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Characterisation of the Flavour and the Chemical Composition of Lager Beer after Ageing in Varying Conditions

Aged beer flavour was studied by ageing a lager beer in different conditions (varying temperature-time profiles, different oxidative conditions and varying pH and ethanol concentrations). This led to beers with a varying aged flavour, which could be explained by differences in the reaction rate of ageing reactions. High temperatures, oxidative conditions and to a lesser extent, a lower pH, accelerated beer ageing. Enhanced (E)-2-nonenal formation probably led to the greater perception of cardboard flavour after ageing at high temperatures. Madeira flavour was only perceived after ageing at 20 °C and ribes flavour was mainly perceived in oxidatively aged beer. In beers with these flavour notes, high concentrations of acetaldehyde, Strecker aldehydes and diacetyl were found and in the Madeira flavoured beer, also of 2-furfuryl ethyl ether and 5-hydroxymethylfurfural. In the end, this study provides an overview of the different aged flavours that can develop in different ageing conditions and the corresponding flavour compounds that make up the chemical composition of these flavours.

Descriptors: beer, flavour, ageing, carbonyl compounds, flavour stability

1 Introduction

During storage of beer, the 'fresh' flavour profile is disturbed and typical aged flavour notes such as cardboard, solvent, ribes, Madeira and caramel, appear. These flavour notes can be found in varying intensities in aged beer. Perception of beer flavour is the result of the interaction of numerous chemical compounds with receptors in the olfactory organ and thus, this flavour evolution during storage is the result of gradual changes in the chemical composition of beer. Many chemical reactions such as oxidation, Strecker degradation, aldol condensation, furanic ether formation, degradation of hop bitter acids, Maillard reactions and hydrolysis of esters, occur during storage and result in the increase of aged flavour compound and the decrease of fresh flavour compound concentrations. However, explaining aged flavour notes from the increase and decrease of the concentration of flavour compounds remains difficult [1]. In a previous study, 26 compounds were tested for their contribution to aged flavour and 11 compounds were suggested to play an important role [2]. Additionally, the flavour of a beer that was thermally aged for 3 weeks at 40 °C, could be mimicked fairly well by addition of a combination of these compounds to fresh beer. However, earlier studies show clearly that beer finishing and packaging operations and storage conditions determine the type of flavour evolution considerably [3, 4]. Furthermore, it is evident that different beers age in varying ways depending on their raw materials and production process [5, 6]. This can be explained by the many different ageing reactions that are influenced in their own specific way by the beer composition, several variables inherent to beer (especially pH and oxygen content [3, 7, 8]) and external conditions (e.g. temperature and light [4, 9]). As a result of differences in reaction rates, concentrations of flavour compounds resulting from these reactions will vary accordingly.

In this study, the flavour changes and the concentration evolution of selected flavour compounds was studied after ageing of lager beer in varying conditions (varying temperature-time profiles, different oxidative conditions and varying pH and ethanol concentrations) in order to obtain better insights in aged beer flavour and the contribution of aged flavour compounds to aged flavour.

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Tables and figures see Appendix

2 Experimental

2.1 Chemicals and beers

All chemicals were purchased from Sigma (St. Louis, MO, USA) with the highest purity available. A commercial lager beer with an original gravity of 11.72 °P, an alcohol content of 5.12 v/v %, a colour of 6.5 EBC and a bitterness of 26 EBU was used for the ageing experiments.

2.2 Preparation and ageing of lager beer

Lager beers were aged in different conditions. An overview of the tested conditions is given in table 1. The temperature-time

profiles were 5 days at 60 °C, 3 weeks at 40 °C, 3 months at 28 °C, 6 months at 20 °C and 10 years at 20 °C. Oxidative reactions were initiated in two ways. Oxygen was introduced in the beer by flushing the headspace of the bottle with oxygen for 20 seconds after opening. Afterwards, the bottle was closed and was turned several times. The total in-pack oxygen content of the resulting beer was 12 ppm compared to 0.1 ppm in the initial beer. The other oxidative condition was initiated by supplementing the bottle with Fenton reagent (addition of 1.402 mM H_2O_2 and 0.00863 mM $FeSO_4.7H_2O$). Afterwards, the bottle headspace was made relatively oxygen free by overfoaming before closing. The pH of beer was adjusted by adding H_2SO_4 (20 %) or NaOH (20 %) for the low (pH 3.8) and the high pH (pH 4.6) respectively. The reference beer had a pH of 4.2. Finally, the ethanol content was adapted to 6.2 % (v/v) compared to 5.2 of the reference, by adding pure ethanol. After the additions, overfoaming was performed before closing the bottles and bottles were turned several times. Oxidative beers and other beers to which additions were made were aged for 3 months at 28 °C. Beers to which additions were made, were compared to a reference beer that was aged in the same temperature-time interval. Initially, 2 references were used: bottles of beer to which no addition was made, but that were opened and overfoamed afterwards, and bottles of beer that were not opened before ageing. Since no noteworthy differences were found between both, the unopened beer bottles are described as the reference beer further in the text.

2.3 Total in-pack oxygen

The total in-pack oxygen content was determined by first shaking the bottles for 5 minutes on an orbital shaker, followed by measuring the dissolved oxygen content with a Mettler Toledo Intap 4000 instrument.

2.4 Sensory analysis

Sensory tests were carried out with a trained panel of at least 8 members. Beers were always presented in black glasses. Besides an evaluation of some fresh flavour aspects and the general ageing character, stale flavour was evaluated for 9 aspects (cardboard, metal, solvent, old hops, ribes, Maillard (caramel, burnt, bread, butter), stale-sulphury, acetaldehyde (green apple) and Madeira) by giving a score from 0 to 8. A score of 0 meant that the particular flavour aspect was not present, while a score of 8 indicated that this aspect was extremely strong. Finally, an overall appreciation score was given on a scale from 1 to 9.

2.5 Analysis of volatile flavour compounds

Esters and higher alcohols were analysed with a calibrated Autosystem XL gas chromatograph (GC) (HS40; Perkin Elmer, Norwalk, USA) coupled with flame ionization detection (FID). The GC was equipped with a Chrompack-Wax 52 CB column (length: 50 m; 0.32 mm ID; layer thickness: 1.2 µm; Varian, Palo Alto, CA). Filtered beer (5 mL) was transferred to vials and subsequently heated for 16 min at 60 °C in the headspace (HS) autosampler before injection (needle temperature: 70 °C). Helium was used as the carrier gas. The oven temperature was kept at 50 °C for 7.5 min, increased to 110 °C at 25 °C/min and was held at that temperature for 3.5 min. The FID temperature was kept constant at 250 °C. Analyses were carried out in duplicate and results were evaluated with Perkin Elmer Turbochrom Navigator.

Carbonyl compounds were measured with HS solid-phase microextraction (SPME) coupled to gas chromatography and mass spectrometry (MS). Three procedures were used that were previously described by Saison et al. [10, 11]. Carbonyls were determined with on-fibre ((E)-2-nonenal and (E,E)-2,4-decadienal) and in-solution (other carbonyls) derivatisation using pentafluorobenzyl hydroxylamine (PFBHA) as derivatisation agent. The other compounds were measured after extraction of underivatised compounds.

Ten minutes of pre-incubation was applied for the three procedures. Subsequent SPME extraction was different for the three procedures. On-fibre derivatisation was performed after loading a PDMS-DVB fibre with PFBHA by exposing the fibre to the headspace of a PFBHA solution (1 mg/mL, 10 min, 45 °C, 250 rpm). The fibre was subsequently exposed to the HS of a vial containing 10 mL beer, 50 µL of an ethanol solution with 100 mg/L *p*-fluorobenzaldehyde, and 3.5 g NaCl (30 min, 45 °C, 250 rpm). In-solution derivatisation was done by exposing a PDMS-DVB fibre to the HS of a vial containing 10 mL beer, 50 µL of an ethanol solution with 100 mg/L *p*-fluorobenzaldehyde, and 0.375 mL of a PFBHA solution (20 g/L) (40 min, 60 °C, 250 rpm). The underivatised compounds were measured following extraction with a DVB-CAR-PDMS fibre of the HS of a vial containing 10 mL beer, 50 µL of an ethanol solution with 200 mg/L 2-heptanol and 100 mg/L guaiacol, and 3.5 g NaCl (30 min, 40 °C, 250 rpm).

GC-MS analysis was carried out using a Trace GC Ultra coupled to a dual stage quadrupole MS, consisting of a curved, small quadrupole as prefilter and a normal quadrupole (both from Thermo, Austin, TX, USA). A Rtx-5SilMS column (60 m x 0.25 mm I.D.) with a film thickness of 1 µm was used. The GC was equipped with a split-splitless injector which was held at 250 °C. Compounds were analysed following 2 min desorption and splitless injection. During the GC run, a constant flow rate (1.5 mL/min) of the carrier gas (Helium) was maintained. The GC program for both derivatisation procedures was the same and started at 60 °C for 2 min, then increased in 4 steps: 60 to 165 °C at 50 °C/min; 165 to 200 °C at 2 °C/min, 200 to 260 °C at 4 °C/min and 260 to 290 °C at 5 °C/min and was held at 290 °C for 6 min. The GC program for the underivatised compounds started at 30 °C, the oven temperature was raised in 3 steps after 2 minutes: 30 to 70 °C at 10 °C/min followed by 1 minute at 70 °C; 70 to 190 °C at 4 °C/min and 220 to 270 °C at 25 °C/min and was finally held at 270 °C for 6 minutes. The mass spectra were obtained by electron impact ionisation at 70 eV and the ion volume and the transfer line were held at 250 °C and 290 °C respectively. The detector measured from m/z 35 to 400 and results were analysed using Xcalibur software (Thermo, Austin, TX, USA). Analyses were performed in triplicate.

2.6 Statistical analysis

Sensory data were analysed for statistical significance by parametric *t*-tests for paired samples, using the software XLSTAT 2007.6 for

MS Excel. Partial Least Squares Regression (PLS2) was carried out using the Unscrambler 9.7 software.

3 Results and discussion

A lager beer was aged in various conditions (varying temperature-time profiles, different oxidative conditions and varying pH and ethanol concentrations) and resulting beers were tasted and analysed afterwards. Sensory results and the concentration evolution of a selection of flavour compounds will first be discussed separately. Afterwards, correlations between sensory and analytical results will be discussed in order to explain the sensory results from the chemical composition.

3.1 Sensory analysis of beers aged in different conditions

3.1.1 Temperature-time profile

A lager beer was aged with varying temperature-time profiles: 5 days at 60 °C, 3 weeks at 40 °C, 3 months at 28°C, 6 months at 20 °C and 10 years at 20 °C. Obviously, the latter beer was not produced in the same batch process and probably, in the 10 year time period, changes have been applied to the production process. Still, it was thought that including this beer can give added value to the study since extreme conditions may give more explicit results.

The beers were tasted after ageing and sensory results are presented in figure 1. The fresh beer had an ageing intensity of zero and aged flavour notes were absent. Therefore, this beer was not included in the figure. The overall appreciation score on the other hand was 6.6. From the ageing intensity and overall appreciation scores, it is clear that higher temperatures accelerate beer ageing enormously. The beer that was aged at 60 °C for 5 days was even quoted as the second most aged beer. This emphasises the importance of storing beer as cold as possible.

Next to differences in the general ageing score, it was obvious that separate flavour notes were influenced completely different depending on the applied temperature. In general, reaction rates will increase at higher temperatures. However, this increase depends on the activation energy of a specific reaction which differs between distinct chemical reactions. Consequently, reactions rates of separate reactions will not increase equally and ageing will give rise to an alteration of the relative concentration of staling compounds depending on the applied temperature [1]. In this study, it was clear that ageing at 60 °C predominantly gave rise to the perception of cardboard flavour. Other flavour notes hardly developed during the 5 days of ageing. Relatively, the cardboard flavour was also more prominent in the beers aged at 40 °C or 28 °C compared to ageing at 20 °C. The greater appearance of cardboard flavour during ageing at higher temperatures was already observed in previous studies [4, 9]. However, *Greenhoff & Wheeler* [12] claimed that the nature of flavour changes, in particular the cardboard character, displayed at 60 °C was similar to that at 18 or 37 °C. In two other studies, the beers were not only tasted after ageing, but flavour evolution was also monitored. Both studies showed a faster increase of the cardboard flavour in the beers aged at higher temperature, but after a certain period, the intensity declined [13, 14]. Furthermore, it appeared that solvent flavour was more or less perceived evenly strong in all aged beers, even in the beer that was aged for 10 years. Ageing at 20 °C resulted in a beer with a flavour profile consisting of similar intensities for all 5 aged flavour notes presented. Especially the Maillard-like flavour was intensely perceived compared to beers stored at higher temperatures, which is in accordance with results of *Kaneda et al.* [9]. Furthermore, it was apparent that Madeira flavours were only formed in beers aged at 20 °C for a longer storage period, especially after ageing for 10 years. This is not unexpected, since this flavour is primarily known from its appearance in aged ports and Madeira wines. Finally, apart from the Madeira flavour, ribes and Maillard-like flavour notes were strongly perceived.

3.1.2 Oxidative conditions

Beer flavour is known to be affected by reactive oxygen species (ROS) (i.e. singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\bullet-}$), hydroperoxyl radical ($^{\bullet}OOH$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2)) [15]. These ROS can be activated from molecular oxygen in its unreactive ground state ($^3O_2$) by catalytic activity of electron donors (e.g. metal ions such as Fe and Cu), energy or light [15]. In this study, the effect of oxidation on beer ageing was examined by increasing the total in-pack oxygen content (from 0.1 ppm in the reference beer to 12 ppm) on the one hand, and adding Fenton reagent (a mixture of H_2O_2 and $FeSO_4$) on the other hand. Afterwards, beers were tasted and results are presented in figure 2.

Ageing was accelerated significantly in beers stored with oxygen and Fenton reagent. This is confirmed by many studies [3, 13, 16]. Oxidative ageing has long been regarded as the only reason for beer ageing. However, studies by *Currie et al.* [17] and Furusho et al. [13] which dosed low amounts of oxygen in the bottle (0.5 ppm, and 0.42 and 0.69 ppm respectively compared to the controls of 0.05 and 0.16 ppm respectively) followed by ageing, observed only minor effects on aged flavour. Currie et al. [17] concluded that low concentrations of oxygen (< 0.50 ppm) are desirable, but that attempts to reduce oxygen to the ppb concentration range appears unlikely to significantly increase flavour stability. In this study, the dosage of oxygen in beer appeared to result in a slightly higher ageing intensity compared to the beer supplemented with Fenton reagent. However, the difference was small.

Furthermore, separate flavour notes were influenced differently. In the beer aged with oxygen, ribes flavour was especially apparent and also Madeira and Maillard-like flavour notes were enhanced substantially. The cardboard and solvent flavour were unaffected by the presence of oxygen. The addition of Fenton reagent resulted in a more or less equal increase of ribes, Maillard, Madeira and cardboard flavours. Solvent flavour was again unaffected. Earlier, samples stored with oxygen were described as sweet caramelised [7] and a close correlation has been found between ribes flavour and headspace air [16]. However, ribes flavour could also develop quite strong in a number of beers with low headspace-air content. Oxygen thus enhances ribes flavour formation, but it is not required.

3.1.3 pH

In order to study the effect of pH on beer ageing, the pH was adapted to 3.8 and 4.6 starting from a pH of 4.2 in the unmodified lager beer. From the overall score and general ageing intensity, it seems that the aged flavour decreases as the pH increases. These differences were statistically compared with the paired *t*-test, and it appeared that only a significant difference ($\alpha < 0.05$) could be established between the pH 3.8 and the pH 4.6 beers, and to a lesser extent ($\alpha < 0.1$) between pH 3.8 and 4.2 for the overall ageing scores. *Grigsby et al.* [7] were the first to show that a correlation exists between the pH of beer and its 'oxidisability'. Beers aged at extremely low pH (pH 2-3) were shown to have very poor flavour stability. In contrast, beers with a high pH (4.5-4.8) have been reported to possess better flavour stability [7]. Furthermore, Kaneda et al. [18] showed that lowering the pH from 4.3 to 4.1 or 3.8 significantly accelerated staling. Currie et al. [17], on the other hand, who tasted beers that were aged at pH 3.75, 4.06 and 4.25 could not ascertain a significant difference between the beer aged at pH 4.25 compared to pH 4.06. They suggested that keeping the beer above pH 4.0 may be desirable for flavour stability, but that small decreases will only result in marginally inferior flavour stability.

Differences in the appearance of separate flavour notes were small. Only cardboard flavour was perceived more at lower beer pH. Other flavour notes were more or less equally perceived in all beers. *Guyot-Declerck et al.* [19] performed aroma extract dilution analysis (AEDA) on beers aged at pH 4.2 and 4.6 and found out that cardboard perception was higher after ageing at pH 4.2.

3.1.4 Ethanol content

Another parameter that was varied was the ethanol content, since ethanol is involved in several ageing reactions. It appeared that the influence on beer ageing was small from the overall appreciation and the ageing score (Fig. 2). Also when separate flavour notes were studied, only slight differences were observed. The only difference worth mentioning was the slightly increased perception of Madeira flavour. *Perpète & Collin* [20] showed that ethanol significantly increases aldehyde retention in a study on low-alcohol beers. Higher alcohol contents consequently lead to lower perception of the worty character. This difference was however not observed in the obtained results and it is questionable if the increased retention is substantial when the alcohol content is already quite high. Finally, it should be mentioned that a relatively small amount of ethanol (1 v/v %) was added because of limitations of the remaining headspace volume.

3.2 Evolution of staling compounds in beers aged in different conditions

The concentrations of a selection of staling compounds were determined in the differently aged beers and are presented in table 2 and 3. Apart from the concentrations, the thresholds (TH) of the compounds are presented. These thresholds were determined in a previous study in the same lager beer as the one studied here [2]. The concentration evolutions will be discussed shortly in order to obtain an overview of the origin and dependence of the compounds on the tested conditions.

Acetaldehyde was mainly influenced by increased oxidative conditions, especially by the addition of Fenton reagent. It was previously suggested that hydroxyl radicals react non-selectively with ethanol, the most abundant compound in beer after water, resulting in the 1-hydroxyethyl radical [1]. This radical can further react to acetaldehyde. H_2O_2 is more stable, and also other reactions are more probable to occur. However, according to Kaneda et al. [15], the hydroxyl radical can be generated by reaction of H_2O_2 with Fe^{2+}, called the Fenton reaction. The main effect of adding Fenton reagent might thus be the formation of acetaldehyde. In this study, the formation of acetaldehyde was accelerated moderately at higher temperatures. The ethanol content appeared to have only a minor influence, which might be attributable to the limited concentration increase compared to the reference, and the greater importance of ROS. Finally, formation of acetaldehyde was the highest at pH 4.2. The formation of hexanal was mainly influenced by the presence of oxygen, but a lower pH also led to higher concentrations in the aged beer.

The formation of (E)-2-nonenal (T2N) was accelerated tremendously at higher temperatures as can be deduced from the high concentrations in the thermally beers. This indicates that T2N may be specific for thermally aged beers, while it is less formed in naturally aged beers (20 °C). This assumption is confirmed by Van Eerde & Strating [21] who observed a substantial increase of T2N at 40 °C in several beers within a few days, whereas at 20 °C, this was not seen, even after 4 months of storage. A similar result was obtained by Narziss et al. [22] and *Nordlov & Winell* [23]. This might explain why T2N is indicated as very important in some studies [24], while others observe only a limited or no increase during ageing [25, 26]. Oxidative conditions also increased the concentration of T2N during ageing. In contrast, other authors observed an increase of T2N independent of the oxygen concentration in bottled beer [22, 27] and they doubted the occurrence of oxidative formation reactions leading to T2N in beer. Therefore, non-oxidative mechanisms were suggested, such as aldol condensation of acetaldehyde and heptanal [28]. However, it is not clear whether the amounts formed in this reaction under normal storage conditions, are sufficiently high to contribute [6]. Nordlov & Winell [23] proposed the dissociation of nonenal-sulphite adducts at low pH values. However, Kaneda et al. [29] showed a very low activity of T2N with sulphites and *Dufour et al.* [30] showed the irreversible nature of the sulphite addition to the double bond. Another proposition for the formation of T2N, the release from imine adducts [31], seems more plausible. This hypothesis describes the formation of T2N during malting and mashing, and subsequent binding by amino compounds leading to the presence of T2N-imine adducts in beer. Acid hydrolysis at the lower beer pH (±4.2) compared to wort pH (±5.2) subsequently leads to the release of T2N. This reaction mechanism might also explain the higher concentrations of T2N in the beer aged at pH 3.8 compared to pH 4.2 and 4.6. Kaneda et al. [18] on the other hand, suggested that T2N formation is independent of the dissociation from adducts, but based on the fact that the decreasing pH accelerates flavour staling reactions such as free radical reactions.

In this study, forced ageing enhanced the formation of all Strecker aldehydes considerably. Oxygen even seemed to have a greater promoting effect. *Methner et al.* [32] confirmed the large effect of

temperature. *Schieberle et al.* [33] observed a large enhancement of the concentration of Strecker aldehydes during ageing at 45 °C with oxygen. When oxygen was omitted, but ageing was still performed at 45 °C, this enhancement was considerably less. This suggested the greater impact of oxygen compared to temperature. The promoting effect of oxygen on Strecker aldehyde formation has been observed in several other studies [22, 34] and might be attributable to oxidation of the respective alcohols [1]. However, oxidative reactions may also lead to a greater release of Strecker aldehydes from precursors formed in the boiling kettle. Most likely, these precursors are Amadori rearrangement products which can be oxidised to 2-hexosulose-imines in the presence of transition metal ions and air oxygen [35]. In this study, the effect of pH and ethanol content on the formation of Strecker aldehydes was negligible in the studied ranges, except for 2-methylpropanal, which was formed more at higher pH. Benzaldehyde showed a different trend over the differently aged beers. This can be explained as benzaldehyde can be formed from phenyl acetaldehyde [36], but not by direct Strecker degradation.

Furfural, 5-hydroxymethylfurfural (5-HMF) and 5-methylfurfural behaved similar in the varying ageing conditions. Maillard reactions are known for their correlation with high temperature processes. As in previous studies [37-39], this was confirmed by the presented results. Oxidative conditions and ethanol content on the other hand, seemed without any effect. Furthermore, the lower the pH value, the more formation of furanic aldehydes was observed as also observed previously by *Shimizu et al.* [37]. This pH dependency was even more pronounced for acetylfuran. Although the initiation of the Maillard reaction is improved at high pH, subsequent reactions are either acid- or base-catalysed [40]. The formation of diacetyl, was mainly enhanced in the presence of oxygen, and to a much lesser extent in the presence of Fenton reagent. *Vanderhaegen et al.* [1] suggested that the oxidation of acetoin or 2,3-butanediol may be responsible.

2-furfuryl ethyl ether (FEE) is formed from a condensation reaction of furfuryl alcohol and ethanol and increases linearly with the concentration of its precursors [41]. Consequently, in this study, FEE formation was enhanced in the beer with the higher ethanol level and the conditions that increased the formation of furfural (which can be considered as a good marker for the formation of furfuryl alcohol) (i.e. high temperature and low pH). Moreover, the condensation reaction is acid-catalysed [40]. Finally, it should be mentioned that the FEE concentration was very high in the beer aged for 10 years at 20°C.

Methyl isobutyl ketone, ethyl-2-methylbutyrate and ethyl-3-methylbutyrate can directly or indirectly be obtained from the oxidative degradation of hop bitter acids and consequently, their concentrations were higher in the oxidatively aged beers. Additionally, it was observed that a lower pH led to higher levels, which suggests that bitter acids are oxidised more easily at lower pH. Ethyl-2-methylbutyrate and ethyl-3-methylbutyrate are actually formed from a condensation reaction between the corresponding acids and ethanol [1]. Consequently, high ethanol levels resulted in higher concentrations of these compounds. Finally, it seemed that increasing the storage temperature did not accelerate the oxidation of hop bitter acids that much.

The formation of β-damascenone (β-DS) was highly dependent on the temperature. In beers aged at low temperatures, even after a long storage time, concentrations were quite low. It can thus be proposed that a thermal degradation mechanism is responsible. Low pH values also enhanced the level of β-DS greatly which may be attributed to a faster acid-catalysed glycoside hydrolysis [42]. Additionally, β-DS can also be formed by oxidative degradation of carotenoids [1]. However, no increase of β-DS was observed in higher oxidative conditions.

Although it is assumed that ethyl nicotinate and ethyl pyruvate are formed similarly from the condensation of ethanol and the corresponding acid, they behaved differently and higher ethanol content seemed of little importance.

γ-nonalactone can be formed by intramolecular esterification of 4-hydroxynonanoic acid and was only formed in low amounts in the thermally aged beers. An oxidative environment on the other hand, accelerated its formation.

Hydrolysis of isoamyl acetate occurred somewhat faster at higher temperatures and also oxygen and a lower or higher beer pH induced hydrolysis. The presence of Fenton reagent and ethanol on the other hand, did not. These results are in accordance with *Stenroos et al.* [43] who observed a substantial decrease of isoamyl acetate during ageing at 38 °C compared to 20 °C and also high air contents led to a somewhat faster decrease.

3.3 Impact of the selected staling compounds on aged flavour

In a previous study, the aged flavour of a lager beer aged at 40 °C for 3 weeks could be explained relatively well [2]. In this study, it was shown that aged beer flavour is variable depending on finishing operations and storage conditions because of differences in the relative concentrations of staling compounds. Therefore, it was attempted to explain the sensory results of the differently aged beers from the analytical data in order to obtain a better insight in aged flavour. In the discussion, the concept of the flavour unit (FU), calculated as the ratio of the concentration of a compound and its TH value [44] is used to indicate its contribution to flavour. Previously, it was stressed that care should be taken when extrapolating TH values to other studies because of the influence of differing endogenous concentrations and masking flavours [2]. However, the same lager beer was used and TH values are thus very representative. Still, THs and FUs should be considered as indicative rather than absolute since interactions can occur and because of large personal variations [2]. Since flavour compounds are not likely to contribute considerably to flavour when their FUs stay beyond 0.1 FU, only compounds that exceeded 0.1FU were considered.

Two visualisations were made of the combination of analytical and sensory results to obtain a clear insight. In figure 3, FUs of the most important compounds are presented in spider plots to get an insight in the flavour active ageing compounds in the respective beers. The FUs of Strecker aldehydes were added up, since they showed a similar concentration profile in the differently aged beers, they can interact greatly and for clarity reasons. Because of the great

complexity and great amount of data, multivariate statistics are useful to obtain a visualisation of the main variability of the data set. Additionally, an understanding of the relationship between two data sets can be gained with partial least squares regression (PLS). Therefore, PLS2 (sensory vs analytical results) was used in order to obtain insight in their correlations. For the analytical data, FUs were used and after centering the data, a PLS2 plot was created as presented in figure 4. The scores plot containing the samples (not shown), showed the initial beer in the upper left corner and the beer aged at pH 4.6 quite close. The beers that were aged most were spread at the opposite site according to their separate sensory ageing characteristics. The more distant X-variables (sensory) are located from the centre, the more variability they explain and the more they contributed to the creation of the plot. The more distant Y-variables are from the centre, the more they might explain the sensory results. The plot largely confirms the greater importance of T2N, Strecker aldehydes, FEE, acetaldehyde and diacetyl as compared to 5-HMF and β-DS, as proposed by *Saison et al.* [2].

As can be deduced from the plot, cardboard flavour correlated well with T2N. The flavour of the beer aged for 5 days at 60 °C was mainly made up of cardboard. This may be explained by the increase of about 4 FUs. However, although figure 3 clearly indicated that many compounds such as Strecker aldehydes, 5-HMF, FEE and β-DS increased substantially, it is remarkable that other aged flavours were not substantially perceived. They may be masked by the cardboard flavour. Moreover, Strecker aldehydes were already shown to contribute to cardboard flavour [2]. The beer aged for 3 weeks at 40 °C had lower concentrations of Strecker aldehydes, FEE, diacetyl and 5-HMF, but the sensory characteristics, solvent, ribes and Maillard-like were not perceived less. On the contrary, on average, higher scores were given for these flavour notes. The beer aged at 20 °C for 6 months had a comparable FU profile in figure 3, except for T2N. However, when its sensory profile was studied, ribes, Maillard and Madeira flavour notes were more apparent. It may be that T2N and similar compounds such as TT24DD, are quite prominent and may render beers more harsh, leathery and papery. Lower concentrations of T2N may allow other flavour notes to become more apparent.

Although the concentration of acetaldehyde increased with about 8 and 5 FUs for the Fenton and the oxygen beer respectively, none of the tasters assigned a considerable intensity to the acetaldehyde flavour (sour apple-like) (results not shown). This confirms previous suggestions that acetaldehyde mainly contributes to aged flavour as a supporting background for other aged flavours [2, 45], such as ribes and Maillard-like flavours. Furthermore, diacetyl and Strecker aldehydes were also enhanced greatly in these beers.

From the PLS2 plot, it seems that ribes and Maillard-like flavours, when given a high intensity, were regularly both given a high intensity. It is therefore difficult to discuss them separately. Ribes flavour was especially apparent in the beer that was spiked with oxygen. The spider plot in figure 3 shows that high concentrations of Strecker aldehydes, diacetyl, T2N and acetaldehyde were found in this beer. Adding Fenton reagent resulted in lower concentrations of diacetyl, Strecker aldehydes and T2N, but a higher concentration of acetaldehyde. In this beer, a lower ribes and a more balanced sensory profile concerning the 5 attributes shown in figure 2 was perceived. In these oxidatively aged beers, no increase of specific Maillard compounds such as 5-HMF was observed, but Maillard-like flavour was definitely present. This may be attributed to the combination of Strecker aldehydes and diacetyl as shown by Saison et al. [2]. In the beers aged for 3 months at 28 °C (pH and ethanol variations), a modest Maillard-like flavour was apparent and ribes flavour was virtually absent. These beers showed very low acetaldehyde contents, while Strecker aldehydes were definitely present. The latter were already shown to cause a shift to sweet and bread-like off-flavours during storage [32]. In the end, it may be that a specific ratio of the levels of these compounds may lead to ribes or Maillard-like flavours, but it is very likely that other compounds than the ones measured in this study also contribute. Ribes-like flavour for example, was already linked to the occurrence of polyfunctional thiols in beer [46].

The Madeira flavour was especially apparent in the beer aged for 10 years at 20 °C. Also in this beer, an increase of 3 FUs acetaldehyde was seen. However, especially the very high concentration of FEE catches the eye. Furthermore, high concentrations of Strecker aldehydes, 5-HMF, diacetyl, but not of T2N and β-DS, were observed. The outcome was a beer with less harsh and more warming sensory notes such as Madeira, Maillard and ribes. In this beer, acetylfuran also reached a FU of 0.11. Although this is still quite low, it indicates that flavour compounds that are thought to be unimportant may contribute through interactions in this highly aged beer. In conclusion, Madeira flavour seems to be the results of a very complex combination of many compounds that also involves others than the ones presented.

Solvent flavour was previously shown to be related to FEE formation [2, 41]. In this study, the variability of the solvent flavour was relatively low between aged beers as can be deduced from the PLS plot and thus, not many assumptions can be made.

Finally, beers aged at different pH or ethanol content did not vary greatly and sensory results were not significantly different. However, some tendencies were observed. Differences were observed in the cardboard flavour of beers aged at varying pH's, which may be explained by the higher T2N level with lower pH. Furthermore, β-DS and FEE concentrations differed. They might also correlate with cardboard flavour, but also with the varying solvent flavour. The slightly higher ethanol content resulted in an increase of the same compounds (i.e. T2N, FEE and β-DS) and mainly caused the beer to be slightly more Madeira-like. It is probable that not only increased concentrations of these compounds contributed, but also ethanol itself may contribute considerably. This is not surprising since Madeira flavour is mainly known in beverages of higher ethanol content such as Ports and Madeira wines.

4 Conclusion

Aged flavour of beer was studied in more detail by ageing lager beer in varying conditions. It was evident that different ageing conditions caused aged beer flavour to vary greatly. This could be explained by differences in the formation rate of aged flavour compounds. Ageing at higher temperatures, stronger oxidative conditions and to a lesser extent, lower pH, clearly accelerated

beer ageing. Ageing at 60 °C resulted in a prominent cardboard flavour and at lower storage temperatures, cardboard gradually decreased. Especially the formation of T2N was enhanced greatly at higher temperatures and its concentration correlated strong with cardboard. Strecker degradation, Maillard reactions and β-DS formation were also enhanced at higher temperatures, but these compounds seemed to support cardboard flavour or were masked by higher concentrations of T2N. In contrast, at lower temperatures, ribes and Maillard-like flavour notes were more manifest. Solvent flavour seemed to be influenced less markedly by storage temperature, and Madeira flavour was only perceived in beers aged at 20°C, especially after storage for 10 years. This flavour seemed to be especially correlated with FEE and also with acetaldehyde, diacetyl, Strecker aldehydes and 5-HMF. These results indicate that care should be taken when considering forcing tests which are necessary in order that research on flavour stability can be done over realistic time frames. Oxidative conditions predominantly accelerated the development of ribes flavour, but also of Maillard flavours. Particularly the formation of acetaldehyde was enhanced in these beers, but also Strecker degradation and diacetyl formation. Lower beer pH resulted in slightly increased cardboard flavour, but differences were low and were mainly suggested to be caused by a difference in T2N, FEE and β-DS content. Ageing beer with a slightly higher ethanol level led to a higher perception of Madeira flavour and a slightly higher FEE content.

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Appendix

Table 1 Overview of the conditions that were tested for their effect on beer ageing

Temperature-time profiles	3 months 28 °C		
	Oxidative conditions	pH	Ethanol content
5 days 60 °C	Oxygen Flush	3.8	5.2 v/v % EtOH (Ref)
3 weeks 40 °C	Fenton reagent	4.2 (Ref)	6.2 v/v % EtOH
3 months 28 °C	Non-oxidative (Ref)	4.6	
6 months 20 °C			
10 years 20 °C			

Table 2 **Concentrations of a selection of staling compounds in beers that were aged according to varying time-temperature profiles. Concentrations are expressed in ppb unless stated otherwise**

	TH	Initial	5 days 60 °C	3 weeks 40 °C	3 months 28 °C	6 months 20 °C	10 years 20 °C
Acetaldehyde (ppm)	*1.1*	0.6	1.2	1.0	1.0	1.3	3.9
Hexanal	*88*	0.5	2.0	2.0	0.9	1.3	2.7
(E)-2-nonenal	*0.03*	0.03	0.16	0.11	0.05	0.08	0.09
2-methylpropanal	*86*	11	72	48	29	46	72
2-methylbutanal	*45*	2.9	16.7	6.2	3.6	4.9	17.4
3-methylbutanal	*56*	9	17	14	10	18	28
Benzaldehyde	*515*	1.2	1.6	1.8	1.5	1.8	13.4
Phenyl acetaldehyde	*105*	22	48	35	29	38	43
Methional	*4.2*	1.6	3.6	2.5	2.2	2.6	2.5
Furfural	*15157*	19	916	287	171	273	1094
5-HMF (ppm)	*36*	5	38	13	8	11	47
5-methylfurfural	*1174*	0.2	6.4	2.1	1.0	1.2	9.7
Acetylfuran	*513*	7	26	25	18	10	57
Diacetyl	*17*	6	20	12	11	20	40
Methyl isobutyl ketone	*2560*	4	4	4	5	5	12
Ethyl-2-methylbutyrate	*27*	0.4	0.4	0.5	0.4	0.6	2.0
Ethyl-3-methylbutyrate	*91*	0.7	1.0	1.1	0.9	1.5	5.3
Ethyl nicotinate	*4555*	4	18	18	11	–	164
Ethyl pyruvate	*22525*	39	50	66	86	112	61
2-Furfuryl ethyl ether	*11*	2	31	13	7	13	90
β-damascenone	*203*	123	380	303	242	195	88
γ-nonalactone	*607*	30	33	35	36	31	30
Isoamyl acetate	*510*	356	284	273	295	360	8

Table 3 **Concentrations of a selection of staling compounds in beers that were aged for 3 months at 28 °C after varying several parameters: oxidative conditions, pH or ethanol concentration. Concentrations are expressed in ppb unless stated otherwise**

	TH	Initial	Fenton reagent	Oxygen Flush	Ref (pH 4.2)	pH 3.8	pH 4.6	6.2 v/v % EtOH
Acetaldehyde (ppm)	*1.1*	0.6	9.5	5.9	1.0	0.6	0.8	1.0
Hexanal	*88*	0.5	2.4	3.2	0.9	1.3	0.9	1.2
(E)-2-nonenal	*0.03*	0.03	0.06	0.09	0.05	0.10	0.04	0.07
2-methylpropanal	*86*	11	41	102	29	24	44	35
2-methylbutanal	*45*	2.9	11.2	14.4	3.6	4.2	3.6	3.9
3-methylbutanal	*56*	9	24	39	10	11	12	13
Benzaldehyde	*515*	1.2	4.3	13.2	1.5	1.9	1.0	1.3
Phenyl acetaldehyde	*105*	22	57	70	29	26	29	24
Methional	*4.2*	1.6	2.4	4.1	2.2	2.0	1.8	1.6
Furfural	*15157*	19	165	214	171	292	91	154
5-HMF (ppm)	*36*	5	7	9	8	13	7	9
5-methylfurfural	*1174*	0.2	1.1	1.1	1.0	1.9	0.6	0.9
Acetylfuran	*513*	7	11	15	18	29	8	16
Diacetyl	*17*	6	30	76	11	7	7	10
Methyl isobutyl ketone	*2560*	4	3	10	5	6	2	5
Ethyl-2-methylbutyrate	*27*	0.4	0.7	0.9	0.4	0.6	0.4	0.5
Ethyl-3-methylbutyrate	*91*	0.7	1.6	1.8	0.9	1.2	0.8	1.2
Ethyl nicotinate	*4555*	4	19	20	11	7	13	6
Ethyl pyruvate	*22525*	39	23	70	86	242	30	83
2-Furfuryl ethyl ether	*11*	2	7	7	7	16	5	11
β-damascenone	*203*	123	267	259	242	337	215	297
γ-nonalactone	*607*	30	45	43	36	38	29	31
Isoamyl acetate	*510*	356	301	246	295	251	254	296

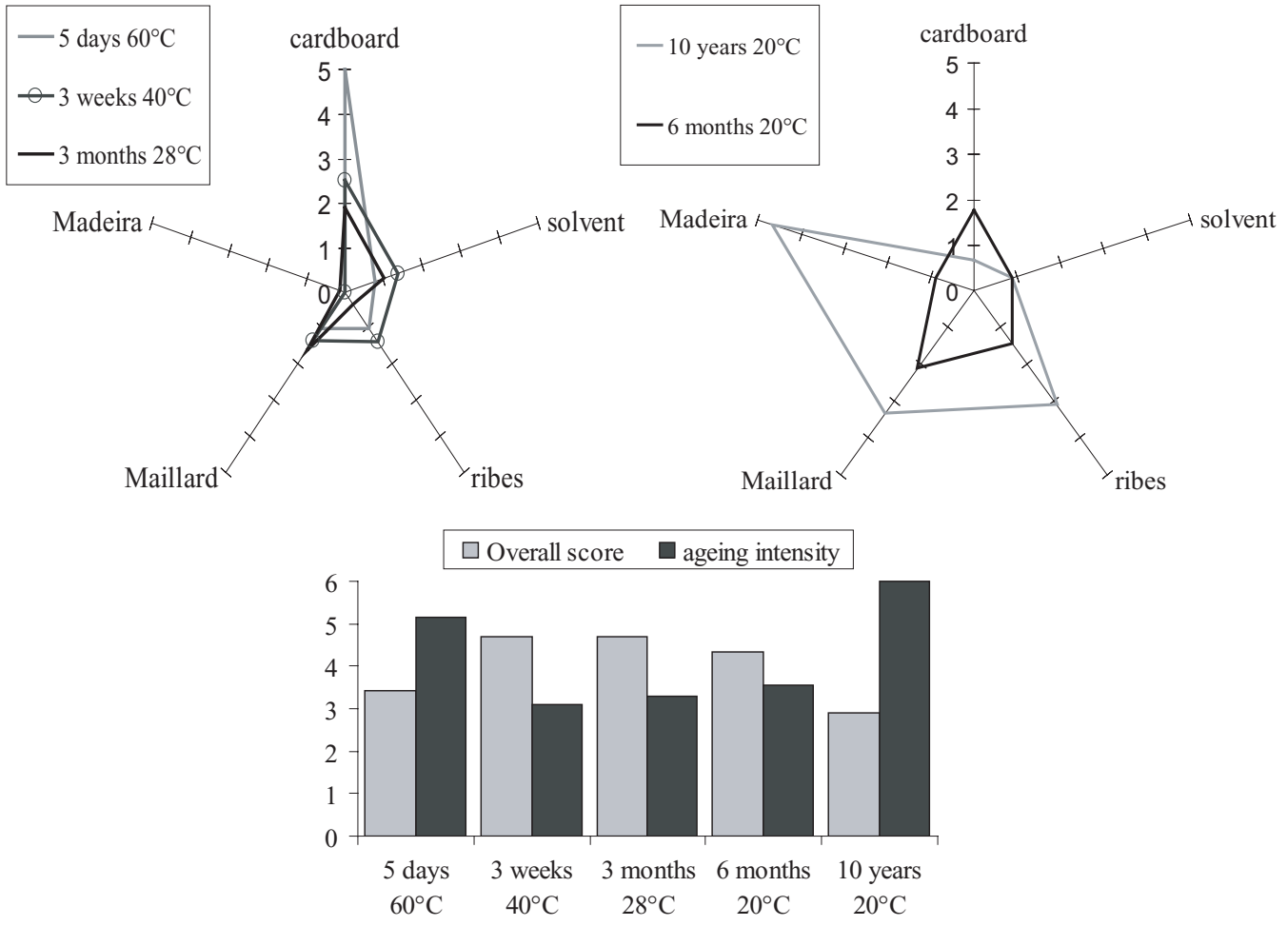

Fig. 1 Sensory results of beers that were aged according to varying temperature-time profiles

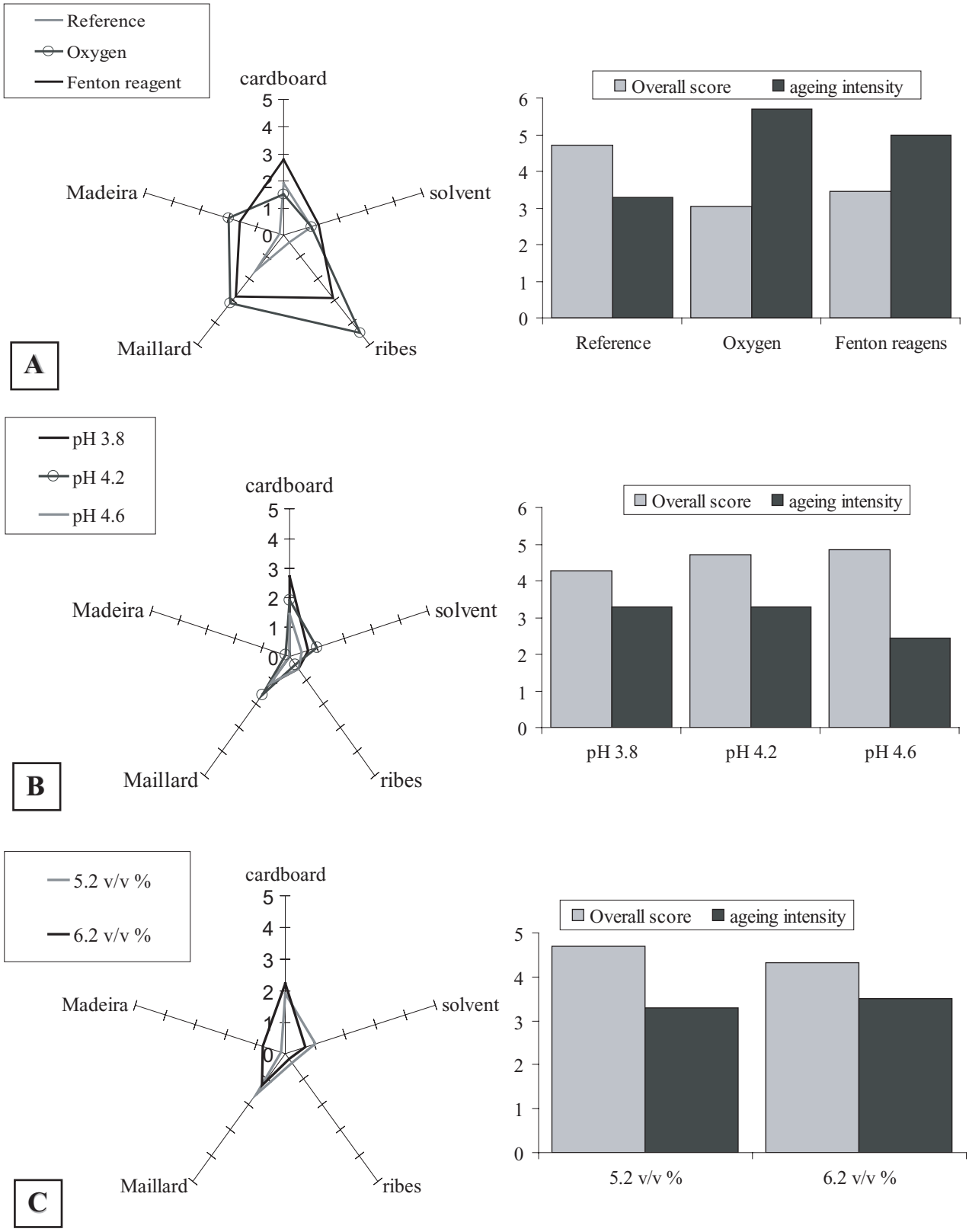

Fig. 2 Sensory results of beers that were aged for 3 months at 28 °C under varying conditions:
(A) oxidative conditions;
(B) pH;
(C) ethanol content

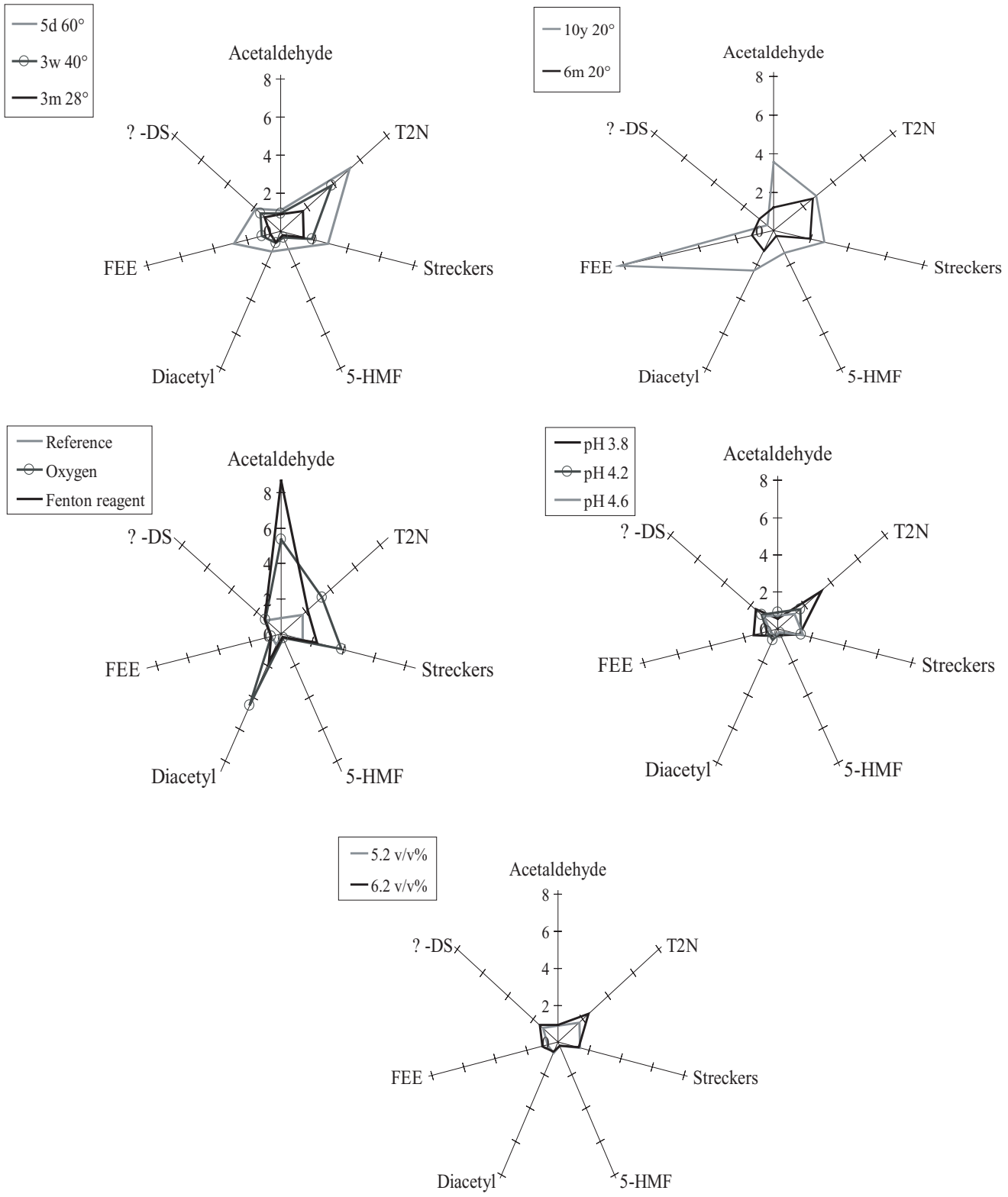

Fig. 3 Spiderplots of the flavour units of ageing compounds in differently aged beers

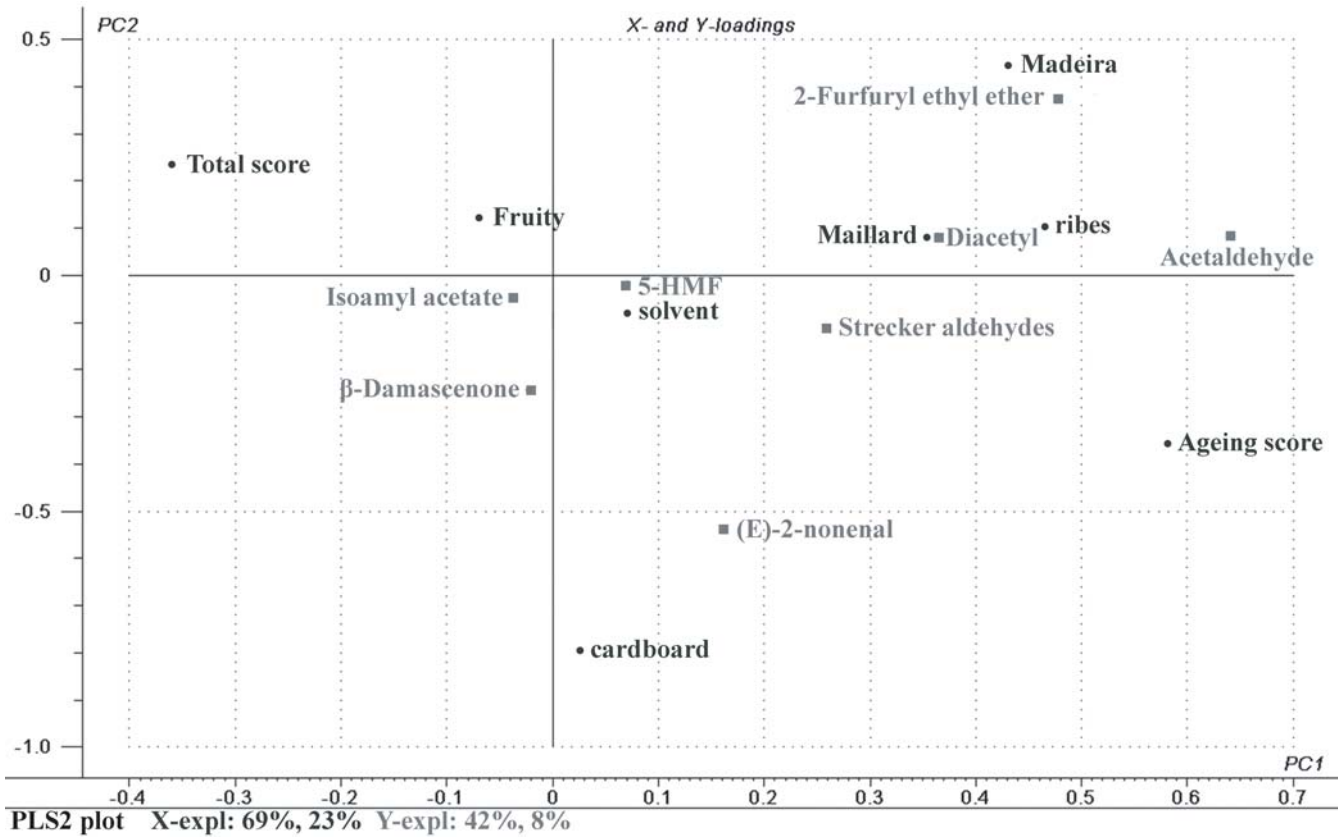

Fig. 4 PLS2 plot of beers aged in varying conditions (X: sensory data; Y: analytical data).

The plot presents the scores of the plot (sensory and analytical variables)