Food Chemistry Advances 1 (2022) 100151

Contents lists available at ScienceDirect

Food Chemistry Advances

journal homepage: www.elsevier.com/locate/focha

Evolution of sensory analysis attributes and volatile aging markers in bottle fermented craft beers during storage at different temperatures.

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ARTICLE INFO

Keywords:
Beer aging
Volatiles aging markers
Craft beer
Oxygen absorbing caps

ABSTRACT

Beer oxidation is strictly linked to its shelf life. Chemical variation in aldehydes, higher alcohols, hops bitter substances and esters, also could have a role key in stale flavor. Bottle refermentation, is the method by which beer in the bottle is made sparkling and is often used in craft brewing. Before the process an amount of oxygen in the headspace can be a potential source of oxidation. We investigated the effect of oxygen and temperature during storage of refermented craft beer in bottles with standard and oxygen scavenger caps. Beer was stored for 13 weeks at 6, 22 and 45 °C for forced aging, and monitored by technological analysis, SPME (-) and - MS (-), Electron Paramagnetic Resonance (EPR). At 45 °C, the samples showed a decrease of International Bitterness Units and an increase in color and concentrations of oxidized molecules, and other related with the high temperature (aldehydes and furanic compounds respectively). No differences were observed between samples stored at 6 and 22 °C. Sensory analysis showed differences in the perception of paint, sweet, cardboard and freshness attributes in the samples stored at 45 °C. No differences were observed in the use of standard and oxygen scavenger caps.

1. Introduction

Beer is a dynamic food matrices subjected to continuous chemical reactions during storage. Some of these cause a change in the perception of the product expectation by the consumer (Vanderhaegen, Neven, Verachtert & Derdelinckx, 2006). The impact of the molecules derived from these reactions varies according to the brewing style and storage conditions (Baert, De Clippeleer, Hughes, De Cooman & Aerts, 2012; Guido et al., 2007; Lehnhardt, Gastl & Becker, 2018; Saison, De Schutter, Uyttenhove, Delvaux & Delvaux, 2009; Vanderhaegen et al., 2006). Changes include sensorial area, flavor deterioration, color increase and decrease of bitterness perception (Vanderhaegen et al., 2006).

During storage, flavor deterioration includes both degradation and formation reactions (Lehnhardt et al., 2018; Vanderhaegen et al., 2006). The degradation of volatiles compounds results in a loss of perceived beer flavor, when they reach a value below their odor threshold. Besides, the formation of volatiles substances can give rise to new compounds with an unpleasant flavor or to the perception of other molecules (Jaskula-Goiris et al., 2019).

Flavor chemical variation includes: aldehydes, higher alcohols, hops bitter substances and esters (Vanderhaegen et al., 2006). Aldehydes, produced through the Maillard reactions, Strecker reaction, enzymatic and non-enzymatic oxidation of lipid and oxidation of higher alcohols, can be found over their odor threshold in aged beer (Vanderhaegen et al., 2006). Maillard reactions include complex reactions between reducing sugars, proteins, peptides, amino acids and amines, usually associated with heat stress and color enhancement (Baert et al., 2012). Typical Maillard aging compounds are furfural, 2-acetylfuran and furfuryl ethyl ether. Strecker degradation is the transamination between an amino acid and an α-dicarbonyl compound, but only few Strecker aldehydes can affect the beer flavor, for instance phenylacetaldehyde and benzaldehyde. Various reaction mechanisms have been proposed for the synthesis of Strecker aldehydes. Since many involve the presence of oxygen (Chu & Yaylayan, 2008; Hofmann, Münch & Schieberle, 2000), these aldehydes are considered volatile markers associated with oxidative phenomena in beer (D D. Saison et al., 2010).

The oxidation of linoleic acid, the most abundant fatty acid in wort, is the source of *trans*-2-nonenal (t2N) responsible for cardboard flavor of aged beer (Baert et al., 2012; Kuchel, Brody & Wicker, 2006; Vanderhaegen et al., 2006). During mashing, this fatty acid is oxidized to hydroperoxy fatty acid (9-LOOH) through an enzymatic route cat-

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https://doi.org/10.1016/j.focha.2022.100151
Received 2 August 2022; Received in revised form 18 November 2022; Accepted 22 November 2022

alyzed mainly by lipoxygenase 1 (LOX-1), and subsequently broken down to t2N. Another possible way to obtain 9-LOOH is the oxidation of linoleic acid started by perhydroxyl radicals (HOO·) (Baert et al., 2012; Kuchel et al., 2006; Vanderhaegen et al., 2006).

Oxidation of higher alcohols to the corresponding staling aldehydes has been proposed by Hashimoto (1972). The reaction mechanism requires light and melanoidins as catalysts, and can be inhibited by some polyphenols. 1-hydroxyethyl radical is the most abundant radical found in beer, it derives from the interaction between ethanol and hydroxyl radical, producing acetaldehyde as the main degradation product. A similar reaction route has also been proposed for the other alcohols as a viable alternative to obtain the longer chain aldehydes that impact aged beer flavor (Andersen & Skibsted, 1998; Vanderhaegen et al., 2006).

The degradation of hops bitter acids causes a decrease of bitterness and the formation of stale molecules or their precursors. Iso-α and β-acids produce carbonyls compounds with various chains lengths as main degradation products (C6 to C7 2,4-alkedienals, C4 to C7 2-alkenals), the reaction take place in presence of ROS (Kaneda, Kano, Osawa, Kawakishi & Kamada, 1989) or suitable electron acceptors (Huvaere et al., 2003). α and β-acids degrade to organic acids, which are precursors in the synthesis of staling esters (Williams & Wagner, 1979).

Ester compounds have a positive impact on beer flavor. During storage, enzymes with esterase activity are released by the autolysis of yeasts and reduce the concentration of some esters, as isoamyl acetate and phenetyl acetate, and flavor profile. Conversely, condensation reactions can occur between ethanol and organic acids, with the formation of unpleasant ester molecules (Vanderhaegen et al., 2006). During storage the concentrations of other carbonyl compounds tend to increase, such us β-damascenone (Ferreira et al., 2022; Silva Ferreira, Bodart & Collin, 2019) and γ-nonalactone (Eichhorn, Komori, Miedaner & Narziss, 1989; Daan Saison et al., 2010), and they are considered as volatile markers of aged beers (Gijs, Chevance, Jerkovic & Collin, 2002; Saison et al., 2009).

Despite the beer shelf life being influenced by both upstream processes and storage conditions, oxygen is still indicated as one of the major causes associated with aging. This pushed commercial breweries to search methods to reduce oxygen concentrations during the bottling step (Decker, Elias & McClements, 2010). However, craft breweries often employ the refermentation bottling method.

Craft beer, by the Italian law refers to an independent brewery able to produce low volumes of beer (200.000 hL/year), not microfiltered or pasteurized (Saison, De Schutter, Delvaux & Delvaux, 2008).

In bottle refermentation method, sugar and yeasts are added to flat beer before bottling, for sparkling and foam formation (Štulíková et al., 2020). This method is preferred by many microbreweries because it adds flavor complexity to the final product and avoid money investment by using semiautomatic bottling line (Štulíková et al., 2020). The drawback is the presence of oxygen, before the refermentation process; as a certain amount of oxygen in the headspace which is almost completely replaced by the CO_2 during refermentation. To reduce the oxygen concentration in the unfilled space, oxygen scavenging bottle caps have been developed by several companies (Dey & Neogi, 2019; Edens, Farin, Ligtvoet & Van Der Plaat, 1992). These oxygen scavengers have a special liner that absorbs oxygen molecules in the headspace, with the aim to prevent the oxidation process, ensuring flavor stability and extending shelf life (Dey & Neogi, 2019; Edens et al., 1992). Wietstock, Glattfelder, Garbe and Methner (2016) found a low concentrations of Strecker aldehydes in a commercial pilsner capped with an oxygen barrier line, stored at 28 °C for 12 weeks. This special coating showed the same migration rate as normal caps towards the hops volatile compounds present in a commercial pilsner (such as linalool and geraniol) (Wietstock et al., 2016).

Chemical aging markers are a suitable method to measure the effect of aging in beer (Rodrigues et al., 2011; Saison et al., 2008; Vanderhaegen, Delvaux, Daenen, Verachtert & Delvaux, 2007). In this approach, the pre-concentration and isolation of the monitored compounds acquires a central role in the analysis. In this perspective, the use of Solid Phase Microexctraction (SPME) coupled with gas chromatography/mass spectrometry (GC/MS) technique is today an established methodology to characterize complex matrices such as beer (Anderson, Santos, Hildenbrand & Schug, 2019; Lehnhardt, Becker & Gastl, 2020), with many advantages over other conventional approaches (Almeida Santos, Gomes da Silva & Cabrita, 2020; Anderson et al., 2019; ben Hammouda, Freitas, Ammar, Da Silva & Bouaziz, 2017; Branco et al., 2020; Lehnhardt et al., 2020).

Also the Electron Paramagnetic Resonance (EPR) spectroscopy has been proposed as an analytical method to relate the resistance of beer to forced oxidation with its shelf life (Barr et al., 2001). In particular, when the beer samples are thermally treated at 60 °C in the presence of *tert*-butylphenylnitrone (PBN), 1-hydroxyethyl radicals are trapped by PBN forming relatively stable paramagnetic adducts which can be detected by EPR spectroscopy (Kocherginsky, Kostetski & Smirnov, 2005).

In this work, we reported the effect of oxygen scavenger caps in the sensorial and volatiles aging markers during storage of a re-fermented craft beer. Beers were kept at 6, 22 and 45 °C in order to force aging (Čejka, Čulík, Horák, Jurková & Olšovská, 2013), and analyzed until 13th week by, standard technological analysis, HS-SPME-GC/MS and EPR. Additionally, to highlight differences between experimental conditions, sensory profile of the beers at the end of the forced aging process were assayed.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma (Milan, Italy), VWR (Radnor, USA) and TCI (Tokyo, Japan) with the highest purity available.

2.2. Brewing process

A Batch of 120 L of beer was produced on the pilot plant facility of Porto Conte Ricerche Srl (Alghero, Italy). As grist, 20 kg of Pilsner malt (Weyermann, Bamberg, Germany) and 1 kg of Carapils malt (Weyermann, Bamberg, Germany) were used. Malts were ground in a two-roll mill spaced 1 mm. Mash-in was done by 75 L of water added with 20 g of $CaSO_4$ (Mr. Malt, Udine, Italy) and 10 g of $CaCl_2$ (Mr. Malt, Udine, Italy). Mash was conducted at 66 °C for 60 min and then heated at 78 °C and kept for 10 min for mash-out. First wort was transferred to kettle and spent grain was washed using water at 78 °C to reach 130 L of total volume. Then wort was boiled for 60 min and *Saaz* hop (Mr. Malt, Udine, Italy) was added at the start of boiling in order to obtain 30 International Bitter Units (IBU). The boiled wort was separated from the hot trub in the whirlpool, then cooled at 13 °C. Dry yeast Saf-Lager W34–70 (Fermentis, Marcq-en-Baroeul Cedex, France) was added directly into fermenter (0.5 g/L). Fermentation was carried out at 13 °C for 10 days, then temperature was reduced to 4 °C for 3 weeks. 330 mL glass bottles were filled with flat beer and 6 g/L of glucose and 0.05 g/L of yeast F2 (Fermentis, Marcq-en-Baroeul Cedex, France) were added. Half of the bottles were corked by standard caps (SC) and the other half with oxygen scavenging caps (OSC) (LD Carlson, Kent, OH – USA) and kept at 22 °C for 14 days to obtain conditioned beer. Standard quality parameters are reported in Table S1.

2.3. Aging process

Conditioned SC and OSC beers were stored for 13 weeks in 3 temperature-controlled cells: 6 °C (control sample); 22 °C (simulating a standard store condition); 45 °C (forced aging). A storage period of 13 weeks at 45 °C correspond approximately to a stocking time of 1.7 years at 20 °C (Čejka et al., 2013). Samples were subjected to technological and chemical analyses according to the scheme shown in Table 1.

Table 1
Scheme of temperature storage and sample times (tn) of beers corked with standard caps and oxygen scavenging caps (OSC).

		Weeks											
		1	2	3	4	5	6	7	8	9	10	11	12	13
T (°C)	Packaging	(t1)	(t2)	(t3)	(t4)	(t5)		(t6)		(t7)				(t8)
6	SC	√				√				√				√
	OSC	√				√				√				√
22	SC	√		√		√				√				√
	OSC	√		√		√				√				√
45	SC	√	√	√	√	√		√		√				√
	OSC	√	√	√	√	√		√		√				√

2.4. EPR experimental

Three SC and OSC craft beer conditioned samples stored for 2 and 7 weeks at 45 °C were analyzed. The samples at t_0, without any storage were taken as references.

5 μL of a PBN solution 2.5 mmol L^{-1} in absolute ethanol were taken to dryness under a flux of nitrogen. 250 μL of decarbonated beer samples were used to solubilize the PBN dried samples described above. The beer samples (100 μL), with a final PBN concentration of 50 mmol L^{-1}, were transferred to capillary tubes and inserted in the EPR cavity. EPR spectra were recorded at 60 °C for at least 150 min, acquiring spectra every 5 min.

EPR measurements were carried out with a Bruker EMX spectrometer operating at the X-band (9.40 GHz) equipped with an HP 53150A frequency counter and with a variable temperature unit. The EPR instrument was set under the following conditions: modulation frequency 100 kHz; modulation amplitude 1.06 G; receiver gain 5×10^5, microwave power 20 mW, time constant and conversion time 163.84 ms.

The lag time was determined by fitting the experimental points with the Boltzmann sigmoidal Eq. (1):

$$Y = Bottom + (Top - Bottom)/(1 + exp((V50 - x)/slope)) \quad (1)$$

and with a modified Boltzmann sigmoidal equation (Fadda, Molinu, Deiana & Sanna, 2021) (Eq. (2)):

$$Y = Bottom + (Top - Bottom)/(1 + exp((V50 - x)/slope)) + rise * x \quad (2)$$

EPR spectra of the radicals were simulated with Bruker WIN-EPR SimFonia software (WinEPR SimFonia, version 1.25, 1996).

According to Marques, Espinosa, Andrews and Foster (2017) the intensity of the PBN adduct after 150 min of thermal treatment and the area under the curve intensity vs. time were considered when it was not possible to determine the lag time.

2.5. Standard quality attributes

Original extract (% w/w), real extract (% w/w), apparent extract (% w/w) and alcohol (% v/v) were measured with a PBA-B generation M (Anton Paar, Graz, Austria). The following analyses were performed according to the official Analytical European Brewery Convention methods (EBC, 1998): Color (EBC-U) by EBC method 9.6; pH by EBC method 9.35; Bitterness (IBU) by EBC method 9.8; Foam stability was measured with a NIBEM-OPH foam stability tester (Haffmans, Zeist, The Netherlands) according to EBC method 9.42.1.

2.6. Analysis of beers volatiles aging markers by hs-spme-gc/ms

5 mL of degassed beer from each sample were transferred in 10 mL headspace vials containing 1.5 g of NaCl and 10 μL of internal standard (1-butanol 25 g L^{-1}), then sealed with PTFE–silicone septa and stored at 7 °C in a refrigerated compartment. Analysis of volatile compounds was carried out using the headspace solid phase microextraction (HS-SPME-GC/MS) technique by means of Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB-CAR-PDMS) fiber (Supelco, Bellefonte, PA, USA) (Riu-Aumatell, Miró, Serra-Cayuela, Buxaderas & López-Tamames, 2014). For SPME analysis each sample were incubated for 10 min at 40 °C, then extraction was carried out exposing the fiber to the headspace for 40 min. Both incubation and extraction were performed in agitation. Fiber desorption was done in the injector for 2 min at 250 °C with a split flow of 7.2 mL min^{-1}. The fiber was activated each day following the manufacturer instruction. The chromatographic analysis was performed using TRACE GC coupled with an ISQ single quadrupole (Thermo Scientific, Hudson, USA). The analytes were separated on a SLB-5 ms capillary column (60 $m \times 0.25$ mm × 0.25 μm film thickness) (Supelco, Bellefonte, PA, USA) using helium as carrier gas at 1.2 mL min^{-1} constant flow rate. Oven temperature program started at 50 °C, held at this temperature for 5 min, and then increased at 5 °C min^{-1} to 250 °C and held for 3 min Charry-Parra, DeJesus-Echevarria and Perez (2011). Transfer line and ion source were set at 250 °C and 270 °C respectively, quadrupole scan range was set at 33–300 amu and in single ion monitoring mode (reported in Table S2), the ionization energy was 70 eV (Aberl & Coelhan, 2012). Chromatographic data were acquired by means of Tracefinder (Thermo Scientific). Calibration curves of target volatiles compounds were obtained with the standard addition method on the same beer stored at 6 °C, based on the approach reported by Saison et al. (2008). Two bottles were analyzed for each sample.

2.7. Sensory analysis

The sensory evaluation of the beers was conducted following a modified Quantitative Descriptive Analysis (QDA) methodology (Yang et al., 2021).

Six experienced assessors males were trained on the use of a 7 points scale for evaluating the selected attributes. Panel performance was monitored throughout training to examine its reproducibility and discriminative ability, both individually and as a group; attribute references were developed over several sessions, acetaldehyde, dimethyl sulfide, *trans*-2-nonenal and benzaldehyde flavor reference standards were purchased from Aroxa (Cara Technology, Surrey, UK).

Throughout the training sessions, the panel developed a protocol for tasting, smelling and palate cleansing to ensure optimum identification and performance. Each session involved the evaluation of 6 samples, OSC and SC, stored at 6 22 and 45 °C.

The samples were labelled with a 3-digit random code and presented in a completely randomized order.

The assessors were given three-minute breaks between samples to avoid palate fatigue, with a 10 min comfort break at the midpoint of evaluation (after 3 samples).

During breaks, the assessors used water (Smeraldina SpA, Tempio, Italia) and unsalted crackers (Mulino Bianco Barilla SpA) to cleanse the palate and to minimize sample carry-over.

All samples were served in covered glasses at a temperature of 8 ± 2 °C, to minimize expectation error and to reduce bias from appearance.

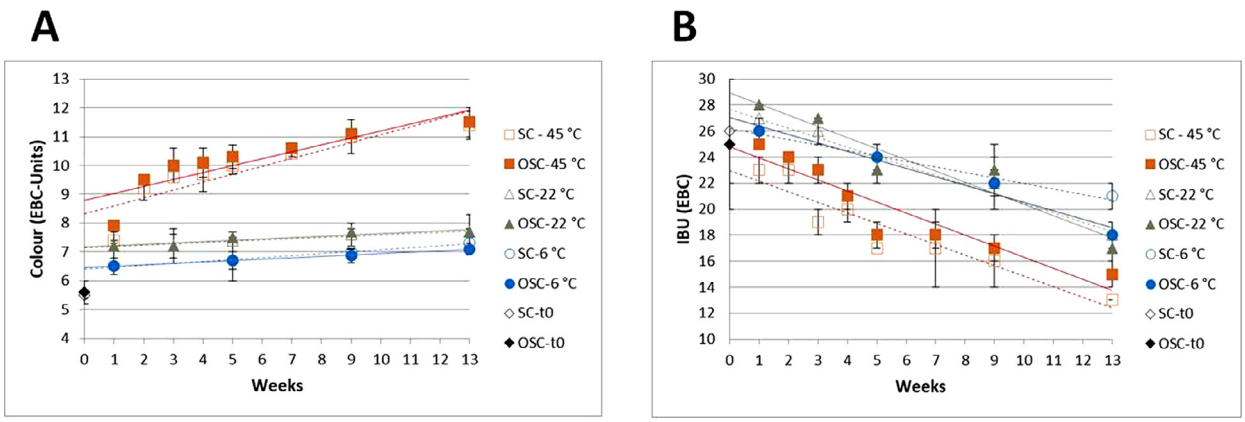

Fig. 1. A: Color measurement in 13 weeks of storage. **B:** IBU measurement in 13 weeks of storage. Samples with (OSC, solid) and without (SC, open) oxygen absorbing caps stored at 6 °C (blue circle), 22 °C (green triangle) and 45 °C (red square). Data are reported as means ± standard deviation (SD).

Repeatability and discrimination ability, were monitored with the software Panel Check, according to Tomic, Nilsen, Martens and Næs (2007).

2.8. Statistical analysis

Statistical analysis was performed with GraphPad Prism8 for Windows software (GraphPad Software Inc. La Jolla. CA92037, USA). A one-way ANOVA was used to compare the results of the lag time calculated with Boltzman and the Boltzmann modified equations (Eq. (1) and Eq. (2)) on SC and OSC samples at T0, a Student's *t*-test ($P \leq 0.05$) was used for means comparison. A one-way ANOVA analysis was also used to the results of the I_{150} (a.u.) and AUC values. Means separation for: technological parameters; HS- HS-SPME-GC/MS; EPR and sensory analysis was calculated by Tukey's test $P \leq 0.05$.

3. Result and discussion

3.1. Standard quality attributes analysis

The standard quality parameters of color and IBU are shown in Fig. 1A and B respectively.

After 13 weeks, the highest values in color were obtained in OSC and SC samples stored at 45 °C (11.5 and 11.4 respectively), while no differences were observed between the samples stored at 6 °C (7.1 and 7.3) and 22 °C (7.7 and 7.7). Also, difference between OSC and SC is not statistically relevant in the monitored conditions (Tukey's test $P \leq 0.05$) (Fig. 1A).

IBU values showed an opposite trend: a faster decrease at 45 °C (17 and 18 IBU for SC and OSC) than at 22 and 6 °C (23 and 24 IBU respectively) (Fig. 1B). This was also confirmed by sensory analysis shown in Figure 7, which show a lower intensity for bitter attribute in beers stored at 45 °C.

The data obtained agree with Aguiar, Pereira and Marques (2022) and Caballero, Blanco and Porras (2012) who reported that high storage temperature has an effect on color increase and isoα-acids degradation which are responsible for the beer bitterness and its characteristic flavor.

3.2. Volatiles analysis

The beers volatiles aging markers investigated by HS-SPME-GC/MS are reported in Table S2.

Yeast fermentation produce ethanol and carbon dioxide as well as various secondary metabolites such as esters, which could be highly impactful on overall beer flavor. This yeast ability to produce specific secondary metabolites varies by strain. In general Ale yeasts produce more esters than lager yeasts (Bamforth, 2009). For this reason, in order to reduce ester content of our experimental beer and focalize attention on oxidative marker, especially in sensory analysis, a lager yeast in bottom fermentation, was used in this work.

Ethyl acetate is the most predominant ester in beer because ethanol is the main alcohol produced by yeast, but other esters such as isoamyl acetate, 2-phenylethanol and 2-phenylethyl acetate are detectable.

In the present work concentrations of ethyl acetate and isoamyl acetate remains constant during 13 weeks of storage in all samples (Fig. S1 A-B), and a decrease in the concentration of 2-phenylethanol and 2-phenylethyl acetate was observed (Fig. S1 C-D). The reduction of some olfactory characteristics, debt by chemical reactions such us esters hydrolysis, esterification (formation of ethyl acetate) and alcohols oxidation are often related with the beer aging (Vanderhaegen et al., 2006).

No significant differences among different storage temperature and use of standard or oxygen scavenging bottle caps were found.

During wort production, there are some high temperature steps (mashing and boiling) that are critical points for compounds involved in the aging markers formation such as: furfural, 2-acetyl furan and furfuryl ethyl ether.

Since furfural and 2-acetyl furan rarely exceed their threshold values, can be considered as analytical rather than sensorial markers (Malfliet et al., 2008). Instead, furfuryl ethyl ether can be considered both analytical and sensorial marker (Vanderhaegen et al., 2004).

After 13 weeks, concentrations of furanic compounds increased dramatically in samples kept at 45 °C following a linear trend (Fig. 2A-C). After only one week of storage at 45 °C we observed a sharp increase in furfural concentration (from 20 to 12 ppb to 1.6 and 2.0 ppm respectively for SC and OSC), while no relevant difference was found in samples stored at 22 °C and at 6 °C.

Likewise, after one week of storage, the concentrations of 2-acetylfuran increased from 3.15 ppb to 25.8 ppb for SC and from 2.4 ppb to 30.6 ppb for OSC at 45 °C, while samples stored at 22 °C and 6 °C showed no relevant differences.

Furfuryl ethyl ether concentrations at 13 weeks increased more (from 0.25 to 34.1 ppb and from 0.19 to 67.3 ppb for SC and OSC) at 45 °C, than in samples stored at 22 °C (1.2 ppb and 0.9 ppb) and 6 °C (0.3 ppb and 0.3 ppb).

It is useful to emphasize that furfuryl ethyl ether exceeded its threshold value (6 ppb) after only 3 weeks of storage at 45 °C (7.1 and 7.8 ppb for SC and OSC samples respectively). As shown in Fig. 4, this compound is associated to the solvent-like stale flavor, found by judge in sensory analysis, discussed in paragraph 3.4.

The obtained values showed that the formation of furanic compounds during beer storage is temperature dependent without any effect arising from the use of oxygen absorbing caps. This agrees with Ferreira et al. (2022), that reported a constant increase of furanic com-

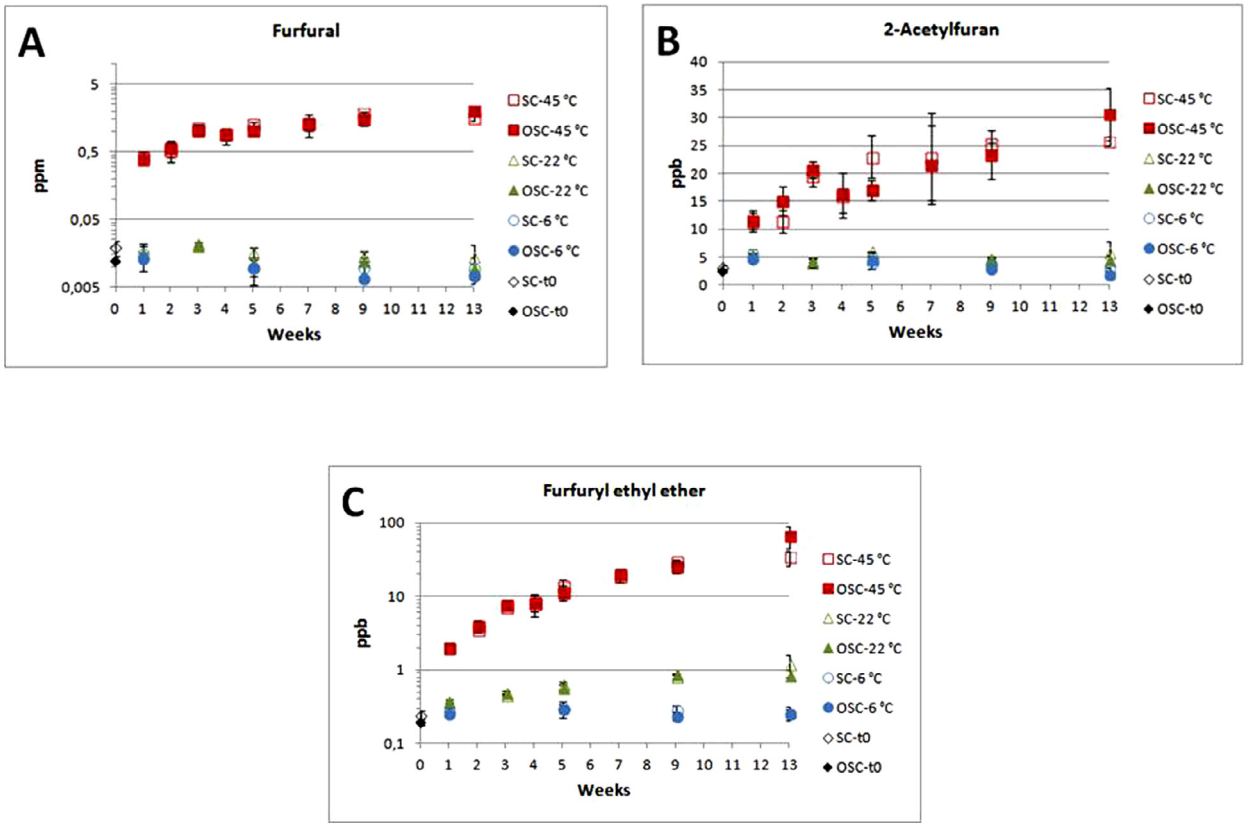

Fig. 2. A: Concentration of furfural during 13 weeks of storage. **B:** Concentration of 2-acetyl furan during 13 weeks of storage. **C:** Concentration of furfuryl ethyl ether during 13 weeks of storage. Samples with (OSC, solid) and without (SC, open) oxygen absorbing caps stored at 6 °C (circle), 22 °C (triangle) and 45 °C (square). Concentrations data are reported in log scale. Data are reported as means ± standard deviation (SD).

pounds 130 times higher in beers kept at 37 °C than at 4 °C and Vanderhaegen et al. (2006) that reported no oxygen effect in furanic compounds formation during beer aging.

Benzaldehyde and 2-phenylacetaldehyde, come from amino acids degradation process (Strecker's aldehydes). After 13 weeks, OSC and SC samples showed higher concentration of benzaldehyde (Fig. S2 A) when stored at 45 °C (15.4 ppb and 9.8 ppb for OSC and SC, respectively) than 22 °C (0.9 ppb and 2.3 ppb) and 6 °C (1.4 ppb and 1.3 ppb). Also, the concentration of phenyl acetaldehyde (Fig. S2 B) is higher in OSC and SC at 45 °C (101 ppb and 71 ppb) than 22 °C (10 ppb and 13 ppb) and 6 °C (10 ppb and 13 ppb).

γ-nonalactone and β-damascenone are also related to the aging process and increase following thermal stress. The concentration of γ-nonalactone and β-damascenone (Fig. S3 A-B) in SC and OSC samples showed large variability in the samples stored at 45 °C. The concentration of γ-nonalactone in OSC and SC at 45 °C (124 ppb and 90 ppb) is significantly higher than OSC and SC at 6 °C (34 ppb and 43 ppb respectively). Concentration of β-damascenone in OSC and SC at 45 °C (4.6 ppb and 3.00 ppb) is significantly higher than in OSC and SC at 6 °C (0.63 and 0.68 ppb).

3.3. EPR analysis

Fig. 3A shows the EPR spectra of the radical species generated during the thermal treatment of craft beer samples at 60 °C. In the experimental spectrum (Fig. 3A-b) two species can be detected; the principal one (Fig. 3A-c) is the typical six lines PBN spin adduct with hyperfine coupling parameters a_N = 16.1 G and a_H = 3.5 G, g = 2.00548, that corresponds to the PBN–1-hydroxyethyl radical adduct. Another species can be observed (Fig. 3A-a) with a_N = 14.5 G, a_H = 13.7 G, g = 2.00558.

These two species have EPR parameters very similar to those previously reported during the thermal treatment at 90 °C of hydro-alcoholic myrtle extracts (Sanna, Mulas, Molinu & Fadda, 2019). The slight differences are due to the differences in the% of ethanol in the solvents: craft beers have 5.5% of alcohol, while for the myrtle hydro-alcoholic extracts it was in the range 60–90%. The four lines in Fig. 3A-a can be interpreted as due to *tert*-butyl aminoxyl radical, which is thought to derive from the hydrolysis of PBN in acidic conditions. The hydrolysis of N-*tert*-butyl-α-phenylnitrone gives *tert*-butyl hydroxylamine, which is oxidized to the *tert*-butyl aminoxyl radical.

After examining Fig. 3B it is possible to state that the lag time can be determined only for the reference samples, that is for the samples not stored at 45 °C. The lag time values are 82.6 and 88.6 min for the samples OSC and SC respectively. These values were determined using as a fitting curve the Boltzmann sigmoidal equation described in the experimental section (Eq. (2)). Lag time values and the other parameters, that is I_{150} (PBN adduct intensity at 150 min) and AUC (are under the curve intensity vs. time) are reported in Table 2.

It was previously observed in the literature that in some cases, because of the shape of the intensity vs. time curve, the experimental determination of a lag time value is impossible (Marques et al., 2017). In fact, in these curves there are no inflection points and the intensity grows continuously with elapsing time. In these cases, other parameters were proposed to compare the flavor stability of different beer samples, that is the intensity after 150 min and the area under the curve from the beginning of the experiment up to 150 min Marques et al. (2017).

From Table 2 it is clear that a distinction between t_2 and t_6 samples is impossible when considering the I_{150} values. Slightly higher I_{150} values are obtained when comparing SC and OSC samples both at t_2 and t_6, with OSC samples having slightly higher values. When the area under (Caon et al.) the curve is considered it is possible to observe that the

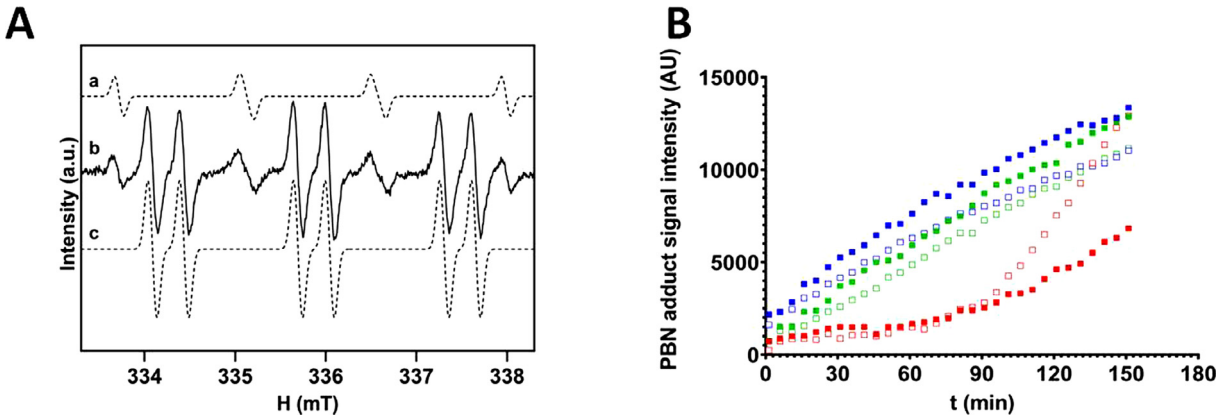

Fig. 3. **A:** Experimental (b, solid line) and simulated (a and c, dotted lines) spectra of the PBN–1-hydroxyethyl radical adduct (c) and *tert*-butyl aminoxyl radical (a) detected during the thermal treatment at 60 °C of craft beer samples. The experimental spectrum was obtained after 150 min of thermal treatment at 60 °C on a craft beer sample. The parameters used for simulating the spectra are reported in the experimental section. **B:** Intensity of the PBN adduct vs. time for samples with OSC (solid) and with SC (open) stored at 45 °C for 0 (red), 2 (blue), and 7 weeks (green).

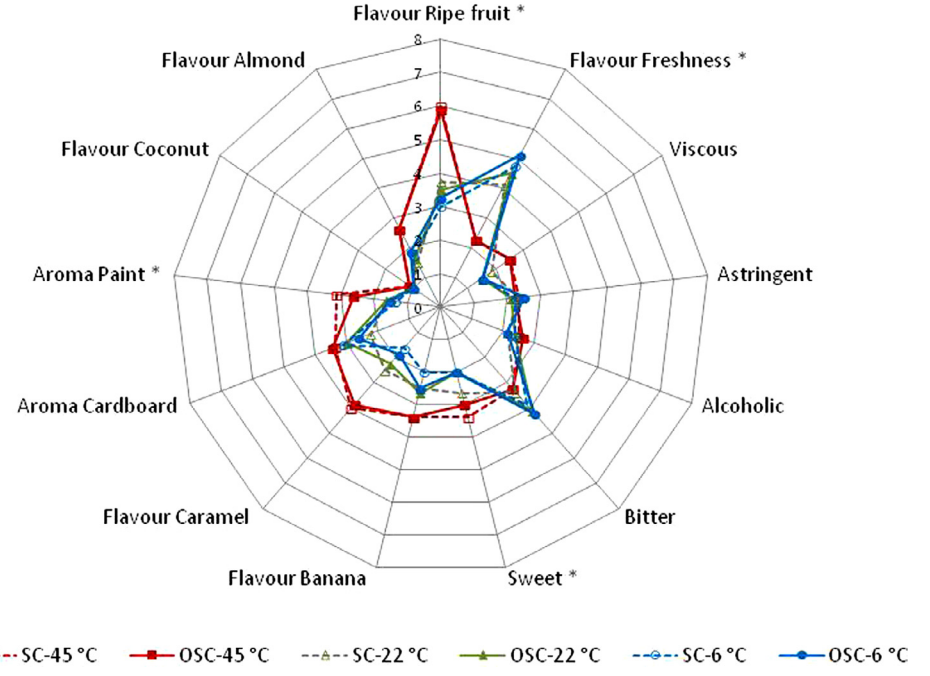

Fig. 4. Radar plot of the mean rating scores for SC (dotted lines) and OSC (solid line) stored for 13 weeks at 6 °C (blue), 22 °C (green) and 45 °C. Asterisks denote sensory parameter observed with significant difference between the samples ($p \leq 0.05$).

Table 2
EPR parameters measured for the craft beer samples.

Sample	lag time (min)	lag time (Boltzmann modified)	I_{150} (a.u.)	AUC (a.u.)
SC t_0	88.8 ± 4.2 **a**	89.4 ± 12.9 **a**	12,931 ± 1362 **a**	575,413 ± 37,208 **d**
SC t_2	–		11,040 ± 321 **a**	1,034,302 ± 8080 **b**
SC t_6	–		11,170 ± 644 **a**	895,433 ± 10,101 **c**
OSC t_0	82.6 ± 2.4 **a**	77.6 ± 6.6 **a**	6827 ± 130 **b**	403,777 ± 9551 **e**
OSC t_2	–		13,379 ± 207 **a**	1,256,695 ± 30,964 **a**
OSC t_6	–		12,872 ± 134 **a**	1,070,722 ± 59,968 **b**

Means of lag time and lag time (Boltzmann modified) with different letters within the same column are statistically different according to Student's *t*-test ($P \leq 0.05$). Means of I150 (a.u.) AUC (a.u.) with different letters within the same column are statistically different by Tukey's test ($P \leq 0.05$).

highest value is measured for t_2 samples both for SC and OSC, and when comparing SC and OSC, the latter exhibit higher values except for the t_0 samples.

During thermal treatment of beer samples at 60 °C the precursors of the radical species, which eventually accumulated during the production and the storage of beers, are converted into radical species, which in their turn transform into the 1-hydroxyethyl radical, derived from ethanol. This latter radical species can be trapped by PBN or react with the endogenous antioxidants.

The precursor of the radical species can also react more slowly during the storage of beers, generating radical species and depleting the endogenous antioxidants. Considering that the highest AUC value is

reached for the samples stored at 45 °C for 2 weeks, we can hypothesize that at longer storage times the precursors of the radical species, after completely depleting the endogenous antioxidants, have enough time to react with each other originating a lower number of radicals which are trapped by PBN.

3.4. Sensory analysis

At the end of 13 weeks, the SC and OSC samples stored at the three different temperatures were subjected to sensory analysis by means of a QDA test.

According to Ferreira et al. (2022), it was that the oxidation reaction rate increases with increasing temperatures, the results shown in Fig. 4 indicated significant differences among storage temperature trials.

Increasing value are found in samples stored at 6, 22 and 45 °C for paint, ripe fruit, sweet and cardboard flavors, all considered oxidation derived off-flavor. Contrariwise, freshness attribute was found higher at lower temperature, showing that storing beer at low temperatures helps to protect freshness, and original chemical sensory profile.

No significant differences ($p > 0.05$) were observed between SC and OSC samples, indicating that the panel could not significantly discriminate between them.

4. Conclusion

The influence of oxygen and storage temperature, in a craft bottle-fermented beer, capped with standard and oxygen scavenging bottle caps were studied. As expected, a high storage temperature of 45 °C always resulted in a decrease in International Bitterness Units and an increase in color and oxidation processes (higher furan compounds and aldehydes), confirming that high temperatures drastically reduce shelf life even in bottle-fermented beers.

Storage temperatures of 22 and 6 °C showed no statistically significant differences in standard quality attributes and volatile analysis, in the experimental time.

The Electron Paramagnetic Resonance analysis performed on samples stored at 45 °C for 7 weeks show that oxygen scavenging caps do not stabilize or protect beers towards oxidation during storage. Since the highest amount of radicals was detected after two weeks of storage at 45 °C, for both OSC and SC, it is possible to hypothesize that the major oxidation processes take place during this time interval.

Sensory analysis showed that ripe fruit, cardboard, sweet and paint flavor scores increased at higher storage temperatures, while freshness was lower, confirming the chemical analysis data, but greater significant differences were found between temperatures of 22 and 6 °C, highlighting the importance of storage temperatures. No differences were found between the use of traditional caps or oxygen scavenging bottle caps in our experimental conditions.

Funding

This work was supported by the Sardinia Regional Government: research grant Bi. Ar Sardegna – Sviluppo sostenibile della birra artigianale in Sardegna.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Antonio Valentoni: Data curation, Investigation, Methodology, Writing – original draft. **Antonio Santoru:** Data curation, Investigation, Methodology. **Manuela Sanna:** Data curation, Investigation. **Mauro Fanari:** Writing – original draft. **Maria Cristina Porcu:** Data curation, Investigation. **Angela Fadda:** Data curation, Investigation. **Daniele Sanna:** Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. **Luca Pretti:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Writing – original draft.

Data availability

No data was used for the research described in the article.

Acknowledgements

The authors would like to thank the Associazione Italiana Sommellier (Sassari) for sensorial analysis support. And Birrificio Mezzavia for the technical suggestion.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.focha.2022.100151.

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