

Anaerobic and Aerobic Beer Aging

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Abstract

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Yellow, orange, red and brown pigments are formed by air oxidation of single polyphenols or by thermal degradation of sugars to caramels. Caramels increase their colours during anaerobic heating or decrease them by air oxidation. Epicatechin and caramel undergo reversible redox reaction followed by degradation and/or polymerisation at beer aging. That is why both of these colour compounds, besides acting as acid/alkali indicators, can also represent redox indicators that gradually become irreversible. These reactions are accelerated by transient metals or buffering solutions and are therefore more distinct in tap or brewing water than in deionised water. The kind of the brewing water then predetermines not only the beer attributes but also the course of beer aging. Coloured pigments can be partially bleached by reducing agents such as yeast oxidoreductase enzymes and the colour can be then recovered by oxidation; this depends on their polymerisation degree. Methylene blue and methyl red can be used as artificial oxidation-reduction indicators for the study of the redox potential changes because they act reversibly or irreversibly under aerobic or anaerobic conditions, respectively.

Keywords: caramel; epicatechin; beer aging; reductone; organic radical; light sensitivity

The changes of food colour during storage are explained by various mechanisms, which commonly include enzymatic or non-enzymatic oxidation of polyphenols and/or melanoidin substances formation (FRIEDMAN 1996). The influence of coloured caramel products formed during mashing and hopped wort boiling should be also taken into account in the case of beer (NØDDEKAER & ANDERSEN 2007).

Enzymatically or non-enzymatically oxidised catechin shows a typical absorption band with a maximum around 430 nm. Such compounds can react with other substances such as acetaldehyde, ascorbic acid, or degradation products of saccharides, which also influences their spectral characteristics (KIM *et al.* 2003; LABROUCHE *et al.* 2005; TOTLANI & PETERSON 2006).

The products of advanced polyphenol polymerisation usually show a typical monotonously decreasing curve in visible region similar to those coming from sugar caramelisation. The formation

of hydrogen peroxide during polyphenol oxidation by oxygen has also been described (AKAGAWA *et al.* 2003).

In most cases, these changes are studied under aerobic conditions, but the beer colour increase continues even after all oxygen has been taken up. The majority of authors agree that the decomposition products of saccharides, melanoidins, and oxidised polyphenols can cause beer aging by the oxidation of amino acids and higher alcohols, as proposed in early classical theories (HASHIMOTO & ESHIMA 1977; IRWIN *et al.* 1991).

It is difficult to identify the absolute significance of the individual process stages for flavour stability because many data presented in the literature are faulty, most notably with regard to the robustness and relevance of the organoleptic analysis (BAMFORTH 2004).

The exclusion of oxygen from the beer and headspace of the package were expected to stop beer ag-

ing but this has never been observed. The pigments formed from reductones and oxidised polyphenols play an important role in the course of aging and can be labelled as indicators of aging.

Many authors have described in detail sugar degradation during the beer production and aging (SÁNCHEZ *et al.* 2003; BRAVO *et al.* 2008). The addition of biguanide or *o*-diaminobenzene inhibits sugar degradation including furfural and hydroxyfurfural formation during aging (BECH & BRAVO 2007). Aldehydes can also be formed during wort boiling, masked through the fermentation and then released (SUDA *et al.* 2007).

All the processes are closely connected to the beer aging including the coloured pigments formation. The aim of this study is to show the colour changes of natural or synthetic redox indicators during beer aging and their meaning for electron transport between the beer components under aerobic or anaerobic conditions.

There are many examples of aerobic or anaerobic destructive reactions which can be visualised by organic dye destruction. Indigocarmine is destroyed only under aerobic conditions in the presence of strong radical initiator and the process is supported by maltose or ethanol. The same compound can be destroyed in sulfite solution without adding radical initiator, only in the presence of oxygen (SAVEL 2001).

Polyphenols (*o*-diphenols) or sugar reductones are natural parts of beer. They can be oxidised by oxygen or other oxidation agents to radicals which can recombine themselves and attack the molecules of other organic compounds. The presence of catalysts, as well as light or high temperature, accelerates the process. Numerous intermediates are formed during Maillard reaction, increasing the number of oxidation-reduction units (YAYLAYAN 1997).

The reducing compounds prevailing in beer reduce the oxidation agents (including oxygen), which can be associated with the formation of radicals enabling electron transport. Degradation activity due to Fenton reaction is promoted by maltose addition (SAVEL 2003). Other compounds are formed by radical recombination. Recently, it was found that many enzymes convert their substrates into organic radicals to allow challenging reactions to occur (JARRET 2008).

Oxidation agents accelerate the process. Especially radical initiators such as peroxodisulfate provide a broad spectrum of sugar fragments in acid or alkaline conditions.

Reductones are oxidised by the initially formed radicals. The radicals strongly attack reductones and, after their degradation, sensitive beer compounds are decomposed. This is the basis of the lag time concept. The sensitive compounds can also survive the radical attack in the reduced form until part of the reductones is consumed; this is followed by the degradation process.

Although aerobic oxidation is supposed to be the most dangerous aging process, both key reactions such as Maillard and Strecker oxidation can run under anaerobic conditions; oxygen mostly accelerates them, which is a typical attribute of the beer aging. The products of Maillard reaction or quinones obtained from polyphenols can provide the oxidation agents needed for the Strecker oxidation.

MATERIAL AND METHODS

Maltose, ascorbic acid, methylene blue, methyl red (sodium salt) and (–)-epicatechin were purchased from Sigma Aldrich together with the components for the preparation of phosphate buffer solutions (1/15 mol/l) including phosphoric acid.

Stock solutions contained ascorbic acid (1.0% w/w), methylene blue or methyl red solutions (1 g/l), all dissolved in deionised water. The working solutions were prepared by dilution or dosage of stock solutions into the samples, mostly in a ratio of 1:100.

The epicatechin pigments were prepared from (–)-epicatechin solution (100 mg/l) by heating (90 min at 60°C) in soft tap water containing Ca^{2+} and Mg^{2+} (0.7–0.8 mmol/l), Fe (0.1 mg/l), Cu (below 0.05 mg/l), pH 6.9. The tap water is an example of soft brewing water.

Caramels were prepared by heating sucrose (100 g) up to the melting point to acquire light brown colour and by mixing the melted caramel with deionised or tap water (100 ml). Cold solutions were diluted with deionised or tap water (2:100) to obtain a solution containing the extract (1.2% w/w) with a colour grade of 6–7 (in deionised water) or 12–13 (in tap water) EBC units.

Oxygen was removed from the liquid samples by bubbling nitrogen through the solutions (20 min) and the cells were then tightly sealed. Air saturated samples were bubbled with air for 10 minutes.

Spectrophotometer Hach-Lange DR 5000 equipped with cylindrical glass cells (cuvettes) with an

optical path of 1 cm was used for recording the absorption spectra.

The device for the exposure of the sample to visible light consisted of a tube with an inlet and outlet of tap water for sample cooling. The cuvette with the sample was inserted into the cooling tube and the sample was exposed to two halogen lamps 2×50 W placed at a distance of 1 cm from the cuvette in flowing cooling water.

RESULTS

Formation of pigments by heating epicatechin and caramel

The epicatechin solution (100 mg/l) was prepared by mixing the solid substance in deionised or tap water, and the solutions as well as caramel solutions were filled into cylindrical glass cuvettes. Oxygen was removed from one half of the samples by bubbling nitrogen through the solutions and, after closing the cuvettes with rubber stoppers, all cuvettes were warmed at 60°C for 90 min in a water bath, which simulated pasteurisation or other thermal loads in the beer production. After cooling down, the absorption spectra of the solutions were measured (Figure 1).

The formation of pigments was strongly influenced by the kind of water and aerobic/anaerobic conditions for both polyphenols and caramels. Tap water representing a kind of brewing water

was necessary for the formation of pigments in the case of polyphenols. The reason is the presence of metals as well as the slightly higher pH or higher buffer capacity in comparison to deionised water. Caramel pigments could be prepared from both deionised and tap water, but those from tap water had a higher colour.

Formation of coloured oxidation products of epicatechin by boiling in maltose solution

Epicatechin (100 mg/l), maltose (10% w/w), or mixtures of both substances were boiled under the air for 2 h under a reflux condenser, which simulated wort boiling. After cooling down, the samples were pipetted into cylindrical glass cuvettes, oxygen was removed from one half of the samples, and the cuvettes were sealed with rubber stoppers. Visible spectrum was recorded after filling and after storage in the dark at 45°C, which simulated aging of the products (Figure 2). Oxygen reoxidised the reduced epicatechin pigment which was followed by further polymerisation and degradation.

The colour of the epicatechin pigments could be partially diminished by boiling with maltose, probably as a result of the reduction processes caused by the reductones formed. The pigments were then reoxidised at aerobic aging, which was seen by a partial recovery of the pigments colour. The same reaction mechanism proceeds in the

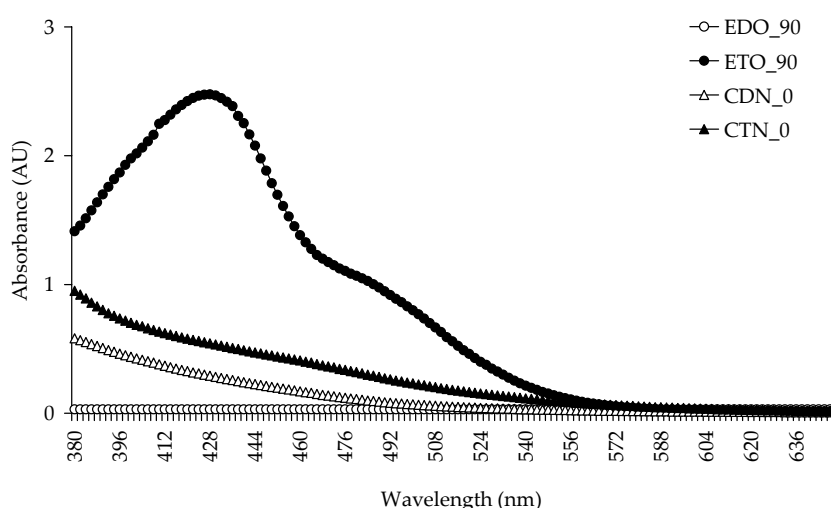


Figure 1. Absorption spectra of caramel before heating (CDN_0 – caramel in deionised water, nitrogen, CTN_0 – caramel in tap water, nitrogen), the spectra after heating (90 min at 60°C) were almost identical (data not shown) Epicatechin solutions in deionised water after heating under nitrogen or air were colourless, EDO_90 – epicatechin in deionised water, oxygen, 90 min at 60°C, ETO_90 – epicatechin in tap water, oxygen, 90 min at 60°C

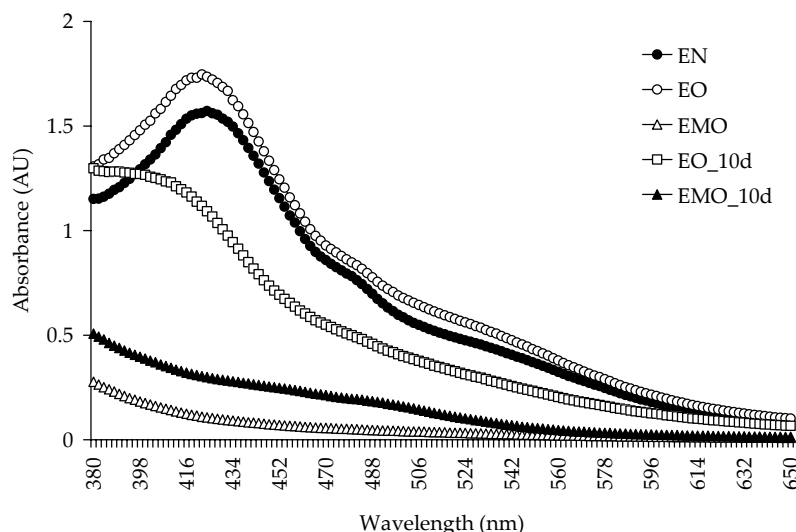


Figure 2. Absorption spectra of epicatechin (E) solutions (100 mg/l) boiled under air for 2 h without or with maltose (10% w/w) (EM) followed by cooling and bubbling with nitrogen (N) or oxygen (O) before and after storage for 10 days at 45°C (10 d)

brewhouse at wort boiling, which is followed by another reduction step (reduction by yeast during fermentation), the colour being recovered later in the course of aging.

Colour changes of epicatechin and caramel pigments as a result of aging at various pHs

The solutions of epicatechin or caramel pigments prepared respectively by heating epicatechin or caramel in tap water were mixed with deionised water or phosphate buffer solutions in a ratio of

1:1. The samples were aged under aerobic or anaerobic conditions in the dark at 45°C for up to 10 days and the absorbance at 430 nm was measured (Figures 3 and 4).

The colour of epicatechin pigments decreased during aging under both aerobic and anaerobic conditions. The surprising colour decrease was probably caused by further changes of pigments including their degradation. We could also observe the colour increase in the case of other polyphenols such as caffeic or ferulic acids (unpublished data).

The colour of caramel pigments increased at anaerobic aging. In the initial phase of aerobic ag-

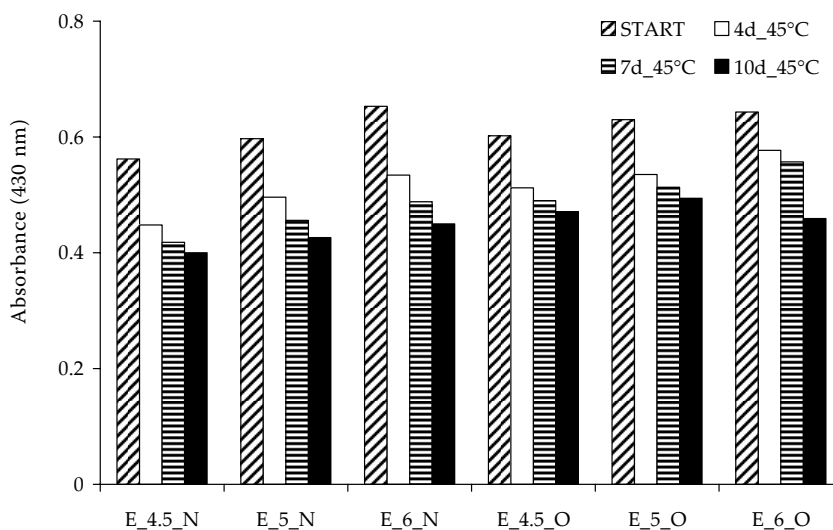


Figure 3. Colour changes of oxidised solutions of epicatechin (E_pH) in buffer solutions (pH = 4.5, 5, 6) after heating (0–10 days at 45°C) under anaerobic (N) or aerobic (O) conditions

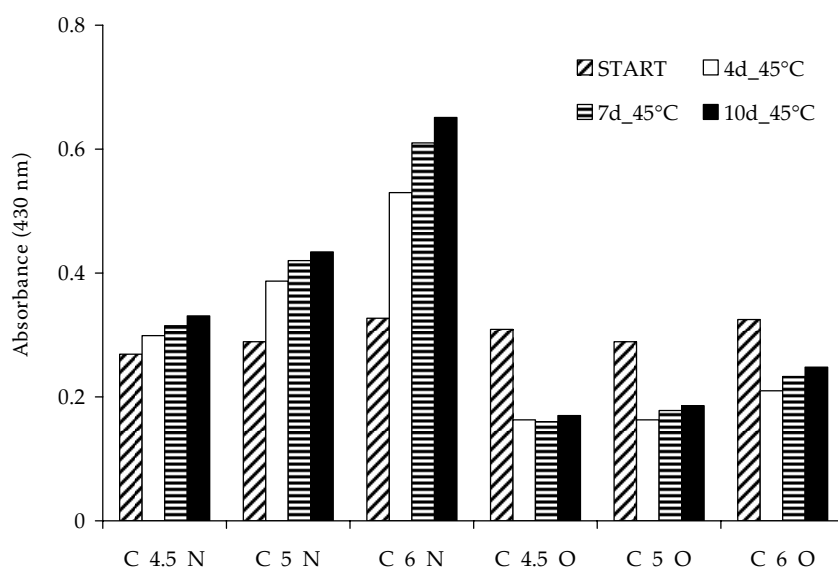


Figure 4. Colour changes of caramel solutions (C_pH) in buffer solutions (pH = 4.5, 5, 6) after heating (0–10 days at 45°C) under anaerobic (N) or aerobic (O) conditions

ing, the colour of the caramel pigments decreased, probably as a result of oxidative destruction, which was followed by a slight increase in colour.

The colour changes were strongly influenced in both cases by pH value; higher changes of colour were more distinct at higher pH values.

Colour changes of epicatechin and caramel pigments as a result of aging under reducing conditions

Ascorbic acid was added (to the final concentration of 100 mg/l) to the epicatechin and caramel pigments solutions prepared by heating epicatechin or caramel in tap water, and the samples were aged under aerobic or anaerobic conditions in the dark at 45°C for up to 10 days (Figure 5).

Depending on the conditions of aging, the colour of epicatechin pigments can at first increase and then decrease with time. Ascorbic acid reduces epicatechin pigments under both aerobic and anaerobic conditions, which could be seen by the colour decrease, but the colour recovered again later under aerobic conditions. Epicatechin pigments acted as a reversible redox indicator in this model situation.

In the case of caramels, the colour increased at anaerobic aging which is supposed to be the result of polymerisation reactions. Contrary to anaerobic conditions, the colour decreased as a result of caramel pigments degradation at aerobic aging.

Colour changes of beer pigments as a result of aging under various conditions

The results, which were obtained in the model aging situation described above, were verified by aging of fresh unpasteurised beer and further aging of one-year-stored beer in the presence of ascorbic acid under aerobic and anaerobic conditions (Figure 6). Both beer samples were repasteurised and then aged in the dark at 45°C for up to 8 days.

As expected, the beer colour increased gradually, but a small decrease could be observed at the beginning of the storage. Ascorbic acid inhibited the colour increase only partially. The combination of polyphenol polymerisation, degradation, and reoxidation of the formerly reduced oxidised polyphenols, which were described separately in detail above, occurs simultaneously in the case of beer.

Beer undergoes colour changes caused by temperature, pH, redox capacity, and the presence of oxygen, all of which are changed during the brewing and fermentation processes.

The significant decrease in colour during fermentation and lagering is linked with the brewer's yeast reductase activity. The subsequent increase of colour during beer aging is caused by the decrease of the reducing capacity or, in some cases, by degradation and polymerisation of the colour products contained in beer. The colour substances that have passed through all these changes become less prone to reversible reduction.

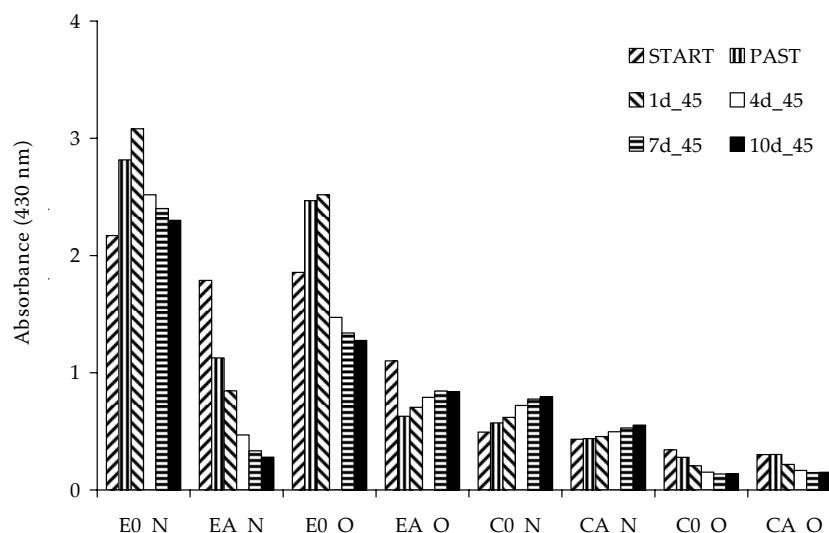


Figure 5. Colour changes of oxidised epicatechin (E) or caramels (C) solutions in tap water, without (E0, C0) and with (EA, CA) ascorbic acid addition (100 mg/l) after pasteurisation (2 h at 60°C) followed by heating (1–10 days at 45°C) under anaerobic (N) or aerobic (O) conditions

Colour changes of artificial redox indicators in beer pigments after exposure to visible light

Methylene blue (MB) and methyl red (MR) were added to beer or caramel solutions to study the redox changes after the exposure to visible light. Before the exposure of the solutions to light, only caramel solution could slowly reduce MB in the absence of oxygen (Figure 7). Methylene blue was quickly reduced after the exposure to visible light

by both caramel and beer under both aerobic and anaerobic conditions. The velocity of the reduction was so high that the blue colour disappeared even if all oxygen in the solution had not been consumed (Figure 7), and further reoxidation and blue colour recovery took place after the lamps had been switched off.

In the absence of oxygen, the blue colour recovered after fresh oxygen had been introduced. Similar reactions could be observed with polyphenols but only to a small extent (data not shown).

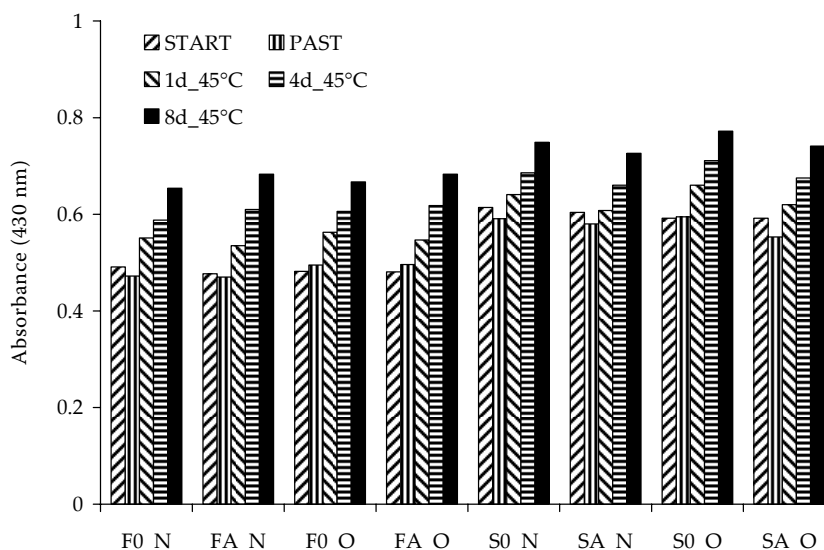


Figure 6. Colour changes of fresh, unpasteurised (F) and aged pasteurised bottled beer (S – stored 1 year at 20°C) followed by pasteurisation and heating (1–8 days at 45°C) with (FA, SA) and without ascorbic acid (F0, S0) under aerobic (O) and anaerobic conditions (N)

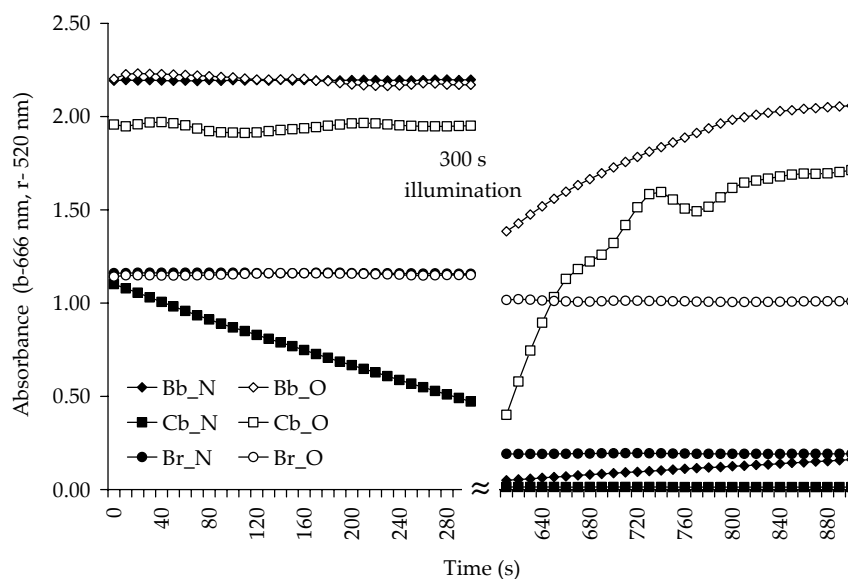


Figure 7. Absorbance of beer (B) or caramel (C) with methylene blue (at 666 nm) or methyl red (at 520 nm) addition (10 mg/l) before (0–300 s) and after 5-min exposure to visible light (610–900 s). Bb – beer + methylene blue, Cb – caramel + methylene blue, Br – beer + methyl red, under anaerobic (N) or aerobic (O) conditions

The same behaviour could be observed during heating of the mixture in the dark, but all the changes were much slower. During this process, electrons were transferred from reductones to oxygen through methylene blue, but irreversibly, because reductones degraded into sugar fragments. The reductone degradation is probably caused by oxygen reactive species that are generated during oxygen reduction.

Methyl red, in contrast to methylene blue, can be degraded only under anaerobic conditions and the process is irreversible. This can be used for the study of electron transport under anaerobic conditions. Methylene blue (MB) and methyl red (MR) are suitable oxidation-reduction indicators because they act differently under aerobic or anaerobic conditions (Figure 7). Methylene blue, which was reduced by caramel even before the exposure to light, presents an example of a reversible indicator oxidisable by air. Methyl red is an indicator irreversibly destroyed by reductones although oxygen can prevent the reaction.

Differential spectroscopy of beer after exposure to visible light and during aging

The light can reduce beer absorbance in the initial part of visible spectrum where shorter wavelengths (higher energy frequency) occur. Only small absorbance recovery during storage after illumination

was observed under aerobic or anaerobic conditions and the degradation process is expected to prevail. The longer was the exposure to the light, the deeper bleaching was observed (Figure 8). At the exposure of beer to light, it showed up that short wavelengths of the spectrum caused a greater absorbance decrease.

The same decrease of absorbance at low wavelengths was observed at the beginning of beer storage at 20°C. As the temperature rose, the decrease diminished and the absorbance increased. Typical shapes of differential spectra were able to reflect and distinguish such subtle changes that had occurred in beer after some minutes of heating at 60°C or one- or two-day storage at 20°C.

According to the previous results, the beer aging process is accompanied by a decrease of absorbance in a range close to 380 nm, while the absorbance recorded at long wavelengths increases. At higher storage temperatures, this decrease of the absorbance at low wavelengths fades away, the absorbance increases within the whole spectrum and the beer samples turn brownish.

DISCUSSION

Electron exchange during aging

Epicatechin and caramel pigments, besides acting as acid/alkaline indicators, represent typical redox

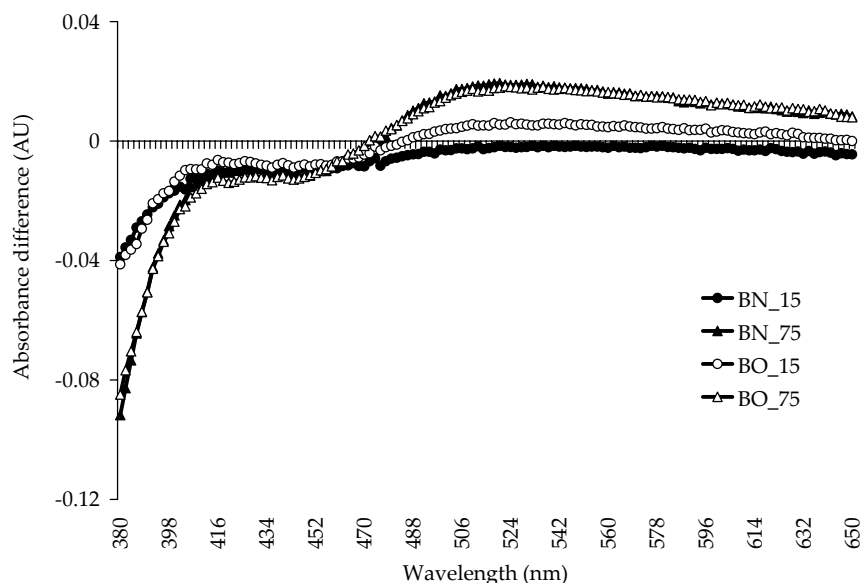


Figure 8. Differential spectra of unpasteurised beer (B) bubbled through with nitrogen (BN) or air (BO) followed by exposure to visible light for 15 min and 75 min

indicators which, although working reversibly in the initial steps of the beer production, gradually become irreversible by various mechanisms (Figures 3–5). The beer colour decreases during the reduction steps of the beer production such as fermentation, but the addition of reducing agents such as ascorbic acid to beer does not stop the colour increase in later stages of beer aging (Figure .6). The colour of beer increased or decreased during heating or under exposure to the visible light (Figure 8).

The colour increase or decrease can be caused by (i) reversible oxidation/reduction of pigments, (ii) their irreversible degradation.

Reversibility or irreversibility represents a substantial feature of aging. The aging of beer is based on the reactions such as:

1 reductone \leftrightarrow reductonyl radical \leftrightarrow dehydroreductone \rightarrow colour products \rightarrow further polymerisation or degradation (colour increase and decrease)

2 polyphenol (*o*-diphenol) \leftrightarrow semiquinone radical \leftrightarrow quinone \rightarrow colour products \rightarrow further polymerisation or degradation (colour increase and decrease)

The amount of redox pairs increases and the degree of irreversibility increases, which gives rise to the reactions rate while aging continues without reaching equilibrium. This theory is supported by our experiments, where the colour products formed by warming of the epicatechin solution could not be fully reversed to the original colourless substances.

Polyphenols and reductones can interact. The model of a partially reversible oxidation reaction

between the oxidised products of polyphenols and reductones corresponds to the irreversible transformation:



where: OP stands for oxidised polyphenols, R for reductones, OR for coloured oxidised reductones, and RP for reduced polyphenols

The formation of oxidative radicals requires a reducing compound which acts concurrently as a radical scavenger. The critical ratio of the oxidation/reduction pair is therefore expected for the maximal rate of beer aging. The pro- or antioxidant behaviour was proved in melanoidins (AMES 2001). Similarly, sulphite is considered to be an antioxidant although it undergoes radical oxidation in the presence of oxygen and metal catalyst; in the course of this reaction, phenylacetaldehyde can be formed from phenylalanine (SAVEL 2001).

The reaction rate of aging can be influenced by chemical or photochemical catalysis. Both a decrease and an increase of absorbance were detected even inside one absorption curve expressed as differential spectra of beer exposed to visible light (Figure 8). New compounds, which arise by these reactions, extend the spectrum of species enabling electron transport.

Differential spectra technique proved to be a useful tool for studying the changes in beer (SAVEL 2005). Visible light of short wavelengths at first

decreased the absorbance of beer; which was also observed during beer storage at 20°C, this initial colour change being followed by colour increase. This seems to imply that all these changes are based on the same principle. The reductive colour pigments are first destroyed; the colour then increases, which is associated with reoxidation or oxidation of polyphenols.

The changes of beer colour during aging comprise reversible changes followed by irreversible ones such as anaerobic reductone polymerisation or degradation as well as polyphenol air oxidation. Oxidised polyphenols and caramels form a balanced oxidation-reduction system in which a slow electron exchange continues even under anaerobic conditions. The temperature, light, and oxidation agents accelerate the process (SAVEL *et al.* 2008).

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