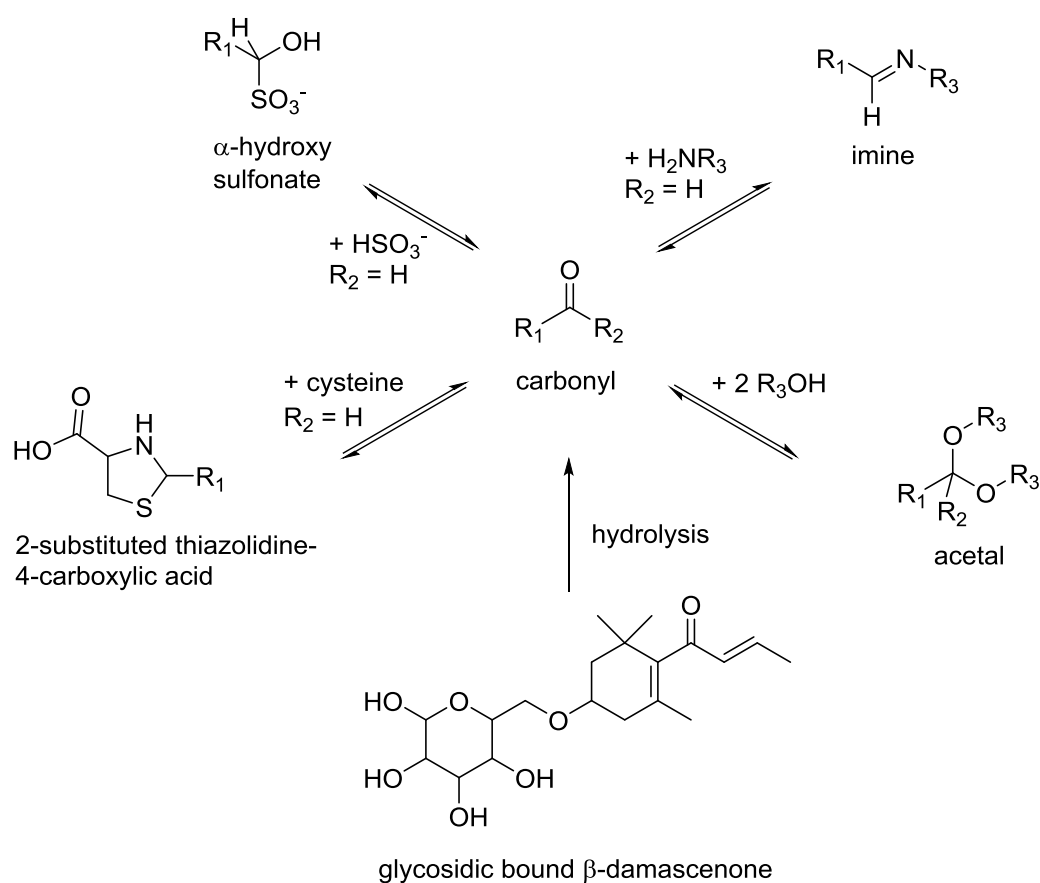
Figure 1: Different pathways for the release of bound-state aldehydes: Bisulfite adduct from Dufour et al. (Dufour et al. 1999), imine adduct from De Schutter et al. (Schutter 2008), 2-substituted thiazolidine-4-carboxylic acid from Baert et al. (Baert et al. 2015), glycosidic bound precursors (here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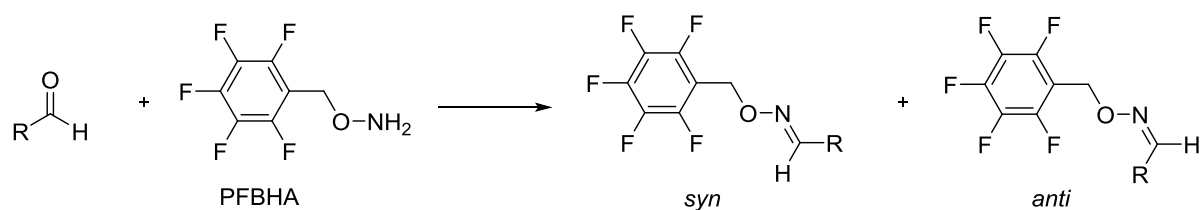

Figure 2: Reaction between carbonyls and PFBHA, yielding two possible oxime isomers (Martos and Pawliszyn 1998)
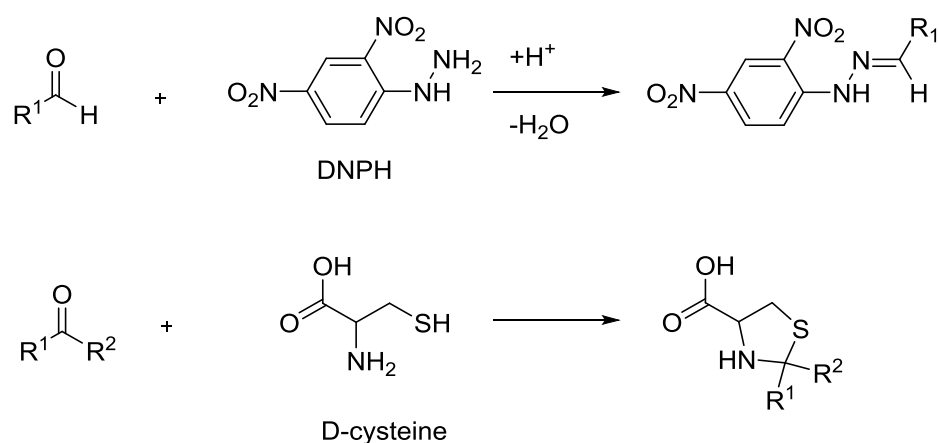
Figure 3: Derivatization with DNPH from Goncalves et al.(Moreira Goncalves et al. 2010) and D-cysteine from Kim et al. (Kim and Shin 2011)

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

Pathway	Compound name	Origin	Critical steps during process	Source
Strecker degradation	2-Methylpropanal	Valine or oxidative degradation of isohumulones	Oxygen uptake	(Vanderhaegen et al. 2006; Baert et al. 2012; Clippeleer et al. 2010; Eichhorn 1991)
	2-Methylbutanal	Isoleucine or oxidative degradation of isohumulones	Oxygen uptake	(Vanderhaegen et al. 2006; Baert et al. 2012; Clippeleer et al. 2010; Wietstock, Kunz, and Methner 2016b)
	3-Methylbutanal	Leucine or oxidative degradation of isohumulones	Oxygen uptake	(Vanderhaegen et al. 2006; Baert et al. 2012; Clippeleer et al. 2010; Wietstock, Kunz, and

				Methner 2016b)
	Phenylacetaldehyde	Phenylalanine	Oxygen uptake	(Vanderhaegen et al. 2006;Baert et al. 2012; Wietstock, Kunz, and Methner 2016b)
	Benzaldehyde	Degradation of phenyl acetaldehyde	Oxygen uptake	(Vanderhaegen et al. 2006; Baert et al. 2012; Wietstock, Kunz, and Methner 2016b)
	Methional	Methionine	Oxygen uptake	Baert et al. 2012, Wietstock, Kunz, and Methner 2016b)
	2-Acetyl pyrazine	α-dicarbonyls and amino acids	/	(Vanderhaegen et al. 2006)
Maillard reaction	2-Furfural	Carbohydrates and amino acids	Time and temperature of storage	(Vanderhaegen et al. 2006; Baert et al. 2012; Eichhorn 1991)
	5-Hydroxymethylfurfural	Carbohydrates and amino acids	Time and temperature of storage	(Baert et al. 2012; Rakete, Klaus, and Glomb 2014)
	5-Methylfurfural	Carbohydrates and amino acids	/	(Eichhorn 1991)

	2-Furfuryl ethyl ether	2-Furfural and ethanol	Time of wort boiling	(Vanderhaegen et al. 2004; Schutter 2008)
Lipid oxygenation	Hexanal	Linoleic acid oxidation (LOX and 13-HPL)	Malting and mashing, time and temperature of storage	(Vanderhaegen et al. 2006)
	Heptanal	Oleic acid oxidation	Malting and mashing, time and temperature of storage	(Meilgaard 1975; Lustig 1995; Eichhorn 1991)
	(E)-2-nonenal	Linoleic acid oxidation (LOX and 9-HPL), but also from aldol addition of acetaldehyde and heptanal	Malting and mashing, time and temperature of storage	(Meilgaard 1975; Vanderhaegen et al. 2006; Lustig 1995; Kuroda et al. 2003)
	(Z,Z)-2,4-decadienal	Linoleic acid oxidation	Malting	(Lustig 1995)
Hop degradation products	Ethyl-3-methylbutyrate	Degradation of acyl side chain of (iso-)humulones	Time and temperature of storage?	(Schnaitter et al. 2016)

		with ethanol		
	Ethyl-2-methylbutyrate	Degradation of acyl side chain of (iso-)humulones with ethanol	Time and temperature of storage?	(Schnaitter et al. 2016)
Other oxidations	β-damascenone	Carotenoids (neoxanthin), glycoside	Tent. raw material content, pH during mashing and boiling	(Gijs et al. 2002; Chevance et al. 2002)
	Dimethyl trisulphide (DMTS)	Degradation of methional, S-methyl-L-cysteine-S-oxide	/	(Gijs et al. 2002; Thiele 2006)
	γ-nonalactone	Lipids	Time and temperature of storage	(Vanderhaegen et al. 2006; Eichhorn 1991)
	Diethyl succinate	Esterification of succinic acid and ethanol	Time and temperature of storage?	(Eichhorn 1991)
	Ethyl nicotinate	Tryptophan	Time and temperature of	(Eichhorn 1991; Lustig 1995, 1995; Eichhorn

			storage	1991; Palamand and Grigsby 1974)
	2-aminoacetophenone	Tryptophan	/	(Palamand and Grigsby 1974; Christoph et al. 1999)
Adduct formation	Imines	Reaction with NH$_2$-groups	Removal with spent grains, hot break, and yeast biomass	(Baert et al. 2012)
	Thiazolidine-4-carboxylic-acids	Reaction with cysteine	Tent. trub separation	(Baert et al. 2015)
	Bisulfites	Reaction with SO$_2$	Tent. removal with yeast biomass and cold break	(Baert et al. 2012)

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

Reference	Forced-aging regimen	Equivalent time of natural aging	Analytical methods used	Sensorial evaluation	Found substances with significantly elevated concentrations/ used indicators
Bravo et al. 2008	3 d, 60 °C	/	SPME HR-GC/MS; GC-O	/	Furaneol, (E)-2-nonenal, phenylacetaldehyde

					, two unknown compounds ("solvent-like" resp. "aloe-like")
Cejka et al. 2013	6 d, 45 °C	Approximatel y 6 w, 30 °C or 6 m, 20 °C	HS-GC precolumn derivatisation (PFBHA)	Staleness: 0 (none)–5 (very strong)	3-methylpropanal, 2-methylbutanal, 3-methylbutanal, phenylacetaldehyde , 2-furfural
Back et al. 1997, Eichhorn 1991, Herrmann et al. 2010	1 d shaking + 4 d, 40 °C	3–4 months at 20 °C	Steam distillation	Impression of aging: 0 (none)–4 (very strong)	Ethyl nicotinate, 2-furfural, 2-methylbutanal, 3-methylbutanal, γ-nonalactone, phenylacetaldehyde , benzaldehyde, 3-methylbutan-2-one, heptanal
Depraeter e et al. 2008	7 d, 40 °C	/	P&T GC-MS	Staleness: 0 (none)–5 (very strong); Individual aspects (0–8): sweet, bitter, after-	2-Furfural, 5-Methyl-2-furfural, furfuryl ethyl ether, 3-methylbutanal, benzaldehyde, hexanal, 4-methyl-pentan-2-one, ethyl-3-

				bitter, pungent, fruity, solvent, cardboard, Madeira, ribes, caramel, sulfury	methylbutyrate, (decrease in ethyl hexanoate and isoamyl acetate)
Gijs et al. 2002	5 d, 40 °C; pH set to 3, 4.2, 5, 6, and 7	"This accelerated aging mimics very well a natural 20 °C storage."	SPE (Amberlite XAD-2 resin); Dynamic headspace–GC (sulfur chemiluminescence detection GC-O	/	At pH 4.2:, ethyl butyrate, methional, 2-methoxypyratine, DMTS, 2-acetylpyrazine, maltol, (E)-2-nonenal, γ-nonalactone, β-damascenone, ethyl cinnamate, and 5 unknown compounds
Greenhoff and Wheeler 1981	22 h, 60 °C	3 weeks, 37°C or 6 m, 18 °C	HPLC precolumn derivatization (2,4-DNPH)	Staleness: 0 (none)–5 (extreme): papery, oily, caramel, solvent, and	2-butanone, butanal, acrolein, crotonal, pentenal, hexenal, heptenal, octenal, nonenal,

				rancid but no differences between T	decenal
Guedes de Pinho and Silva Ferreira 2006, Soares da Costa et al. 2004	7 d, 37 °C	180 d, 4 °C	LLE (dichloromethane); GC-O	Quality scoring: +1 (no defect) to −3 (major defect)	methional, phenylacetaldehyde, β-damascenone, sotolon
H. Kaneda et al. 1995	5 d, 37 °C	22 d, 30 °C or 42 d, 25 °C	Chemiluminescence; DPPH reducing activity	Staleness: 1 (not present)–5 (very strong)	/
Malfliet et al. 2009	60 d, 30 °C in the dark	/	*On-fibre* derivatisation (PFBHA) SPME	Overall-aging-score: 0 (fresh)–8 (strongly aged, undrinkable)	2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, phenylacetaldehyde, 2-furfural, hexanal, (E)-2-nonenal
Noël et al. 1999a	5 d, 40 °C	/	Steam distillation	/	(E)-2-nonenal

Rakete, Klaus, and Glomb 2014	2 weeks, 50 °C (aerated and unaerated)	/	HPLC-MS	/	2-Furfural, 5-HMF, and certain dextrins
Saison et al. 2008, Saison et al. 2010b	5 d, 60 °C; 3 w, 40 °C; 3 m, 28 °C	/	*On-fibre* derivatisation (PFBHA) SPME	Overall appreciation: 1–9; Single aspects (cardboard, metal, solvent, old hops, ribes, Maillard, stale-sulfury, acetaldehyde, Madeira): 0 (not present)–8 (extremely strong)	Acetaldehyde, Hexanal, (E)-2-nonenal,2-methylpropanal, 2-methylbutanal, 3-methylbutanal, benzaldehyde, phenylacetaldehyde, methional, 2-furfural, 5-HMF, 5-methylfurfural, acetylfuran, diacetyl, ethyl-3-methylbutyrate, ethyl nicotinate, ethyl pyruvate, 2-furfuryl ethyl ether, γ-nonalactone, β-damascenone
Schieberle and	4 weeks, 45 °C,	/	SAFE-GC-O	/	2-methylpropanal, 3-methylbutanal, 2-

Komarek 2005	flushed with O_2		(AEDA)		methylbutanal, methional, phenylacetaldehyde
Vesely et al. 2003	12 weeks, 30 °C	/	*On-fibre* derivatisation (PFBHA) SPME	/	2-methylpropanal, 2-methylbutanal, 3-methylbutanal, pentanal, hexanal, 2-furfural, methional, phenylacetaldehyde, (E)-2-nonenal
Wietstock, Kunz, and Methner 2016b	30 weeks, 28 °C	/	SAFE-GC/MS	/	2-methylbutanal, 3-methylbutanal, methional, phenylacetaldehyde, benzaldehyde, 2-furfural, ethyl nicotinate, γ-nonalactone,
Zufall et al. 2005	12 weeks, 28 °C	/	SPME-GC-FPD, SPME-GC/MS	Modified MEBAK method: 0 (none)–10 (very strong)	/