

Modelling of beer sensory staleness based on flavour instability parameters

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Abstract

Why was the work done: Deterioration in the flavour of fresh beer is a challenging concern for the brewing industry. Despite extensive research on flavour instability, the focus of studies has centred on a limited set of parameters, rather than taking a broader approach.

How was the work done: In this study, the intent was to produce a flavour stable beer. Using a low kilning temperature, malt of low staling potential was used in combination with best brewing practice to produce at pilot scale three batches of unpasteurised top fermented pale beers. Forty-three markers were analysed in the fresh and aged beers (stored at 30°C for 15, 30, 60 and 90 days). Beer staling was evaluated by a trained sensory panel and multivariate data analysis was used to explore how the markers contribute to staleness.

What are the main findings: Repeatability was achieved between replicate brews and, subsequently, staling. Polyphenols, haze, total reactive antioxidant potential (TRAP), iso- α -acids, colour, furfural, 2-methylpropanal and 2-methylbutanal showed a strong correlation with staleness. After 60 days of storage at 30°C, staleness doubled despite volatile aldehydes remaining below their sensory thresholds, suggesting a synergistic effect of carbonyls contributing to staleness. A Partial Least Square (PLS) model was established, modelling the sensory staleness from 2-methylpropanal, furfural, TRAP and the ratio of *trans*-/*cis*-iso- α -acids.

Why is the work important: The staling phenomena was reproduced in beers from parallel brewing trials with only minor variations. The four parameters in PLS modelling indicate that beer staling involves a combination of oxidative and non-oxidative pathways.

Keywords:

brewing, beer staling, flavour stability, markers, staling aldehydes, multivariate data analysis, sensory evaluation

Introduction

Flavour instability remains a challenge for the brewing industry (Bamforth 2011; Filipowska et al. 2021; Mertens et al. 2022). From the sensory perspective during storage, beer flavour deteriorates (Zufall et al. 2005). This has been widely studied and critically reviewed (Saison et al. 2009; Giannetti et al. 2019). Stale characteristics include a decrease in fruity and sulphury aromas and a reduction in the intensity and quality of bitterness. These changes are linked with lower consumer acceptance scores (Guinard et al. 1999) and reduced drinkability (Paternoster et al. 2020).

From the chemical point of view, beer flavour instability has been reviewed by Vanderhaegen et al (2006) and Baert et al (2012). Essentially, oxidation chain reactions are the predominant mechanisms behind the deterioration of beer flavour. Further, temperature induced reactions are implicated in the formation of staling compounds (Bravo et al. 2008) with the heat load applied during wort boiling and clarification impacting on the level of staling compounds in stored beer (Yano et al. 2004). Additionally, the rate of flavour degradation and the formation of staling compounds is dependent on beer pH (Saison et al. 2010). More recently, exposure to vibrations within the supply chain has been suggested as another critical control point in beer flavour instability (Paternoster et al. 2017; Paternoster 2018).

Several staling phenomena have been reported in beer flavour instability. Beer staling coincides with the formation of aldehydes, characterised by low flavour threshold levels which are associated with stale off-flavours (Saison et al. 2009). Isomerised α -acids, responsible for beer bitterness, are unstable (especially the *trans* isomers) and decrease during beer storage (De Cooman et al. 2000; Caballero et al. 2012). The content of polyphenols decreases during beer ageing, due to reversible non-covalent and irreversible covalent binding with proteins resulting in haze formation (Bamforth 2011; Jongberg et al. 2020a, 2020b; Wang and Ye 2021). In terms of flavour stability, several reports have suggested the antioxidative properties of polyphenols in beer (Kaneda et al. 1995; Lermusieau et al. 2001; Piazzon et al. 2010; Zhao et al. 2013), whereas others suggest negligible antioxidative effects or

even pro-oxidative effects (Andersen and Skibsted 1998; Andersen et al. 2000; Aron and Shellhammer 2010).

Fresh beer often contains sulphite, a by-product of yeast during fermentation. This is considered a key endogenous beer antioxidant trapping hydrogen peroxide and binding volatile aldehydes. The antioxidative effect of sulphite can be evaluated by electron spin resonance spectroscopy (ESR), as typically sulphite levels correlate with the lag phase – the time period preceding the initiation of fast radical formation (Uchida et al. 1996; Andersen et al. 2000; Kunz et al. 2012; Andersen et al. 2017). Therefore, in terms of oxidative stability, the levels of sulphite (and ESR lag phase) are considered an indicator of beer oxidative stability.

The formation of many of staling compounds are initiated by reactive oxygen species, which lead to chain reactions responsible for the formation of oxidation products such as carbonyl compounds (Vanderhaegen et al. 2006; Baert et al. 2012; Wietstock et al. 2016) with 1-hydroxyethyl radicals reported as intermediates in the chain reactions (Andersen and Skibsted 1998; Elias et al. 2009). The main pro-oxidants are transition metal ions, especially trace levels of iron and copper ions, which are the catalysts of oxidation via Fenton and Haber-Weiss reactions (Kaneda et al. 1992; Vanderhaegen et al. 2006; Mertens et al. 2022). Overall, the rate of oxidative deterioration of beer is determined by the balance between the pro-oxidants and antioxidants.

Malt plays a pivotal role in beer flavour instability. A low Kolbach index, heat load and colour together with low levels of transition metal ions, α -dicarbonyls and Strecker aldehydes in malt have a positive effect on flavour stability (Gastl et al. 2006; Filipowska et al. 2021). Nobis et al (2021a) observed that the use of malt of high proteolytic modification results in wort high in aldehydes. Further, greater malt modification led to elevated levels of Strecker aldehydes (Nobis et al. 2021b; Lehnhardt et al. 2021). Unlike malt, hops are less important in aldehyde formation during beer storage. De Clippeleer et al (2010, 2014) reported that the concentration of aldehydes formed in aged beer is independent of the mode of bittering, such that the degradation of hop bitter acids is of minor importance.

This study produced a flavour stable beer using current best brewing practice to reduce beer staling. The malt was selected to provide a lower pool of staling precursors (e.g. free amino nitrogen, dicarbonyls or aldehydes). Accordingly, it was anticipated that the beer would demonstrate lower levels of aldehydes during forced ageing. The investigation focusses on the analysis of flavour instability markers to provide a comprehensive insight and understanding of the staling phenomena in unpasteurised top fermented pale beer, enabling sensory staling to be modelled.

Materials and methods

Malt quality parameters

Malt quality parameters were determined using the analytical methods of the European Brewing Convention (2018): moisture content (4.2), homogeneity and partly unmodified grains (4.14), friability (4.15), total nitrogen content (4.17), diastatic power (4.12.1), α -amylase activity (4.13), extract yield (4.5.1), colour (4.7.1), viscosity (4.8), total soluble nitrogen (4.9.2), free amino nitrogen (4.10), pH (8.17), β -glucan (4.16). Determination of the thiobarbituric acid index (TBI) in malt was performed using the adapted method of Coghe et al (2004). Trihydroxy fatty acids in malt were determined according to Jaskula-Goiris et al (2011). Determination of S-methylmethionine, dimethyl sulfide and lipoxygenase activity in malt was performed as described by Dugulin et al (2020).

Wort and beer production

Three identical batches of unpasteurised top-fermented pale beer were produced using the 5 hL scale pilot plant at KU Leuven, Technology Campus Gent. The batches were produced over three consecutive days. The process was performed under oxygen limiting conditions with the installation (including the malt container and milling system) flushed with CO₂ prior to use. Throughout the milling and mashing-in stages a consistent CO₂ supply was maintained, which stopped after mashing-in. Pilsner malt (87 kg) was milled with a wet disc mill – hydromill (Meura, Péruwelz, Belgium) and mixed with 1.91 hL deaerated, reverse osmosis water enriched with 80 mg/L Ca²⁺ ions (as calcium chloride dihydrate, Merck KGaA, Darmstadt,

Germany). At mashing-in, the pH of the mash was adjusted to 5.2 with 30% (v/v) lactic acid (90% (v/v) (S)-Lactic acid, Merck KGaA, Darmstadt, Germany).

The mashing protocol was as follows: 63°C (30 min), 72°C (15 min), 78°C (1 min); temperature rise 1°C/min. Mash filtration was performed via a membrane assisted thin bed filter Meura 2001 (Meura, Péruwelz, Belgium) with a sparging rate of 2.33 L/kg and last runnings at 2.0 ± 0.2°P. The sweet wort extract was adjusted to 13°P prior to boiling for 1 hour. Hop pellets were added: 50 g/hL of Magnum at the onset of boiling (13.0% (w/w) α -acids); 100 g/hL Tettnanger and 120 g/hL Saaz at the end of boiling (respectively 3.0% and 2.5% (w/w) α -acids) targetting 20 mg/L of iso- α -acids in the final beer. Hot trub was removed using a whirlpool and the wort cooled to 20°C and aerated with sterile air filtered via Pall HEPA filter of 0.2 μ m (Pall Corporation, Port Washington, USA). *Saccharomyces cerevisiae* active dry yeast Fermentis S-04 (Fermentis Lesaffre, Lille, France) was pitched at 80 g/hL. Before pitching, the yeast was hydrated with sterile, reverse osmosis water at a ratio of 1:10 (m/v). Static fermentation was performed for 3 days at 20°C in cylindroconical fermenters of 0.85 hL total volume under atmospheric pressure (fermenter dimensions: 65 cm total height, 40 cm width, 25 cm height of the cone; approx. 40% headspace volume). Lagering was performed in 50 L kegs (45 L beer volume) for 14 days at 0°C. The beer was filtered using cellulose sheets - BECOPAD 350 (Eaton, Groot-Bijgaarden, Belgium); carbonated to 5.6 g CO₂/L; and bottled in 250 mL brown glass bottles with oxygen-scavenging crown caps. Bottling was performed using a six head rotating counter pressure filler with double pre-evacuation and high-pressure injection prior to bottle sealing (CIMEC, Nizza Monferrato, Italy). Dissolved oxygen after bottling was below 7 ± 5 μ g/L, measured using Haffmans Inpack TPO/CO₂ Meter c-TPO (Pentair Haffmans, Venlo, the Netherlands).

Forced ageing of beer

Fresh beer was stored at 0°C in the dark. Forced, aged beers were kept in darkness for 15, 30, 60 and 90 days at 30°C in a thermostatically controlled room. On reaching the desired forced ageing, beers were stored at 0°C prior to analysis (up to a maximum of three months).

Beer analysis

The Anton-Paar DMA 5000 Alcolyser density meter and Alcolyzer (Anton Paar, Graz, Austria) were used to determine the following parameters: alcohol, density, apparent extract, real and original extract, apparent and real degree of fermentation. P600 pH meter (Consort, Turnhout, Belgium) was used to measure pH. Haffmans Inpack TPO/CO₂ Meter c-TPO (Pentair Haffmans, Venlo, the Netherlands) was used to determine the total package, dissolved and headspace oxygen levels. Analytical methods (European Brewery Convention 2004) were used for: free amino nitrogen (9.10), total polyphenol content (9.11), flavanoid content (9.12), foam stability (9.42) using NIBEM-T Meter (Pentair Haffmans, Venlo, the Netherlands), colour (9.6) and sensitive proteins (9.40). Cold and permanent haze were evaluated at 0 and 20°C at an angle of 90° and 25° using the Vos Rota 90 turbidity meter (Pentair Haffmans, Venlo, the Netherlands) – for cold haze determination, beers were kept for minimum of 24 hours at 0°C prior to analysis. Determination of proanthocyanidins was according to the method of Bate-Smith (1973). Thiobarbituric acid index (TBI) was analysed as described by Thalacker and Bößendörfer (2005). Total reactive antioxidant potential (TRAP) was determined according to Araki et al (1999). Spectrophotometric analyses were performed using Varian Cary 100 (Agilent Technologies Inc, Mulgrave, Australia).

Oxidative stability of beer

Oxidative stability was assessed by electron spin resonance (ESR) spectroscopy. The measurement of the rate of radical formation and determination of lag times was carried out as described by Uchida et al (1996). Bottled beer was degassed by adding 10 µL of 1-octanol together with vigorous mixing for 5 min. The spin trap was applied as 600 mM of *N-tert-butyl-α-phenylnitrone* (PBN) in ethanol. An aliquot of 1.9 mL of beer was mixed with 100 µL of PBN solution and incubated at 60°C with aliquots transferred to the ESR flow cell at 10 min intervals. The ESR spectra were recorded with a Miniscope MS 200 X-band spectrometer (Magnettech GmbH, Wertheim, Germany). The following instrument settings were applied: microwave power 10 mW; modulation amplitude 0.2 mT; modulation frequency 100 kHz; sweep width 10 mT; sweep

time 60 s. Samples were collected with 50 µL disposable BLAUBRAND® micropipettes (BRAND GmbH, Wertheim, Germany). The spectra were recorded at ambient temperature and relative signal intensities were registered relative to spectra of 2 µM aqueous solutions of 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO) measured as the first and the last sample of the day. Measurements were performed in duplicate.

LC quantification of thiols and sulphite

Quantification of sulphite and free thiols was carried out according to the method of Hoff et al (2013) based on the derivatisation of sulphite and free thiols with ThioGlo 1 fluorescent reagent in water free acetonitrile followed by separation with reversed phase high performance liquid chromatography (RP-HPLC) coupled with fluorescence detector. Calibration curves were prepared with sulphite (in the form of Na₂SO₃) and *N*-acetyl-L-cysteine (NAC) in degassed beer. Linear fitting was used with R²≥0.9900 in both cases. Limit of detection (LOD) for sulphite and total thiols was respectively 0.6 mg/L and 4.9 mg/L. The column used was Jupiter C18, fully porous silica column (LC column 150 x 2.0 mm, 5 µm particle size, 300 Å pore size, Phenomenex), the detector was set to 242 nm excitation and 492 nm emission. Total run time was 16 min with 4 min post-run. Measurements were carried out in triplicate, with the calculation of means and standard deviations.

UPLC quantification of iso-α-acids

Quantification of *cis*- and *trans*-iso-α-acids was performed using Waters Acquity Ultra-High-Performance Liquid Chromatography-Photodiode-Array Detection (UPLC-PDA) (Waters, Milford, USA). Compounds were separated using a Acquity UPLC HSS C18 1.8 µm column (2.1 i.d. × 150 mm; Waters, USA) with chromatographic conditions based on Jaskula-Goiris et al (2011). Milli-Q water adjusted to pH 2.80 (with phosphoric acid, 85% (v/v), Merck KGaA, Darmstadt, Germany) was used as eluent A; and HPLC-grade acetonitrile (Biosolve Chemie, Dieuze, France) was used as eluent B. Experimental conditions were: isocratic elution using 52% (v/v) B and 48% (v/v) A; analysis time: 12 min; flow rate: 0.5 mL/min; temperature: 35°C. UV detection was applied at 270 nm for iso-α-acids. External calibration was used to quantify iso-α-acids based

on the dicyclohexylamine (DCHA) - iso- α -acids ICS-13 complex (66.5% (w/w) iso- α -acids) (Labor Veratis, Zürich, Switzerland). Data reprocessing used Empower 2 software (Waters, Milford, USA). The *trans*- to *cis*- ratios of cohumulones, humulones, adhumulones and total iso- α -acids was determined for each sample and expressed as relative mass ratios between *trans*- to *cis*-components.

HS-SPME-GC-MS quantification of free aldehydes

Free aldehyde quantification was based on *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) derivatisation followed by head-space solid phase microextraction gas chromatography-mass spectroscopy (HS-SPME-GC-MS) according to the method by De Clippeleer (2013) as optimised by Baert (2015). The following marker compounds were quantified: 2-methylpropanal (2MP), 2-methylbutanal (2MB), 3-methylbutanal (3MB), methional (MET), phenylacetaldehyde (PHE), furfural (FUR), hexanal (HEX) and *trans*-2-nonenal (T2N). Experimental conditions are as described by Ditrych et al (2019). The SPME fibre was thermally conditioned according to the supplier recommendations. Derivatisation was achieved by exposing the SPME fibre to PFBHA solution (1 g/L in Milli-Q water) for 10 min. The fibre was exposed to the headspace of the beer sample for 30 min and transferred into the inlet of the gas chromatograph (Thermo Fisher Scientific Inc., Waltham, USA) for thermal desorption of carbonyl compounds in the form of pentafluorobenzoyloximes (PFBOS). The fibre was kept in the inlet for 3 minutes at 250°C with the inlet set to split mode with a flow of 10 mL/min and a split ratio of 12. The GC oven programme was: 2 min at 50°C; an increase of 6°C/min up to 250°C; and 5 min at 250°C. Helium was used as a carrier gas and the flow rate set at 0.8 mL/min. The column used was Rtx-1 Crossbond 100% dimethylpolysiloxane capillary column (40 m length, 0.18 mm i.d., 0.20 μ m film thickness, Restek Corporation, Bellefonte, USA). The transfer line between the gas chromatograph and the mass spectrometer was maintained at 260°C. For component detection, an ISQ Single Quadrupole was used (Thermo Fisher Scientific Inc., Waltham, USA) with a chemical ionisation source type operated at 185°C. Methane was used as a reagent gas with a flow rate of 1.5 mL/min. The

Telectron lens was set to 1.5 V, the electron energy to 70 eV, the emission current to 50 μ A, and the detector gain to 3.00×10^5 . One characteristic ion per PFBOS with a negative charge was used for identification and quantification in the selected ion monitoring mode. Aldehyde reference compounds (Sigma Aldrich Co., St. Louis, USA) in nitrogen flushed Milli-Q water were used for external calibration with linear fitting ($R^2 \geq 0.9800$). Beer samples for analysis were prepared under anaerobic conditions. Degassed beer (4 mL) was transferred to 20 mL amber glass vials, closed with crimp seals with PTFE/silicone septa (Sigma Aldrich Co., St. Louis, USA) and analysed. Data were processed in XCalibur™ (Thermo Electron Corporation, Waltham, USA). Measurements were carried out in triplicate, with calculation of means and standard deviation.

UPLC-MS quantification of cysteinylated aldehydes

Quantification of cysteinylated aldehydes was performed using a Ultra-High-Performance Liquid Chromatography-Mass Spectroscopy (UPLC-MS) (Waters, Milford, USA) (Bustillo Trueba et al. 2019). The following marker compounds were quantified: 2-isopropylthiazolidine-4-carboxylic acid (2MP-CYS), 2-(*sec*-butyl) thiazolidine-4-carboxylic acid (2MB-CYS), 2-isobutylthiazolidine-4-carboxylic acid (3MB-CYS), 2-(2-(methylthio)ethyl) thiazolidine-4-carboxylic acid (MET-CYS), 2-benzylthiazolidine-4-carboxylic acid (PHE-CYS), 2-pentylthiazolidine-4-carboxylic acid (HEX-CYS), 2-(furan-2-yl) thiazolidine-4-carboxylic acid (FUR-CYS). External calibration was based on previously synthesised authentic reference compounds (Bustillo Trueba et al. 2019). Linear calibration was used with $R^2 \geq 0.9900$. The limit of detection (LOD) varied depending on the compound and was in the range 0.2-2.2 μ g/L. Data reprocessing was done using Empower 2 software (Waters, Milford, USA). Measurements were carried out in triplicate, with calculation of means and standard deviation.

Sensory analysis

Sensory evaluation was performed at the Carlsberg Research Laboratory (Carlsberg Group A/S, Copenhagen, Denmark) by a trained panel experienced in evaluating off-flavours in beer. The panellists were male and female with an age range

of 25-65. The panellists participated in regular training sessions where they identified single compound flavour capsules from FlavorActiV™. As part of training, several compounds from the 'stale and aged category' of the beer flavour wheel (Schmelzle 2009) were evaluated. Furthermore, the panellists also have experience in evaluating aged beers at the end of shelf-life and beers that have been aged by forcing (by being exposed to a higher temperature for a defined period), enabling the panellists to recognise different qualities of stale aroma. The panel performance was monitored regularly using beer proficiency kits from FlavorActiV™ as well internal schemes.

The degree of staling was evaluated blind by rating and ranking tests, where the panellists (n=16, n=17) were asked to judge the intensity of overall staleness on a scale from 0 (no detectable staleness) to 100 (strongly aged) for each sample. The forced aged beer for 90 days was used as a blind anchor point on the scale at a value of 85. All samples from the same batch were analysed in two independent sessions – six sessions in total, 8-10 panellists participating in each session. For panellists, who participated in both sessions, mean values of the rated and ranked staleness were calculated and used for data evaluation. Beer samples were served in transparent glasses at about 12°C in aliquots of 60 mL. Water and unsalted crackers were served in between each evaluation to cleanse the palate.

Statistical and multivariate data analysis

Statistical analysis was carried out using SPSS statistical software (IBM Corporation, Armonk, USA). The statistical significance of analytical parameters was calculated using one-way ANOVA with post-hoc Tukey HSD test. Rating of the overall staleness intensity was evaluated by descriptive statistics (mean, SD, median and interquartile range), Shapiro-Wilk normality test and non-parametric Kruskal Wallis ANOVA.

Multivariate data analysis included principal component analysis (PCA) and partial least square (PLS) regression modelling. The PCA was performed using R software (version 3.6.2, The R Foundation for Statistical Computing); data were auto scaled and cross validated. The PLS modelling was performed

using PLS-Toolbox (Eigenvector Research, Inc., Manson, USA) for MATLAB (The MathWorks, Inc., Natick, USA). To establish the PLS regression model, the median values of the determined staleness intensity in the sensory rating test (Y input as the predictor) and the four analytical parameters 2-methylpropanal, furfural, the ratio of *trans*- to *cis*-iso- α -acids and TRAP (X input as descriptors) were selected, each covering the datapoints collected during the period of forced ageing (n=15). The PLS model utilised SIMPLS algorithm with data auto-scaled and Venetian blinds cross-validation procedure used to estimate the performance of the model. Regression coefficient R for the non-cross validated model and the cross validated model R_{CV} were calculated.

Results

Malt quality

Malt with lower levels of free amino nitrogen, Kolbach index, thiobarbituric acid index (TBI) and colour has a positive impact on beer flavour stability, by providing a smaller pool of precursors for the formation of staling compounds (*e.g.* amino acids, α -dicarbonyls) (Gastl et al. 2006; Filipowska et al. 2021). Accordingly, during malt drying, kilning was performed at a maximum of 65°C for an extended period, resulting in lower values of free amino nitrogen and colour. It is noteworthy that the malt used in this work contained sufficient levels of trihydroxy fatty acids and lipoxygenase activity for the formation of lipid oxidation products during brewing and beer storage. These and other quality parameters of the pilsner malt are summarised in [Table 1](#).

Brewing process

Three replicate pilot scale brews of top fermented non-pasteurised beers were produced to investigate the staling phenomena and variations during forced ageing of the beers at 30°C. Analysis of the three beers ([Table 2](#)) confirmed consistency with little batch-to-batch variation. Precautions were taken to limit the production of staling compounds during the brewing process. These included a high mashing-in temperature (63°C) and pH adjustment to 5.2 at mashing-in to limit lipoxygenase activity.

Table 1.

Quality parameters of Pilsner lager malt. The values are presented as means and standard deviations (n=3). dm = dry matter. Reported values are from ¹Briggs et al (2004), ²Kunze (2004), ³Esslinger (2009), ⁴O'Rourke (2002) and ⁵Davies (2006).

Malt quality parameter	Values	Reported values for pale lager malt
Moisture [%]	6.0 ± 0.2	3 - 5 ^{1,2,4}
Extract yield [% dm]	82.6 ± 0.4	77 - 83 ^{1,2,3,5} > 80 ⁴
Colour [EBC]	3.2 ± 0.2	2 - 4.5 ^{1,2,4,5}
TBI [10 g dm]	9.87 ± 0.00	-
Friability [%]	90.6 ± 1.7	> 80 ^{1,4,5} > 85 ³
Homogeneity [%]	98.4 ± 0.5	> 95 ^{4,5}
Partially unmodified grain [%]	1.60 ± 0.52	< 5 ^{4,5}
Whole grain [%]	0.6 ± 0.4	< 3 ³
β-Glucan [mg/L]	148 ± 20	< 250 ^{1,2}
Viscosity [mPas]	1.56 ± 0.02	< 1.6 ^{1,2,4}
Total protein [% dm]	10.9 ± 0.3	9.5 - 11.0 ^{3,4} < 10 ^{2,5}
Total soluble protein [% dm]	4.00 ± 0.16	3.8 - 4.8 ^{2,3} 3.7 - 4.4 ⁵
Kolbach index [%]	36.8 ± 1.4	35 - 41 ² 38 - 44 ² 37 - 45 ^{1,4}
Free amino nitrogen [mg/L]	132 ± 7	160 - 250 ^{1,2,3,4,5}
Diastatic power [°WK]	320 ± 25	> 220 ^{2,3,4,5}
α-Amylase activity [DU]	61.0 ± 5.5	> 30 ^{1,4} > 40 ^{3,5}
pH	6.07 ± 0.05	5.7 - 6.3 ¹
S-methylmethionine [mg/kg dm]	6.00 ± 0.80	3 - 8 ²
Dimethyl sulphide [mg/kg dm]	2.46 ± 0.01	-
Trihydroxy fatty acids [mg/kg dm]	32.90 ± 0.40	-
Lipoxygenase activity [U/g dm]	76.80 ± 4.20	-

Oxidation was limited by brewing under conditions that limited oxygen ingress. Further, wort separation was achieved using a membrane assisted thin bed mash filtration to achieve good separation of staling precursors and limited contact time with heat. Wort boiling provided a low temperature difference and high exchange surface area with the heat supplied by an inline heat exchanger, conventional heating jacket as well as direct steam injection of pure steam. Clarification was performed by whirlpooling, to achieve sufficient separation of staling precursors

(e.g. transition metal ions, lipids or aldehydes bound to insoluble particles) and was followed by immediate wort cooling and aeration with sterile air. Fermentation was performed using a top fermenting yeast strain. After fermentation, the beer was subject to cold lagering at 0°C for 14 days to precipitate chill haze particles, which were removed by cold filtration. For beer packaging, measures were taken to achieve the lowest concentration of oxygen including a double purge of the beer bottles with CO₂, high pressure injection prior to bottle

Table 2.

Analysis of the pilot scale beers. Data represent the mean value of three batches and standard deviation (n=3).

Alcohol (% v/v)	5.29 ± 0.06
Density (g/mL)	1.01 ± 0.00
Apparent extract (°P)	2.63 ± 0.13
Real extract (°P)	4.54 ± 0.12
Original extract (°P)	12.54 ± 0.08
App. Degree of fermentation (%)	79.01 ± 1.00
Real degree of fermentation (%)	65.33 ± 0.79

sealing to limit headspace oxygen and use of crown corks with oxygen scavenging liners. Any ingress of light was reduced by using brown bottles coupled with storage in the dark. Pasteurisation was not used to exclude the possibility of heat induced staling mechanisms.

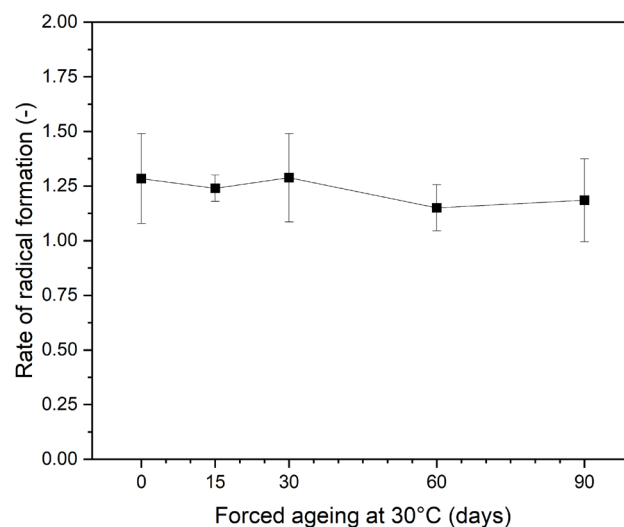
The top fermented beers in this work contained marginal levels of sulphite and thiols with analysis suggesting concentrations below their respective detection limits of 0.6 (sulphite) and 4.9 (thiols) mg/L. Low levels of sulphite can be associated with the use of a top fermenting yeast and a relatively high fermentation temperature of 20°C (Hysert and Morrison 1975; Kaneda et al. 1991). Sulphite is known for its protective mechanism against oxidation especially in lager beers (Kaneda et al. 1996; Uchida et al. 1996; Andersen et al. 2000; Elias et al. 2009; Kunz et al. 2012; Andersen et al. 2017). The poor antioxidative protection through the lack of sulphite was supported by ESR measurements. The fresh (and aged) beers displayed very short lag phases (Supplementary Figure S1), close to the detection limit, before a steady formation of radicals suggesting the beers should easily undergo oxidative changes.

Changes during storage

The beers were forced aged by storage for 90 days at 30°C in the dark in bottles with oxygen scavenging caps. After a negligible lag phase, the rate of radical formation was similar for all the beers, showing that the storage period had no significant effect on the potential to undergo oxidation in the presence of oxygen (Figure 1). In the absence of sulphite, the use of oxygen scavenging crowns would minimise the extent of oxidation in the beers that otherwise

Figure 1.

Rate of radical formation determined by ESR as a function of storage time at 30°C. Data represent mean values and standard deviation (n=3).



would occur through the constant but low rate of ingress of oxygen into the bottles through the cap liners.

Sensory analysis using a rating test showed that a general increase in overall staleness was observed with increasing storage (Figure 2). In total, during 90 days at 30°C of forced ageing, staling almost doubled. The results of the ranking and rating tests agreed (Supplementary Table S1), showing that forcing for 60 and 90 days resulted in beers that were significantly more stale than fresh and forced beers for 15 and 30 days.

Several chemical and physical beer instability markers were analysed to describe the changes during storage. In agreement with previous work (Malfliet et al. 2008; Jaskula-Goiris et al. 2011), soluble proteins and pH did not show statistically significant changes during beer ageing (Table 3). Beer colour increased, whereas thiobarbituric acid index (TBI), total polyphenols, flavanoids, proanthocyanidins and TRAP decreased, which agrees with previous reports (Malfliet et al. 2008, 2009; Jaskula-Goiris et al. 2011).

The levels of cold haze measured at 90° more than doubled during storage, whereas cold haze at 25° fluctuated (Table 3). Permanent haze at 25 and 90°, decreased during storage presumably due to binding and sedimentation of the small haze particles. This reflects the decrease in total polyphenols, flavanoids

Figure 2.

Box and whisker plots for sensory assessment of staleness intensity of the beers (n=3) at different forced ageing times: fresh, 15, 30, 60 and 90 days at 30°C. The top of the box represents the 75th percentile, the bottom of the 25th percentile, the horizontal line represents the median value and '•' denote means. The whiskers represent the highest and the lowest values that are not outliers or extreme values. Groups (A and B) were statistically evaluated by nonparametric Kruskal-Wallis test ($p < 0.05$).

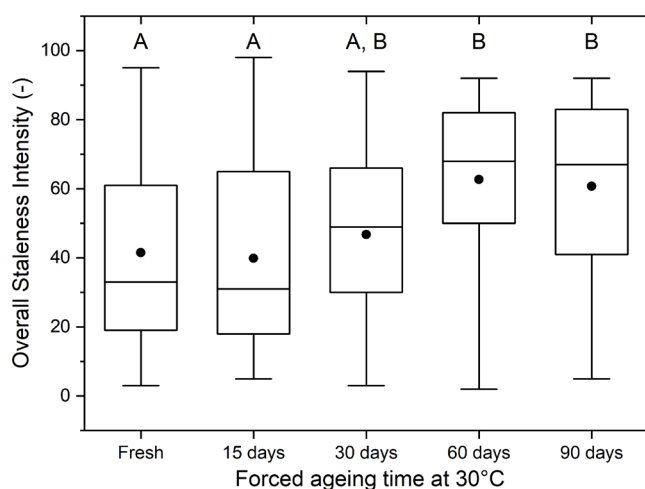


Table 3.

Analysis of fresh and stored beers at 30°C. Data represent mean values and standard deviation (n=3). Statistical significance was evaluated by ANOVA Tukey HSD post-hoc test. The statistical differences ($p < 0.05$) within a given dataset are marked with an asterisk and statistical groups represented by a capital letter in the superscript.

Forced Ageing Period at 30°C	Fresh	15 days	30 days	60 days	90 days
Colour (EBC) *	6.0 ± 0.1 ^A	6.5 ± 0.2 ^B	6.3 ± 0.2 ^{A, B}	6.4 ± 0.2 ^{A, B}	6.7 ± 0.2 ^B
TBI (100 mL of beer) *	33.5 ± 0.5 ^B	33.0 ± 0.6 ^{A, B}	32.9 ± 0.7 ^{A, B}	32.7 ± 0.4 ^{A, B}	32.0 ± 0.2 ^A
TRAP (mM citric acid eq.) *	1.60 ± 0.00 ^B	1.59 ± 0.01 ^B	1.53 ± 0.03 ^A	1.51 ± 0.03 ^A	1.49 ± 0.00 ^A
Total polyphenols (mg/L) *	263.8 ± 4.3 ^B	249.0 ± 8.7 ^B	231.7 ± 9.1 ^A	230.1 ± 2.3 ^A	230.3 ± 2.6 ^A
Flavonoids (mg/L catechin eq.) *	50.6 ± 4.9 ^B	50.2 ± 0.9 ^{A, B}	47.0 ± 1.4 ^A	46.4 ± 1.7 ^A	45.6 ± 1.2 ^A
Proanthocyanidins (mg/L)	50.0 ± 3.4 ^A	47.6 ± 2.6 ^A	40.5 ± 2.7 ^A	42.0 ± 3.3 ^A	41.6 ± 1.9 ^A
Sensitive protein (EBC) *	4.1 ± 0.7 ^{A, B}	3.7 ± 0.7 ^A	3.7 ± 0.3 ^A	5.1 ± 0.2 ^B	4.8 ± 0.3 ^{A, B}
Permanent haze 90° (EBC) *	2.1 ± 0.5 ^B	1.5 ± 0.2 ^{A, B}	1.4 ± 0.2 ^{A, B}	1.3 ± 0.2 ^A	1.1 ± 0.2 ^A
Cold haze 90° (EBC) *	4.2 ± 0.8 ^A	5.2 ± 1.8 ^{A, B}	4.0 ± 0.9 ^A	7.3 ± 0.2 ^{B, C}	9.7 ± 0.4 ^C
Permanent haze 25° (EBC) *	0.7 ± 0.1 ^{B, C}	0.8 ± 0.1 ^C	0.6 ± 0.2 ^{B, C}	0.4 ± 0.1 ^{A, B}	0.2 ± 0.0 ^A
Cold haze 25° (EBC)	5.9 ± 1.3 ^A	8.3 ± 2.3 ^A	5.6 ± 0.4 ^A	7.7 ± 1.4 ^A	6.5 ± 0.8 ^A
Soluble proteins (mg/L)	336.1 ± 27.8 ^A	319.8 ± 19.2 ^A	364.5 ± 46.0 ^A	381.6 ± 27.5 ^A	356.0 ± 27.1 ^A
pH	4.25 ± 0.07 ^A	4.22 ± 0.06 ^A	4.22 ± 0.07 ^A	4.21 ± 0.07 ^A	4.23 ± 0.07 ^A

and proanthocyanidins. The monomer polyphenols are known to oxidise and polymerise, resulting in structures that can bind with haze active proteins forming large haze active polymers (Wang and Ye 2021).

Iso-α-acids

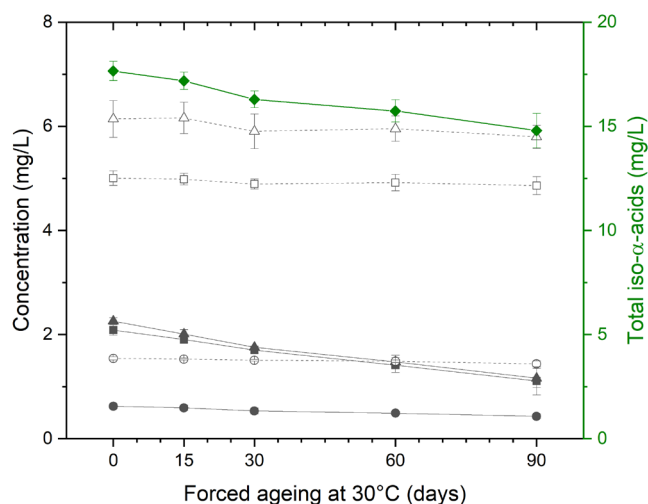
The fresh beer had moderate bitterness with 17.7 ± 0.5 mg/L total iso-α-acids with iso-cohumulones, iso-humulones and iso-adhumulones representing 40, 48 and 12%, respectively. After 90 days of forced ageing, the six isomerised fractions decreased in total by 16% ($p < 0.01$) (Figure 3). The *trans*-isomers were less stable, decreasing by 47, 49 and 31% (respectively) for *trans*-iso-cohumulone, *trans*-iso-humulone, and *trans*-iso-adhumulone; the corresponding values for the *cis*-isomers were 3, 6 and 7%. A marked decrease in *trans*- and *cis*-isomer ratio was observed over time, which was almost linear for iso-cohumulones and iso-humulones (Figure 4).

Volatile and cysteinylated aldehydes

During beer storage, staling aldehydes are anticipated to be formed by several mechanisms including *de novo* formation via lipid oxidation, Maillard reactions, Strecker degradation and direct oxidation of amino acids (Baert et al. 2012; Wietstock et al. 2016). Aldehydes may also be released from non-volatile adducts or 'bound-state aldehydes' (Kaneda et al. 1994; Bustillo Trueba et al. 2021). In this study, volatile aldehydes were

Figure 3.

Concentration of iso- α -acids as a function of storage time at 30°C: *trans*-iso-cohumulone (■); *cis*-iso-cohumulone (□); *trans*-iso-humulone (▲); *cis*-iso-humulone (△); *trans*-iso-adhumulone (●); *cis*-iso-adhumulone (○); total iso- α -acids (◆). Data represent mean values and standard deviation (n=3).

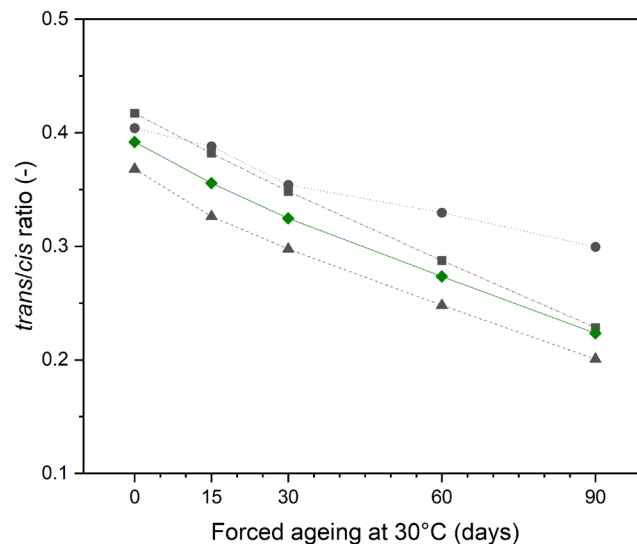


investigated using HS-SPME-GC-MS. Compounds included the Strecker aldehydes 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional and phenylacetaldehyde; hexanal and *trans*-2-nonenal as examples of lipid oxidation; and furfural as a Maillard reaction product. The HS-SPME-GC-MS technique, coupled with on-fibre derivatisation of carbonyl compounds, ensured the precise measurement of volatile aldehydes, which are found in small quantities particularly in fresh beer. The bound-state aldehydes were investigated as their corresponding cysteinylated forms in solution.

Over 90 days of forced ageing at 30°C, the levels of most of the volatile aldehydes increased, although the levels were still lower than the reported sensory threshold values (Saison et al. 2009). The trends for volatile staling aldehydes during forced ageing (Figure 5) are in agreement with previous work with the same brewing protocol and pilot plant, but different yeast strains and malt (Jaskula-Goiris et al. 2011; De Rouck et al. 2013; Bustillo Trueba et al. 2021). Although the maximum concentration for some of the aldehydes (2-methylpropanal, 2-methylbutanal and 3-methylbutanal) are significantly lower than in the previous reports. Of

Figure 4.

Ratio of *trans*-/*cis* iso- α -acids as a function of storage time at 30°C: iso-cohumulones (■); iso-humulones (▲); iso-adhumulones (●); total iso- α -acids (◆). Data represent mean values.



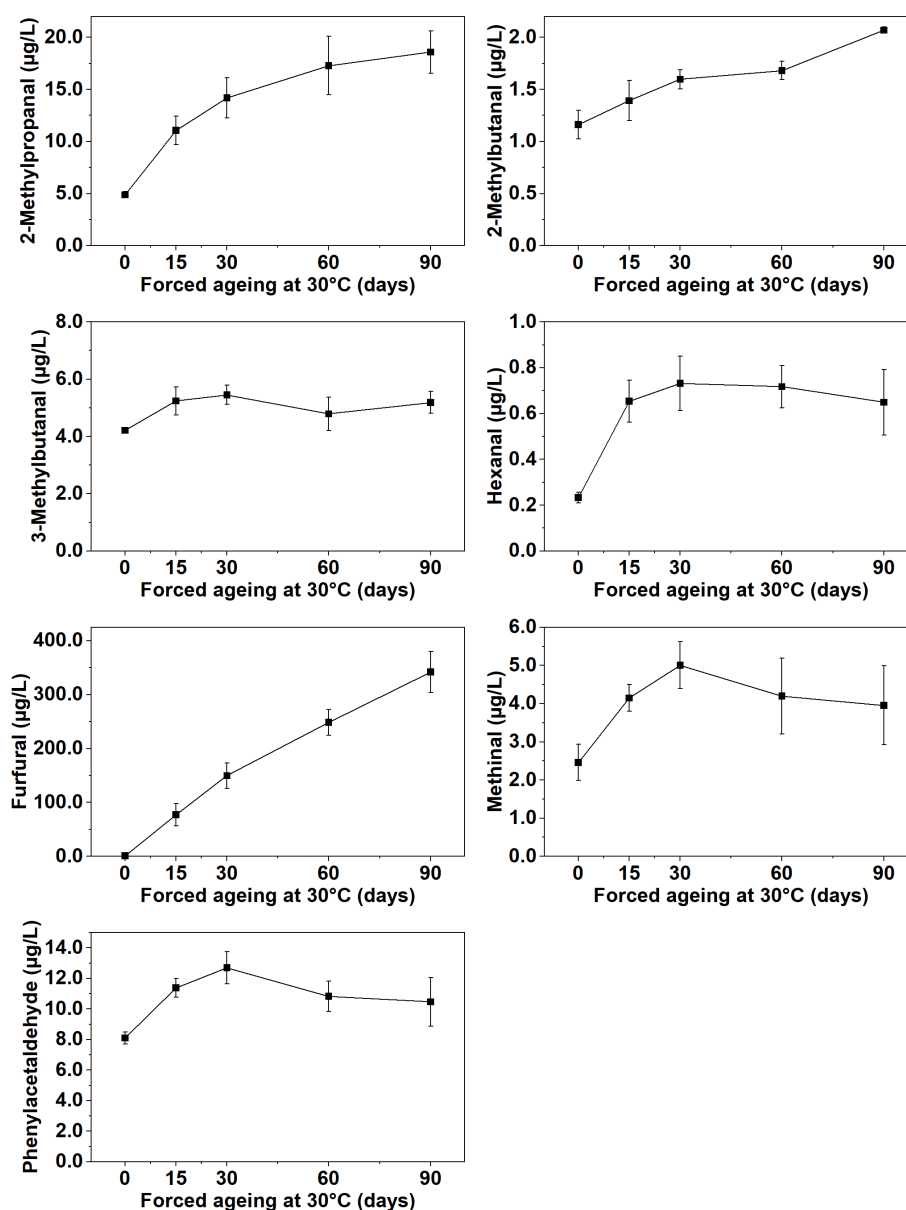
the Strecker aldehydes, there was a marked increase in 2-methylpropanal and 2-methylbutanal during the period of forced ageing. Whereas levels of methional and phenylacetaldehyde increased during the first 30 days of forced ageing at 30°C and then levelled off. A plateau in aldehyde levels during beer staling has been reported previously (De Clippeleer 2013; De Rouck et al. 2013; Bustillo Trueba et al. 2021; Wietstock et al. 2016). Changes in 3-methylbutanal during forced ageing were statistically not significant. However, the concentration of furfural increased linearly during forced ageing reaching ca. 350 $\mu\text{g/L}$ after 90 days. With lipid oxidation aldehydes, only hexanal was quantified with *trans*-2-nonenal below the limit of detection (0.02 $\mu\text{g/L}$) throughout storage.

All seven cysteinylated aldehydes in both fresh and aged beers were below the detection limit (0.2 - 2.2 $\mu\text{g/L}$). The absence of cysteinylated aldehydes supports the recent findings of Bustillo Trueba et al (2021) and indicates that cysteinylated aldehydes *per se* are not implicated in the release of staling aldehydes in aged beer, nor are they good markers of flavour instability.

Figure 5.

Concentration of volatile aldehydes in the beers as a function of storage at 30°C.

Data represent mean values and standard deviation (n=3).



Modelling staleness

Multivariate principal component analysis (PCA) was used to identify the most relevant differences among aged beers and the analytical parameters (Figure 6). Variables displaying little variation during accelerated beer staling (ESR based oxidative stability parameters) or quantified below the limit of detection (sulphite, volatile *trans*-2-nonenal and cysteinylated aldehydes) were excluded from the calculation of the model. Analysis of Residuals vs. Hotelling T^2 plot did not identify any outlier samples.

A biplot of the PCA was constructed of the data comprising of 15 objects representing beers from all staling categories and 33 loadings representing the analysed parameters. The total explained variance of the first two components PC1 and PC2 corresponded to almost 70% (Figure 6). The biplot shows that the objects were clearly grouped in clusters according to the period of forced ageing along the PC1, which explained 58% of total data variation. Loadings such as TRAP, ratio of *trans*-/*cis*- α -acids, 2-methylpropanal, furfural, sum of aldehydes and the sensory rated overall staleness

Figure 6.

Principal component analysis (PCA) bi-plot of data for fresh and stored beers at 30°C. Labels indicate forced ageing in days (0, 15, 30, 60, 90) and the brewing trial (A, B, C). The parameters include: proanthocyanidins (♦ PRO), total polyphenol content (♦ TPC), flavanoids (♦ FLA), total iso- α -acids (♦ AA), *trans*-isohumulone (♦ *tih*), *trans*-isoadhumulone (♦ *tiah*), *trans*-isocohumulone (♦ *tich*), *cis*-isohumulone (♦ *cih*), *cis*-isoadhumulone (♦ *ciah*), *cis*-isocohumulone (♦ *cich*), *trans/cis* ratio of total iso- α -acids (♦ *t/c IAA*), *trans/cis* ratio of isoadhumulone (♦ *t/c iah*), *trans/cis* ratio of isocohumulone (♦ *t/c ich*), *trans/cis* ratio of isohumulone (♦ *t/c ih*), cold haze at 25° (♦ CH25), cold haze at 90° (♦ CH90), permanent haze at 25° (♦ PH25), permanent haze at 90° (♦ PH90), total reactive antioxidant potential (♦ TRAP), thiobarbituric acid index (♦ TBI), soluble proteins (♦ SoP), sensitive proteins (♦ SeP), colour (♦ COL), pH (♦ pH), 2-methylpropanal (♦ 2MP), 2-methylbutanal (♦ 2MB), 3-methylbutanal (♦ 3MB), hexanal (♦ HEX), furfural (♦ FUR), methional (♦ MET), phenylacetaldehyde (♦ PHE), sum of volatile aldehydes (♦ sALD), and sensory rated overall staleness intensity (♦ OSI).

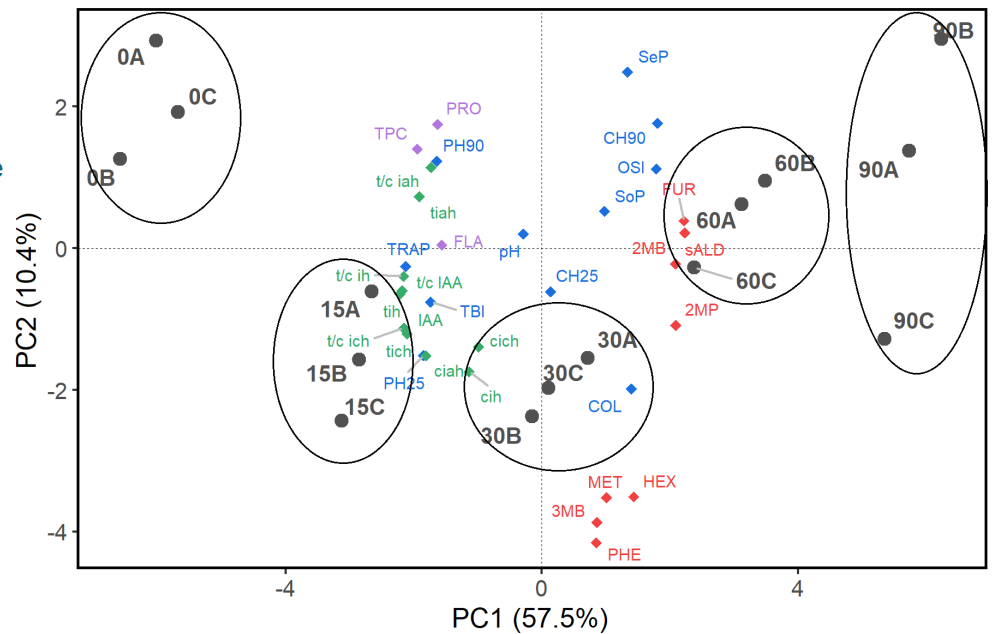
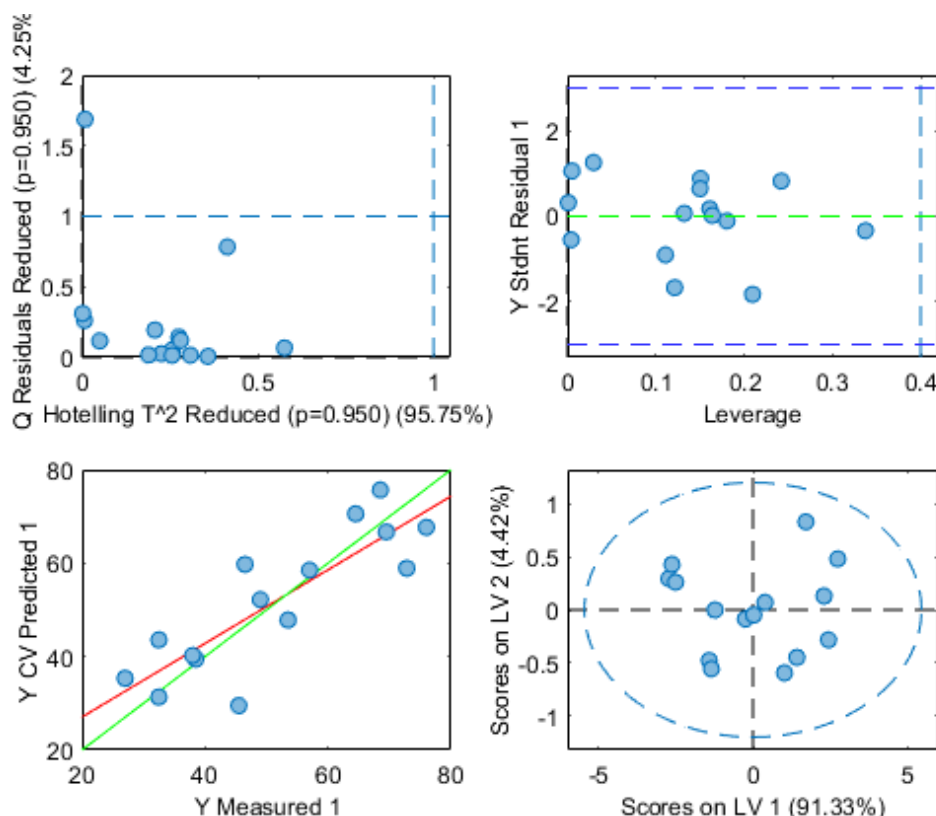


Figure 7.

Partial least square (PLS) regression modelling the intensity of sensory staleness of beer. Based on the X input (descriptors) and data points for TRAP, 2-methylpropanal, furfural, and the ratio between *trans*-/*cis*-iso- α -acids ($n=15$ each); and the Y input (predictor) as the median values of the sensory rated staleness intensity ($n=15$). Green line represents the PLS model: $R=0.83$; red line represents the cross-validated model: $R_{cv}=0.72$.



intensity were especially dependent on PC1. Based on the outcomes of the PCA (Figure 6) and the analysis of Pearson's correlation matrix (Supplementary Figure S2), the parameters which displayed the strongest correlation with sensory determined staleness (furfural 0.90, 2-methylpropanal 0.80, ratio between *trans*- and *cis*-iso- α -acids -0.87, and TRAP -0.88) were selected for PLS analysis modelling the overall staleness intensity (Figure 7). The regression coefficients of the calculated PLS model were $R=0.83$ and on cross validation $R_{cv}=0.72$. These point to high model accuracy. This shows that the selected four markers are linked to flavour changes and can be used for modelling of perceived staleness. Other models based on five and more parameters resulted in outputs of lower accuracy.

Discussion

Good repeatability was achieved from three parallel pilot scale brewing trials with little batch-to-batch variation in the analysis of the beers (fresh and aged) (Tables 2,3). A high degree of repeatability ensures consistent and reliable results, leading to predictable and consistent processes which are necessary for research on beer flavour instability.

Multivariate data analysis together with PCA and PLS modelling provides an understanding of complex relationships and can be used in the investigation of beer flavour instability. In this study, a PLS model was established (Figure 7), modelling the intensity of sensory staleness based on the levels of 2-methylpropanal, furfural, TRAP and the ratio between *trans*- and *cis*-iso- α -acids. The high degree of repeatability in fresh and forced aged beers enhanced the accuracy and reliability of the PLS model which, in turn, identified these four markers as relevant to beer staling. Although using a top fermented pale beer, the model provides confirmation of the previous reports identifying furfural, 2-methylpropanal and the ratio between *trans*- and *cis*-iso- α -acids as markers for the modelling of flavour stability in pale lager beers (Malfliet et al. 2008, 2009; Jaskula-Goiris et al. 2011; De Clippeleer 2013).

There is one Strecker aldehyde, 2-methylpropanal, in the four ageing parameters. The mechanism of the formation Strecker aldehydes during beer

staling remains debatable. Strecker degradation describes the reaction of amino acids (such as valine for 2-methylpropanal) with α -dicarbonyls, α -unsaturated carbonyls and Amadori compounds (Vanderhaegen et al. 2006; Rizzi 2008; Baert et al. 2012). These reactions are non-oxidative and can be classified as 'Maillard reactions', as various α -dicarbonyls can result (Baert et al. 2012). On the other hand, Hofmann and Schieberle (2000) proposed the formation of Strecker aldehydes by direct oxidation of Amadori compounds. Further, in studies on the ageing of model solutions, the formation of Strecker aldehydes was proposed via oxygen reactive species mediated direct degradation of amino acids (Wietstock and Methner 2013; Wietstock et al. 2016).

The dissimilar trends in Strecker aldehydes (Figure 5) suggests a disproportionate contribution of the different pathways in their formation. The short chain aldehydes including 2-methylpropanal, 2-methylbutanal and 3-methylbutanal, are likely to be formed via temperature induced mechanisms, since the content of the oxygen in the packaged beers is extremely low. In addition, with the relatively low degree of attenuation (Table 2), the beers contained sufficient reducing sugars, which could contribute to the formation of Strecker aldehydes via reactions of the amino acids with α -dicarbonyls, α -unsaturated carbonyls and Amadori compounds (Vanderhaegen et al. 2006; Rizzi 2008; Baert et al. 2012).

Furfural exhibited the highest relative change of all the parameters during the forced ageing (Supplementary Table S2). Moreover, the levels of furfural showed the highest correlation with the (sensory evaluated) staleness, even though furfural levels in aged beers were lower than the reported sensory threshold. The contribution of aldehydes to taste, mouthfeel and aroma on addition to fresh beer has been demonstrated, resulting in an increase in harsh, lingering taste, astringency and undesirable odours and aromas (De Clippeleer et al. 2011). Clearly, the results presented here support this, confirming furfural as a good indicator of beer staling (Foster et al. 2001; Malfliet et al. 2008; Jaskula-Goiris et al. 2011; De Clippeleer 2013).

Furfural and 5-hydroxymethylfurfural are the end products of Maillard reactions and are considered

indicators of heat load. During beer storage, furfural is formed via non-oxidative, thermal and pH induced mechanisms (Martins and Van Boekel 2005; De Clippeleer et al. 2010; Baert et al. 2012; Čejka et al. 2013; De Clippeleer 2013). Although Maillard reactions are known to initiate at temperatures above 30°C, the elevated levels of furfural during forced ageing of beer at 30°C have previously been reported (Malfliet et al. 2008; Jaskula-Goiris et al. 2011; Čejka et al. 2013; Bustillo Trueba et al. 2021). Bravo et al (2008) reported an increase in α -dicarbonyls (Maillard intermediates) during beer storage, which can lead to the formation of Maillard products or Strecker aldehydes via Strecker degradation of α -dicarbonyls. The results presented here together with the debatable origin of Strecker aldehydes in aged beer, suggest that further research on the fate of Maillard intermediates during beer ageing would be of value in understanding beer flavour instability.

The bitter iso- α -acids also displayed changes during beer storage (Supplementary Table S2). The data on the degradation of iso- α -acids (Figure 3 and 4) demonstrated that the *trans* isomers were less stable than the corresponding *cis* isomers, which is in accordance with previous reports (De Cooman et al. 2000; Jaskula et al. 2007; Intelmann et al. 2009; Intelmann and Hofmann 2010; De Clippeleer et al. 2010; Brewer 2011; Jaskula-Goiris et al. 2011; Caballero et al. 2012). According to De Cooman et al (2000) the instability of *trans*-iso- α -acids is associated with the *cis*-configuration of the isohexenoyl and the prenyl side chains at C(4) and C(5) creating a region of high electron density, which could initiate auto-oxidation, either via direct reaction involving the double bonds, or via allylic hydrogen abstraction from the doubly activated α -carbon of the isohexenoyl side chain at C(4). However, a recent study employing one/two dimensional NMR and liquid chromatography-mass spectrometry did not confirm the *trans*-specificity of the auto-oxidation mechanisms, since the identified hydroperoxy- and hydroxyl-*allo*-iso- α -acids were the primary auto-oxidation products of both *cis*- and *trans*-iso- α -acids in beer (Intelmann and Hofmann 2010). The *trans*-specific degradation mechanism of the *trans*-iso- α -acids into tricyclohumol, tricyclohumene, isotricyclohumene, tetracyclohumol, and epitetracyclohumol has been investigated by labelling with ^{18}O stable isotope, followed by

computer simulation (Intelmann et al. 2009). The proposed chemical transformation was suggested to be induced by proton catalysed carbon/carbon bond formation between carbonyl atom C(1') of the isohexenoyl moiety and the alkene carbon C(2'') of the isoprenyl moiety of the *trans*-iso- α -acid.

The final parameter with importance in modelling beer staling was total reactive antioxidant potential (TRAP). The assay showed a decrease of 7% in total antioxidative potential over forced ageing and a parameter in the PLS modelling of staleness (Table 3). The TRAP assay of Araki et al (1999) indirectly determines the decline of peroxy radicals generated by thermal degradation (at 45°C) of 2,2'-azobis(2-amidinopropane) dihydrochloride, which interact with antioxidants in beer. This spectrometric measurement is based on bleaching of the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) – a stable, artificial ionic radical. The nature of the ABTS radical cation is selective and is not sensitive towards sulphite (Zhao et al. 2013; Araki et al. 1999), which is a limitation of the TRAP assay in the measurement of the antioxidative properties of beer. Nonetheless, the ABTS cation has been reported to be reactive towards polyphenols (especially catechin, epicatechin, ferulic and gallic acids), ascorbic acid and thiols (Araki et al. 1999; Walker and Everette 2009). The results reported here confirm this as there is a strong correlation ($R=0.85$) between TRAP and total polyphenols (Supplementary Figure S2). Therefore, changes in TRAP values would be expected to be linked to haze formation, where soluble polyphenols may be lost due to precipitation of adducts with proteins.

Conclusions

In this study, every effort was made to produce a flavour stable beer by employing malt with low staling potential and utilising current best brewing practices. Forced ageing of the unpasteurised, top fermented beer, demonstrated low levels of volatile aldehydes, particularly the saturated Strecker aldehydes: 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal. However, although analysis showed the volatile aldehydes remained below their sensory thresholds, the sensory staleness of the beer doubled after 60 days at 30°C of forced ageing, suggesting the synergistic effect of carbonyl compounds in the flavour perception of aged beer.

Comparison of the analytical data of fresh and aged beers from the three replicate brews, shows there was little batch-to-batch variation. This supports consistent and reliable results, such that studies on beer flavour instability can be replicated leading to predictable and consistent outcomes. Further, this allowed an accurate PLS model to be established predicting the intensity of sensory staleness based on four parameters: (i) the levels of 2-methylpropanal, (ii) furfural, (iii) TRAP (total reactive antioxidant potential), and (iv) the ratio between *trans*- and *cis*-iso- α -acids. These parameters are important, serving as markers and providing insight into the mechanisms underlying the pathways to beer flavour instability.

While the changes of TRAP during storage are most likely due to oxidative reactions, the decline in *trans*-iso- α -acids is probably due to a non-oxidative acid catalysed reaction, which changes the *trans*-/*cis*-ratio. The formation of 2-methylpropanal can occur via non-oxidative and oxidative pathways. The beers in this study were shown to lack sulphite and therefore did not have protection against radical formation despite the presence of polyphenols as demonstrated by ESR. With furfural, the linear increase is most likely a consequence of non-oxidative pathways of Maillard intermediates produced during malting and brewing. The chemical changes behind the staling phenomena involve a combination of non-oxidative and oxidative pathways, which are reflected in the changes of 2-methylpropanal, furfural, TRAP and the ratio between *trans*-/*cis*-iso- α -acids during storage. This may explain why staleness can be correlated to a combination of these four parameters.

Author contributions

Maciej Ditrych: conceptualisation, formal and multivariate data analysis, investigation, visualisation, writing (original draft).

Weronika Filipowska: malt analysis, writing (review and editing).

Agata Soszka: formal analysis, writing (review and editing).

Jasper Buyse: brewing and sample preparation.

Saskia Hofmann: sensory analysis, writing (review and editing).

Sidsel Jensen: sensory analysis, writing (review and editing).

Gert De Rouck: project administration, funding acquisition, writing (review and editing).

Guido Aerts: supervision, writing (review and editing).

Mogens Larsen Andersen: conceptualisation, ESR methodology, supervision, writing (review and editing).

Luc De Cooman: conceptualisation, supervision.

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Conflict of interest

The authors declare there is no conflict of interest.

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