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

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

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

1 Introduction

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

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

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

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

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

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

2 Materials and Methods

2.1 Chemicals and samples

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

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

2.2 Sensory analysis

2.2.1 Training of panellists

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

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

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

2.2.3 Quantitative descriptive analysis (QDA) of ageing attributes

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

2.3 Analysis

2.3.1 Gas chromatography-olfactometry (GC-O)

Five millilitres of unfiltered sample was placed in a 20-mL headspace vial and incubated at 40 °C for 5 min. SPME extraction was performed for 30 min at 40 °C with a CAR–PDMS–DVB fibre. The fibre was injected splitless at 250 °C by an autosampler (TriPlus RSH, Thermo Scientific Inc., Waltham, MA, USA) into a gas chromatograph (GC) (Trace 1300 Gas Chromatograph, Thermo Scientific Inc., Waltham, MA, USA) coupled to a single quad mass spectrometer (ISQ QD, Thermo Scientific Inc., Waltham, MA, USA) and a olfactory detection port (ODP 3, Gerstel, Mühlheim an der Ruhr, Germany). The GC was equipped with a DB-5 column (length: 60 m, inner diameter: 0.25 mm, film thickness: 0.25 µm). Helium was used as carrier gas (flowrate: 1.85 mL/min). The initial temperature was 60 °C, held for 4 min. Heating at 5 K/min was undertaken until a final temperature of 250 °C was reached and held for 3 min. Each sample was analysed once by one panellist to assess whether the method worked well for the rapid determination of ageing degree. Peak detection was performed in Xcalibur 3.1.66.10 (Thermo Scientific Inc., Waltham, MA, USA) and identification was performed via the addition of pure compounds and using the NIST database. Odor intensity was rated on a scale from 0 to 3 (0 = not detected, 1 = weak intensity, 2 = medium intensity, 3 = strong intensity).

2.3.2 Gas chromatography–mass spectrometry for volatile ageing indicators

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

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

2.3.3 Calculation of odour activity values

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

2.4 Data analysis

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

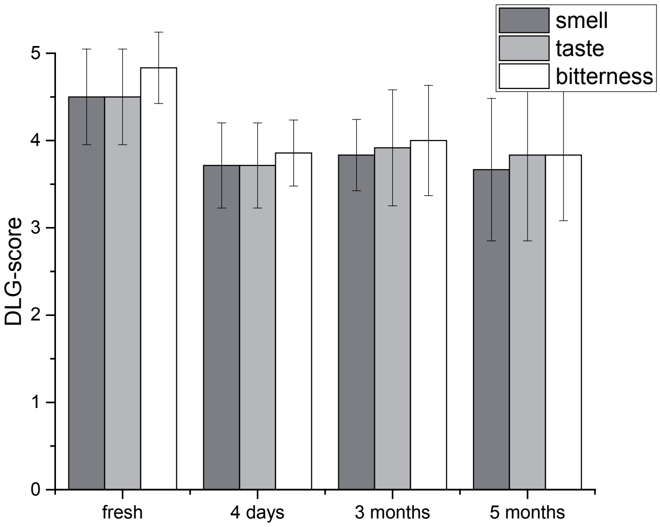

Fig. 1 **DLG scores for fresh, 3-month naturally aged, 5-month naturally aged, and 4-day forced-aged lager beer (n = 7)**

3 Results and Discussion

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

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

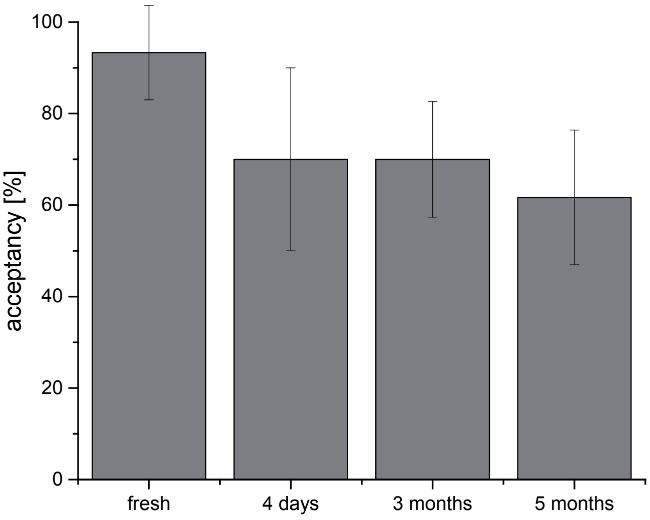

Fig. 2 **Acceptancy test for fresh, 3 month naturally aged, 5-month naturally aged, and 4-day forced-aged lager beer (n = 7)**

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

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

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

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

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

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

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

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

3.2 Sensory analyses – gas chromatography–olfaction

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

To assess whether GC-O is a fast and reliable method for determining the state of ageing in any given sample, the same pilsner and lager as used for sensory analysis were used in this analysis. Table 1 shows the compounds detected by GC-O for the fresh, naturally aged, and forced-aged pilsner and lager beers. The intensity of perceived odours was rated on a scale from 0 (not detected) to 3 (strong intensity). Because this experiment was performed as a pre-trial, each sample was analysed only once. Even though the panelists were trained, the possibility that single compounds remained undetected due to the form of a particular panellist cannot be excluded.

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

3.3 Sensory analyses – QDA

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

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

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

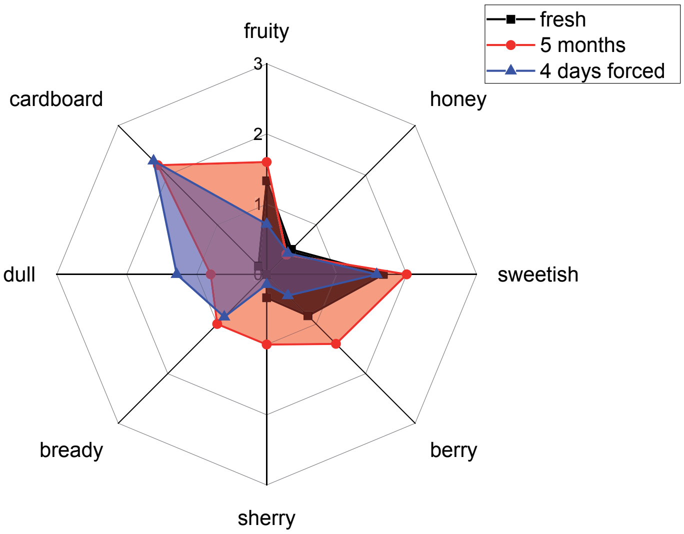

Fig. 3 **Quantitative descriptive sensory analysis of fresh, 5-month naturally aged, and 4-day forced-aged lager beer (n = 7)**

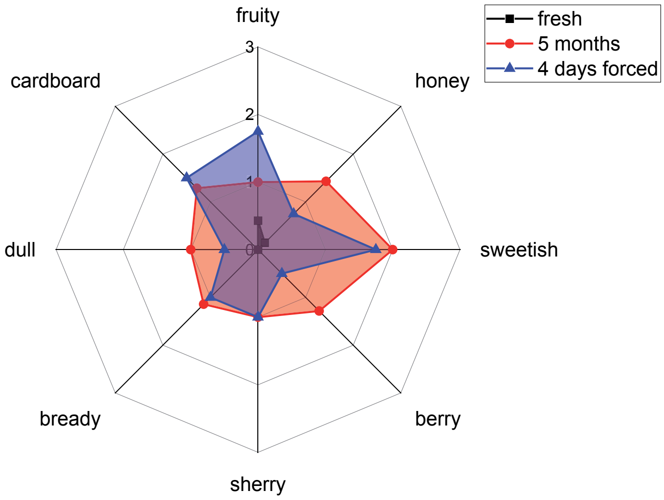

Fig. 4 **Quantitative descriptive sensory analysis of fresh, 5-month naturally aged and 4-day forced-aged pilsner beer (n = 7)**

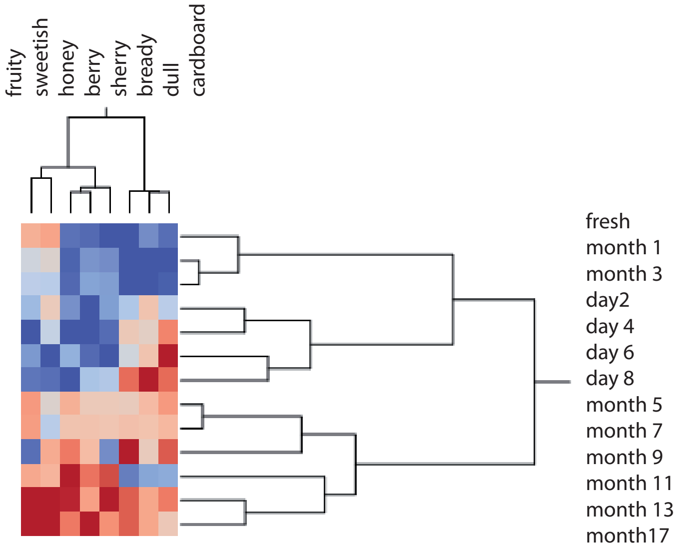

Fig. 5 **Two-way hierarchical clustering of sensory ageing attributes**

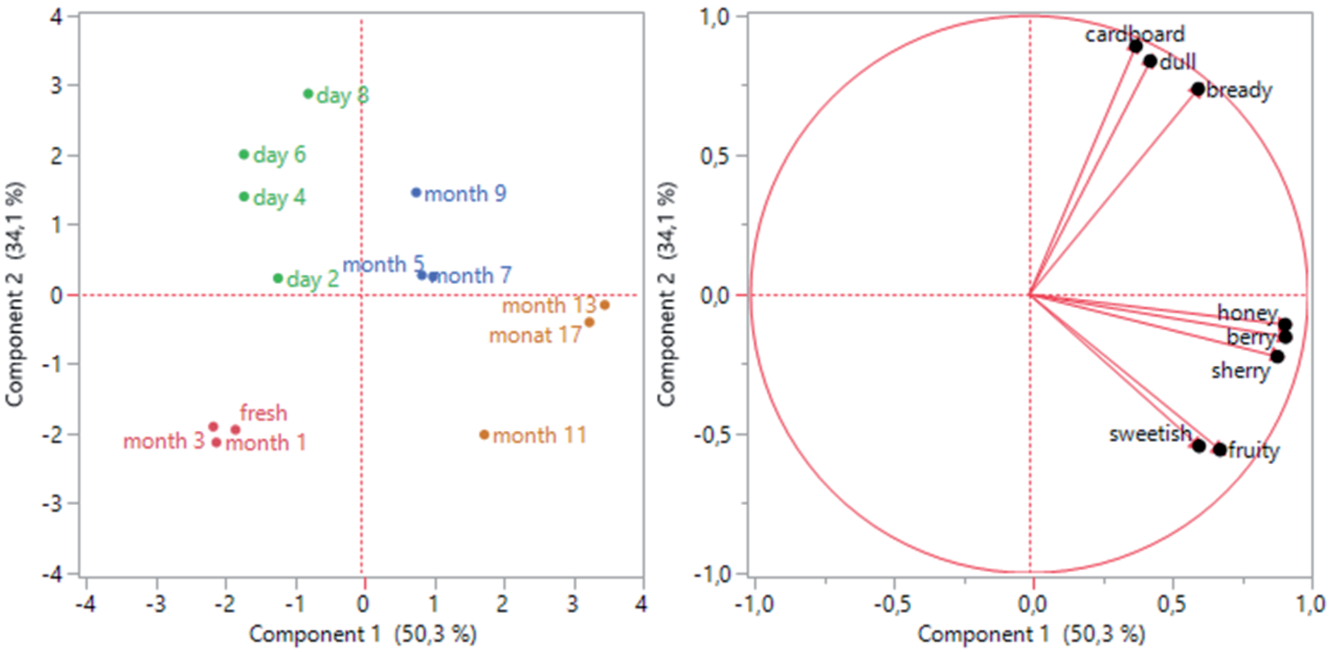

Fig. 6 Principal components analysis of sensory attributes of differently-aged lager beer; left: scores-plot of 13 naturally and forced aged samples; right: loadings-plot of 8 sensory ageing attributes

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

3.4 Sensory analyses – cluster analysis and PCA

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

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

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

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

3.5 Analytical – sum of indicators

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

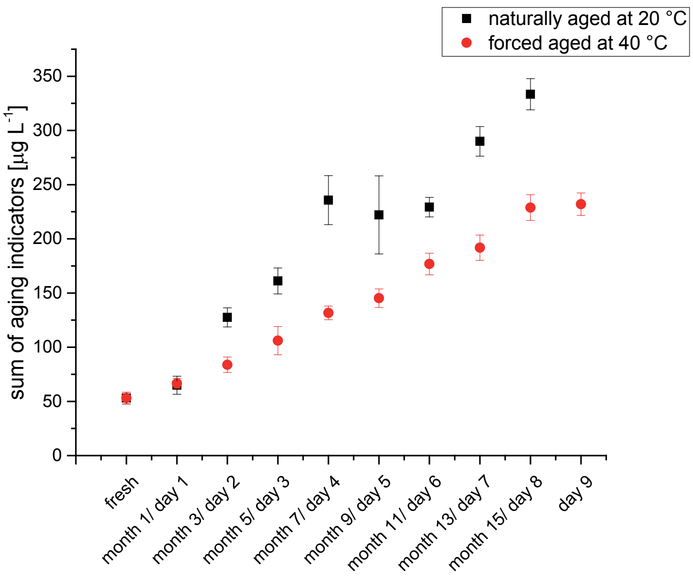

Fig. 7 Sum of ageing indicators after natural ageing at 20 °C and forced ageing at 40 °C

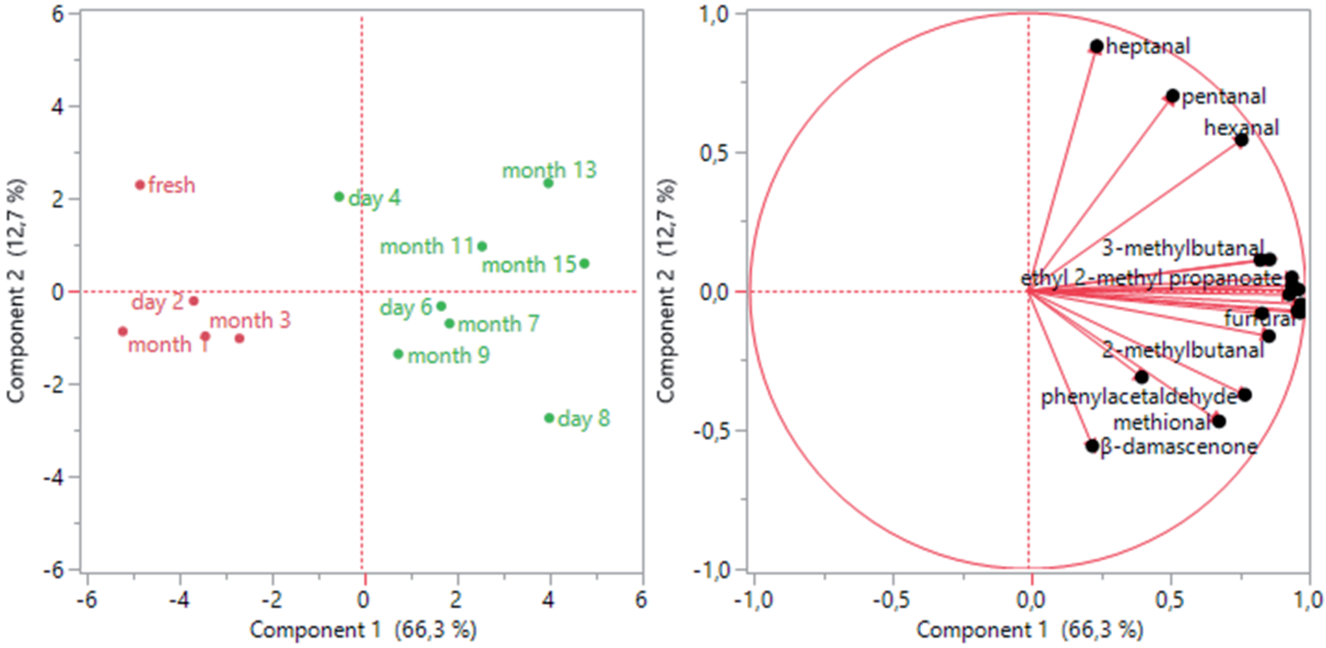

Fig. 8 **Principal components analysis of selected ageing indicators of differently aged lager beer; left: scores-plot of 13 naturally and forced aged samples; right: loadings-plot of 19 chosen ageing indicators**

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

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

3.6 Analytical part – PCA of ageing indicators

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

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

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

3.7 Analytical – time-course trends of selected compounds

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

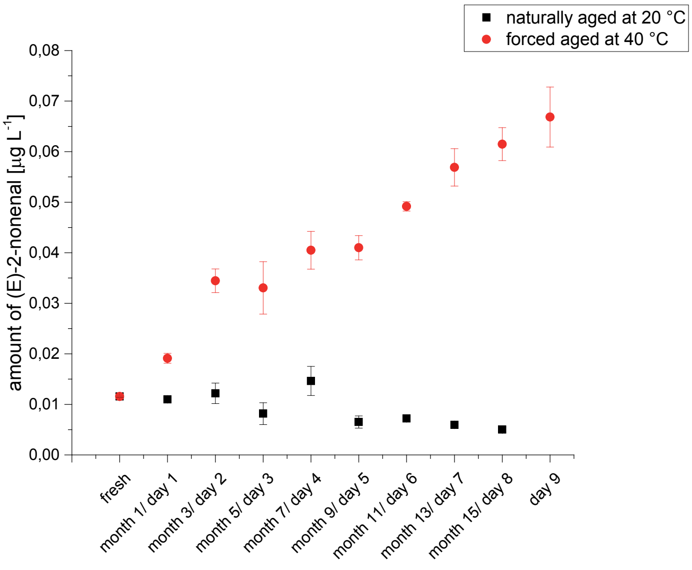

Fig. 9 **Time-course trend of (E)-2-nonenal in lager beer under forced and natural ageing**

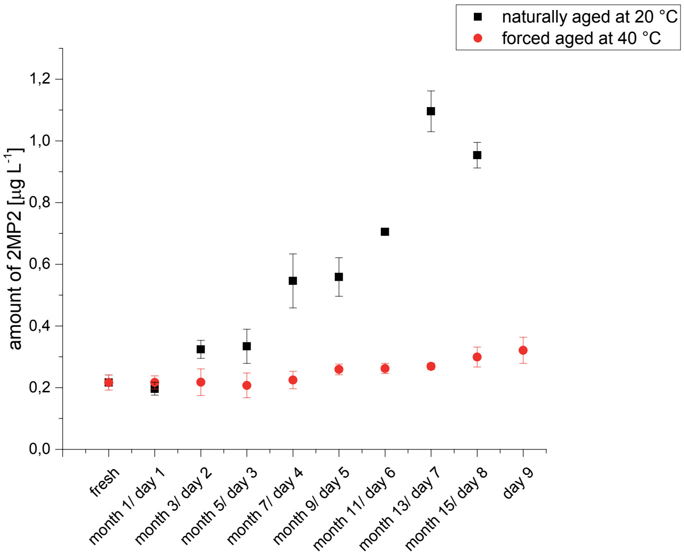

Fig. 10 **Time-course trend of ethyl 2-methyl propanoate in lager beer under forced and natural ageing**

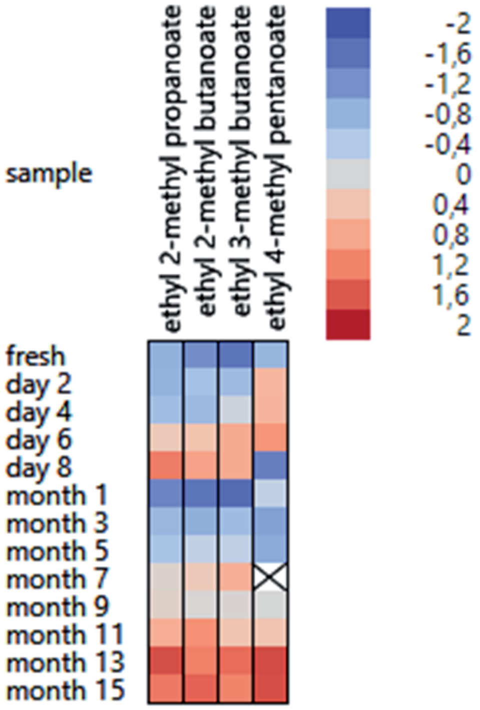

Fig. 11 **Analysed volatile humulone degradation products (2MP2, 2MB2, 3MB2, 4 MP2)**

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

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

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

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

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

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

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

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

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

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

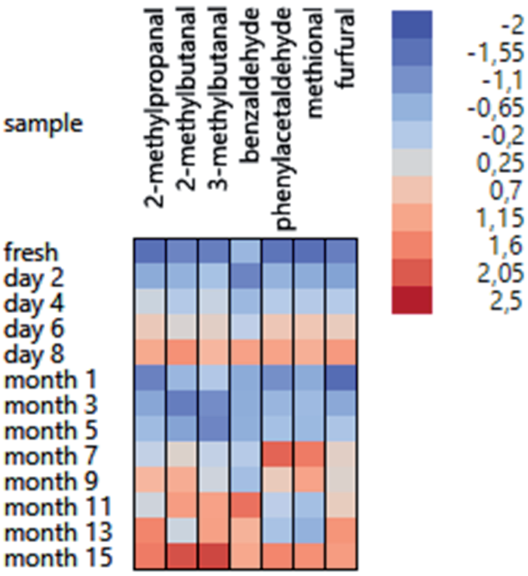

Fig. 12 Analysed Strecker and Maillard reaction products

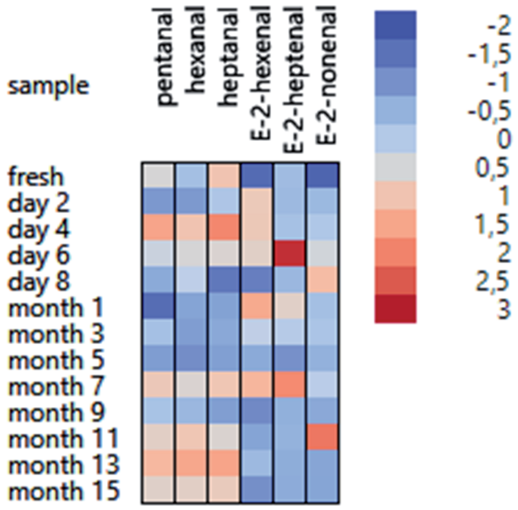

Fig. 13 Saturated and unsaturated linear aldehydes (associated with lipid oxidation)

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

In order to investigate the direct contribution of a certain compound to the aged aroma in lager, OAVs were calculated by dividing determined concentration by flavour threshold. For forced ageing, only (E)-2-nonenal showed an OAV of > 1. At day 2, an OAV of 1.1 was observed and increased until day 9 (OAV = 2.0). For natural ageing, only methional was found above its threshold, at 7 months (OAV = 1.4) and 9 months (OAV = 1.3).

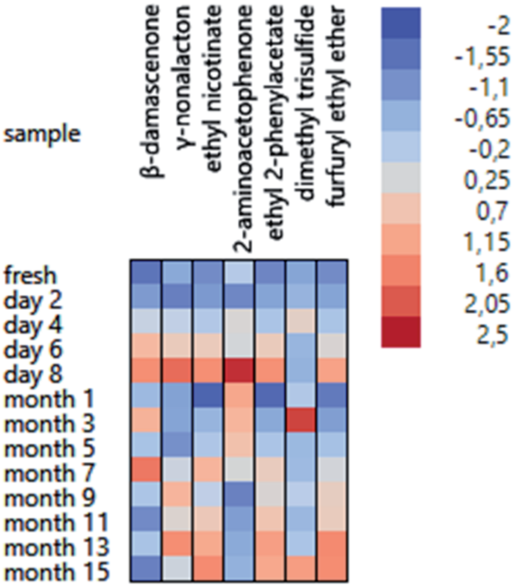

Fig. 14 Other analysed ageing indicators from various reactions (such as lactone formation, esterification, and etherification)

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

4 Conclusion

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

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

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

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

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