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New oxidation destructive analysis (NODA)

Beer pigments compose of caramels, melanoidins and polyphenols pigments, which can be prepared by heating or boiling of mild colored or colorless precursors, such as sugar or (–)-epicatechin. The natural pigments behave as natural pH and redox indicators. During preparation of caramels by sugar heating at 175 °C in oven, various sugars came into slight yellow (maltose), brown (glucose, mannose, ribose, xylose), or dark brown (arabinose, fructose) products. At heating, reductones were formed prior to caramel pigments. Redox indicators were added to beer and the absorbances (at 666 nm for methylene blue, 520 nm for methyl red and 610 nm for indigocarmine) were recorded before and after illumination with visible light (5 min) under aerobic and anaerobic condition. Natural or synthetic indicators can change their color reversibly or irreversibly. Reducing compounds can initialize oxygen free radical formation as well as their scavenging. 1,2-diaminobenzene addition supported the oxygen consumption in beer.

Descriptors: reductone, caramel, antioxidant, prooxidant, 1,2-diaminobenzene, indigocarmine

1 Introduction

Increasing beer color has long been considered to be reliable marker of beer ageing. The absorbance of beer can also decrease at the short wavelengths especially after illumination or hydrogen peroxide addition, which is usually imputed to colored compounds degradation [3, 6, 12].

The consumers use their eyes to assess beer color and recognize the degree of beer ageing. Spectrophotometric methods for beer color measurement recommend various wavelength (e.g. 530 nm was lately replaced by 430 nm). The absorption of beer gradually decreases with increasing wavelength.

Nowadays various tristimulus measurements are replacing the absorption value at single wavelength. A new method called differential spectroscopy has been also developed to recognize gentle color changes, which are caused by electron transport during oxidation of compounds that are naturally present in beer and absorb in the visible region [12].

The beer color is related to redox state of beer which changes during beer ageing. The measurement of beer absorption provided the "red color index" expressed as the ratio of beer absorbance at 465 and 500 nm.

The usage of beer color measurement can be enriched using redox indicators to beer followed by absorbance measurement at suitable wavelength under well defined reaction conditions. There are many methods for the redox potential measurement, which can be divided into chemical, radical and physical methods.

The color increase can be artificially generated by addition of oxidative compounds such as potassium peroxodisulfate, potassium dichromate or potassium permanganate, which is also accompanied by formation of haze and stale flavor compounds. Oxidation agents are efficient under both anaerobic and aerobic conditions but the presence of oxygen made the effect more distinct.

This procedure was called Oxidative Destruction Analysis (ODA). The strong correlation between increasing beer color and decreasing colloidal instability was proved. The formation of the degradation products of sugars such as furfural and hydroxymethylfurfural was also recognized [13].

Sugar pigments and reductones are also formed by thermal sugar degradation during malting or brewing process e.g. on the hot wall of brewing kettle. Aminoacids can catalyze Maillard reaction which provides colored melanoidins. On the other hand the natural reducing compounds such as reductones or polyphenols can inhibit oxidation reactions based on radical or non radical processes. Prooxidant or antioxidant effect of those compounds have been described [1, 2, 10].

Color indicators have been traditionally used for wort and beer redox state determination usually in the presence of air without measurement under anaerobic condition. Some indicators used e.g. α,α′-dipyridyl reclaimed strongly acid condition, which disabled reaction under natural condition, occurring in packaged beer. Classical redox indicator 2,6-dichlorophenolindoindophenol (DCIP) has been used for the estimation of redox potential of wort and beer in the presence of oxygen. The usage of colored indicators in brewing has been reviewed by several authors [8, 9, 11].

Several organic dyes were lately used to recognize not only redox changes under aerobic/anaerobic condition but also to study degradation processes connected to beer ageing. We have used

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Tables and figures see Appendix

three other organic dyes (MB – methylene blue, MR – methyl red, INDC – indigocarmine) suitable for wort or beer redox state determination under aerobic or anaerobic condition (Table 1). Redox indicators were reversibly or irreversibly reduced or oxidized after they had been added to beer. The velocity of color changes depended on the presence of oxygen. Visible light usually accelerated these changes.

Recently we have studied color changes of melanoidins, caramels and oxidised polyphenols which can be considered as natural redox and acid alkali indicators. Enzymatic or nonenzymatic polyphenol oxidation or reduction changes represent model examples of processes in beer production and ageing.

New oxidative destruction analysis (NODA) has been developed as a set of techniques comprising usage of several redox indicators, redox reaction accelerators with UV/VIS spectrophotometry. NODA can be used to study redox reactions measuring reversible or irreversibile color changes of pigments or synthetic dyes after their addition to beer and during its ageing.

The redox state of beer is changed using physical or chemical factors including addition of natural or artificial oxidation/reduction compounds or indicators to wort or beer.

2 Materials and methods

2.1 Stock and working solutions

Various sugars, (–)-epicatechin, methylene blue (MB), methyl red disodium salt (MR) indigocarmine (INDC), 2,6-dichlorophenolindophenol (DCIP), disodium disulfite and 1,2-diaminobenzene (DAB) were purchased from Sigma Aldrich together with components (KH_2PO_4, $Na_2HPO_4.2H_2O$, H_3PO_4, citric acid) for the phosphate or citric acid/ phosphate buffers (pH 4.6, 7.0, 7.6) preparation. The salts or acids (0.1–0.2 mol/L) was dissolved in deionized water to get buffer solution.

Stock solutions of redox indicators were prepared in deionized water all at concentration 1 g/L. The solutions were pipetted into the samples in a ratio of 1:100.

Stock solution of DAB was prepared by vortexing solid matter (100 mg) in 96 % ethanol (5 ml) followed by addition of deionized water (5 ml). The solution was pipetted into the samples in a ratio of 1:100 or 1:10.

Soft tap water contained 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).

2.2 Sugar reductone and polyphenol pigments formation

Sugars were heated at 175 °C for 10, 20 and 30 min in oven and caramel formation observed (Fig. 1).

Reductone and caramel pigments from maltose and other sugars were also formed in buffers or tap water (pH ~7) solutions (10 % m/m). Maltose (10 % m/m) was dissolved in buffers (pH 4.6, 7.0, 7.6) or tap water with and without redox indicator addition. The solutions (5 mL) in test tubes were evacuated to remove air and the tubes were immersed into water (100 °C) or glycerol bath (120 °C) and time needed for redox indicators decolorization was recorded (Table 2).

Polyphenol pigments formation in beer was studied with and without epicatechin addition (100 mg/L) into beer that was then stored at 45 ºC for three days under aerobic or anaerobic condition (Fig. 6).

Anaerobic condition was simulated by bubbling nitrogen (20 min) or oxygen (10 min) through the samples in the cuvettes which were then tightly sealed.

2.3 Visualization of oxygen transfer from headspace to beer in the bottle

Methylene blue (10 mg/L) was added to fresh beer and the bottle crowned. After pasteurization (1 h at 60 ºC) the beer was aged for 1 week at 45 ºC. The bottle was opened and after air entrance the bottle was recrowned (Fig. 3).

2.4 Acceleration of redox reactions by visible light illumination

Redox indicators were added to beer and the absorbances (at 666 nm for MB, 520 nm for MR and 610 nm for INDC) were recorded before and after illumination with visible light (5 min) under aerobic and anaerobic condition (Fig. 4). INDC stock solution was added to disodium sulfite solution in tap water and absorbance (at 610 nm) was recorded before and after illumination with visible light (5 min) (Fig. 5).

2.5 Instruments

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 2x50 W from a distance of 1 cm from the cuvette in flowing cooling water.

Spectrophotometer Hach-Lange DR 5000 equipped with cylindrical glass cells (cuvettes) with an optical path of 1 cm was used for recording absorption spectra (Fig. 2).

Dissolved oxygen measurement was made with fluorescent electrode Hamilton (12 mm diameter) immersed into test tube (diameter 18 mm) containing sample (20 ml) and the concentration of dissolved oxygen was measured.

3 Results and discussion

3.1 Formation of reductones and colored sugar pigments

During preparation of caramels by sugar heating at 175 °C in oven, sucrose and lactose stayed unmelted even after 20 min, while the other sugars came into slight yellow (maltose), brown (glucose,

mannose, ribose, xylose), or dark brown (arabinose, fructose) (Fig. 1). The differences between sugars grew with the time of heating. At heating, reductones were formed prior to caramel pigments.

DCIP was found to be the most suitable for the visualization of reductone formation in maltose solution (Table 2). The intensity of reductone formation from various sugars diluted in tap water was also measured by time of INDC decolorization at 100 °C. The time changed in the sequence: fru (2 min) < ara ≤ glu ≤ rib ≤ malt ≤ man ≤ lac ≤ suc (time >1 h) all without pigments formation, e.g. fructose provided stronger reducing power than sucrose. There is probably some correlation between the degree of the sugar decomposition and the reductone formation which could influence beer ageing.

The pigments were also prepared by maltose solution heating. The higher pH the higher color was obtained. Dark (at pH = 7.6), slight yellow pigments were formed at neutral pH (buffer, tap water) no pigments were formed at pH 4.6.

The colored caramel formation begins in later stages of heating and it is dependent on pH value. The caramelization and Maillard reaction are indispensible part of brewing process including malt kilning and roasting (low water activity) or wort boiling including caramel formation on the wall of the kettle.

3.2 Absorption spectra of redox indicators in beer

Beer pigments compose of caramels, melanoidins and polyphenols pigments, which can be prepared by boiling of mild colored or colorless precursors, such as sugar or (–)-epicatechin. The natural pigments behave as natural alkali and redox indicators.

Synthetic dyes were added to beer to extend the group of indicators especially at long wavelengths (Fig. 2). Redox indicator stock solution was added to pale lager (o.g. 12 % m/m) and absorption spectra was recorded (Fig. 2). DAB addition to beer changed absorption spectrum of beer only a little.

The most of the methods work under aerobic condition without care of their running under anaerobic one. The reaction mechanism of ageing in the absence of oxygen can therefore be hardly understood. The usage of the indicators used in this works enables to compare the course of ageing in the presence or absence of oxygen. The technique of differential spectroscopy might distinguish between natural beer color changes and color changes after indicators addition.

MB was then used for the visualization of oxygen transfer from the bottle headspace to beer (Fig. 3). After all oxygen had been consumed in the bottle with beer and MB decolorized the bottle was opened (air entrance) and then recrowned to show gradual penetrating oxygen to beer.

The blue color interface between oxidized (blue) and reduced form (beer color only) of methylene blue responds to the lack of beer capability to reduce dissolved oxygen after its entrance to beer.

The methylene blue visualization of the oxygen entrance into beer enables following the velocity of beer oxidation by oxygen from air and degradation of reducing compounds which are important for beer freshness preservation. Detailed study of this method has not been published yet because of limited extent of this article and it will be described in a future.

3.3 Acceleration of redox reactions by visible light illumination

Redox indicators were added to beer and the absorbances (at 666 nm for MB, 520 nm for MR and 610 nm for INDC) were recorded before and after illumination with visible light (5 min) under aerobic and anaerobic condition (Fig. 4).

Visible light accelerated electron exchange between redox indicator and beer reductones. Methylene blues was reversibly reduced by beer reductones into its leucoform under both aerobic (aeration) and anaerobic (nitrogenation) conditions, which was then rapidly reoxidized by oxygen. In the absence of oxygen only mild reoxidation of MB was observed.

Methyl red was irreversibly destroyed by beer reductones at illumination under anaerobic condition. The degradation could be completely inhibited by the presence of oxygen. The MR bleaching under anaerobic condition can comprise irreversible reduction and splitting of azo group or its oxidative degradation by hydrogen peroxide in beer.

Slight decrease of absorbance at 610 nm can be explained by reversible INDC reduction by beer reductones or its oxidative degradation by hydrogen peroxide presence in beer. INDC splitting was also proved during maltose oxidation by oxygen in the presence of radical initiator without reductone formation.

The role of indigocarmine and its degradation were also studied in the tap water containing disodium disulfite (Fig.5). Indigocarmine showed complicated reaction during illumination. Although present sulfur dioxide is considered to generally act as antioxidant, it did not stop INDC irreversible degradation in the presence of oxygen. Reactive oxygen species (ROS) formation as intermediate products of oxygen reduction by sulfur dioxide are contrary supposed

Indigocarmine was irreversibly reduced only in the presence of oxygen. Tap water was chosen because the oxidation of sulfite is catalysed by metals, which are naturally present in water. The mechanism of INDC degradation was discussed in the literature repeatedly [7].

Sulfur dioxide although generally acting as antioxidant (ROS scavenger) can act as important prooxidant (ROS formation) in the presence of oxygen.

Other type of antioxidant e.g. epicatechin can inhibit or support beer browning (Fig. 6). The presence of oxygen can make decision whether the same compound works as antioxidant or prooxidant. Epicatechin could trap ROS changing itself into quinones, which could later act as a prooxidant.

Similarly indigocarmine can also be degraded in the presence of oxygen and ascorbic acid as a kind of reductone.

3.4 Colour changes of indicators in beer containing DAB

Redox indicators were added to beer at 20 °C and the absorbances (at 666 nm for MB, 520 nm for MR and 610 nm for INDC) were recorded under aerobic and anaerobic condition without illumination. After two minutes DAB stock solution was added to the mixture and absorbance measurement continued (Fig. 7).

It is impossible to reach MB reduction in beer by its illumination combined with presence of oxygen (Fig. 4). The equilibrium between reductones and sugar dicarbonyls is suppposed to exist. Dicarbonyls were eliminated by DAB addition which might support further reduction of MB. Oxididised reductone could also take part in MR bleaching whereas INDC showed only small changes thanks to combined oxidation reduction. This kind of redox reaction is typical of sugar reductones whereas DAB addition doesn't influence the effect of disodium disulfite.

Sugars dicarbonyl compounds are supposed to take part important role in beer ageing. DAB could inhibit beer ageing via sugar dicarbonyls binding although other mechanisms have been mentioned [4, 14].

3.5 Oxygen consumption in beer containing DAB

To test the theory the DAB solution was added to beer and oxygen concentration was measured (Fig. 8). Sugar dicarbonyls reaction with DAB supported the oxygen consumption in beer. It might be the way how to measure dicarbonyls concentration.

4 Conclussion / Summary

A new oxidative destruction analysis (NODA) is a group of procedures used for study of mechanisms of beer ageing based on measuring reversible or irreversible color changes of natural pigments or synthetic dyes after their addition to beer which undergoes light or temperature shocks under aerobic or anaerobic conditions. Transport of oxygen from the bottle neck to beer in bottle was visualized with the help of methylene blue indicator. The methylene blue visualization of the oxygen entrance into beer enables following the velocity of beer oxidation by oxygen from air and degradation of reducing compounds but detailed study of reproducibility and general applicability has not been realized yet. Reducing compounds of beer besides acting as antioxidant scavengers of reactive radicals can by contraries act as generators of reactive species as a result of reduction of neutral oxygen molecules. There are two groups of those compounds: one (e.g. SO_2) that provide oxidation products which remain stable (anion SO_4^{2-}) and the other whose oxidation products can be further reduced providing another oxidation compounds (oxidised polyphenols and caramels). The equilibrium between reductone and its oxidized form (dicarbonyl) is supposed to exist. DAB addition supported the oxygen consumption in beer probably by moving equilibrium between sugar reductones and dicarbonyls.

5 Literature

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Appendix

Table 1 Redox indicators for aerobic/anaerobic condition

Dye	Reaction mechanism
MB	reversible oxidation/reduction under aerobic/anaerobic condition.
MR	irreversible degradation under anaerobic condition
INDC	reversible anaerobic reduction or irreversible aerobic degradation
DCIP	reversible oxidation/reduction under aerobic or anaerobic condition

Table 2 Decolorization time (min), of various redox indicators during anaerobic heating of maltose solution (10 % w/w)

pH	DCIP	INDC	MB
4.6 (buffe)r	7	>60	60
7.0 (buffer)	2	12	7
7.0 (tap water)	2	>60	4
7.6 (buffer)	1	3	3

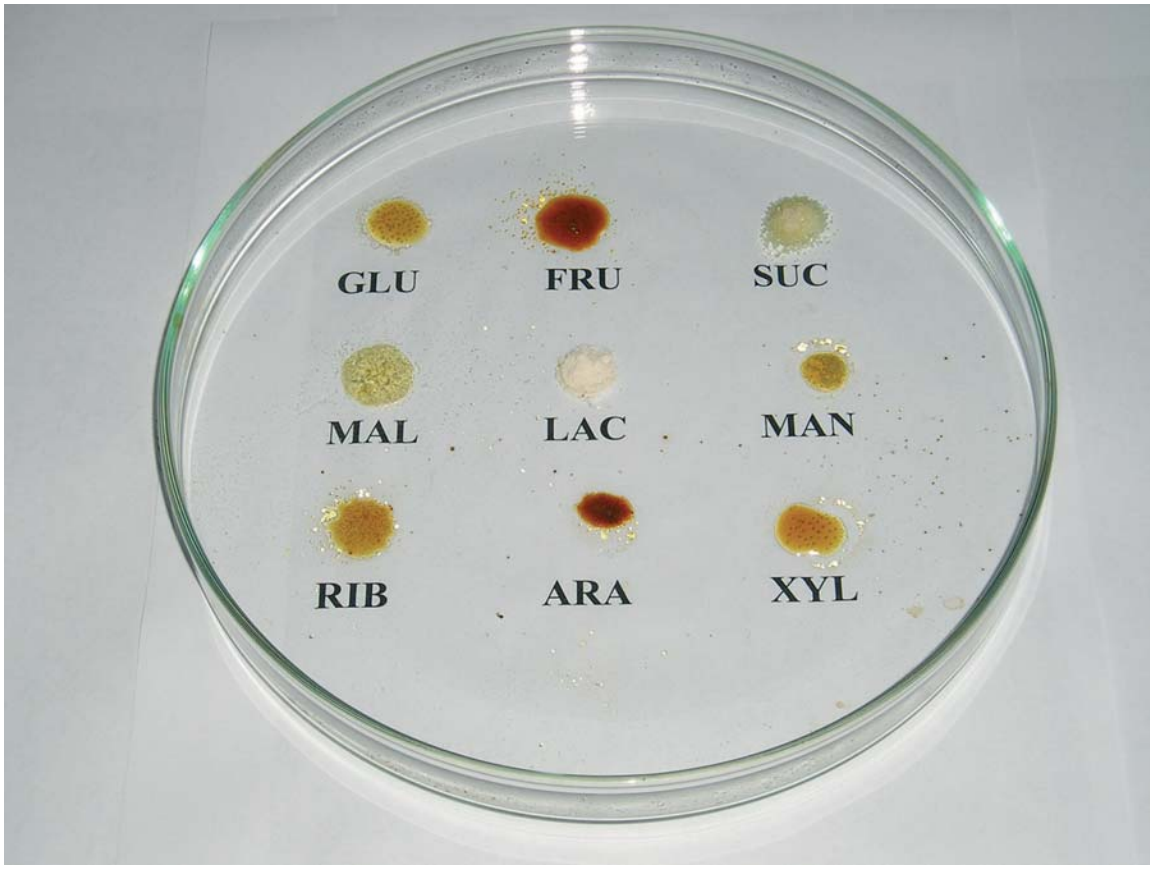

Fig. 1 Caramelization of various sugars heated at 175 °C for 20 min. GLU – glucose, FRU – fructose, SUC – sucrose, MAL – maltose, LAC – lactose, MAN – mannose, RIB – ribose, ARA – arabinose, XYL – xylose

Fig. 2 **Absorption spectra of various indicators (MB – methylene blue, MR – methyl red, INDC – indigocarmine) dissolved in beer (each at 10 mg/L)**

Fig. 3 **Visualization of oxygen transfer from bottle neck to beer through reoxidation of the previously reduced form of methylene blue for two volumes of headspace loaded with air**

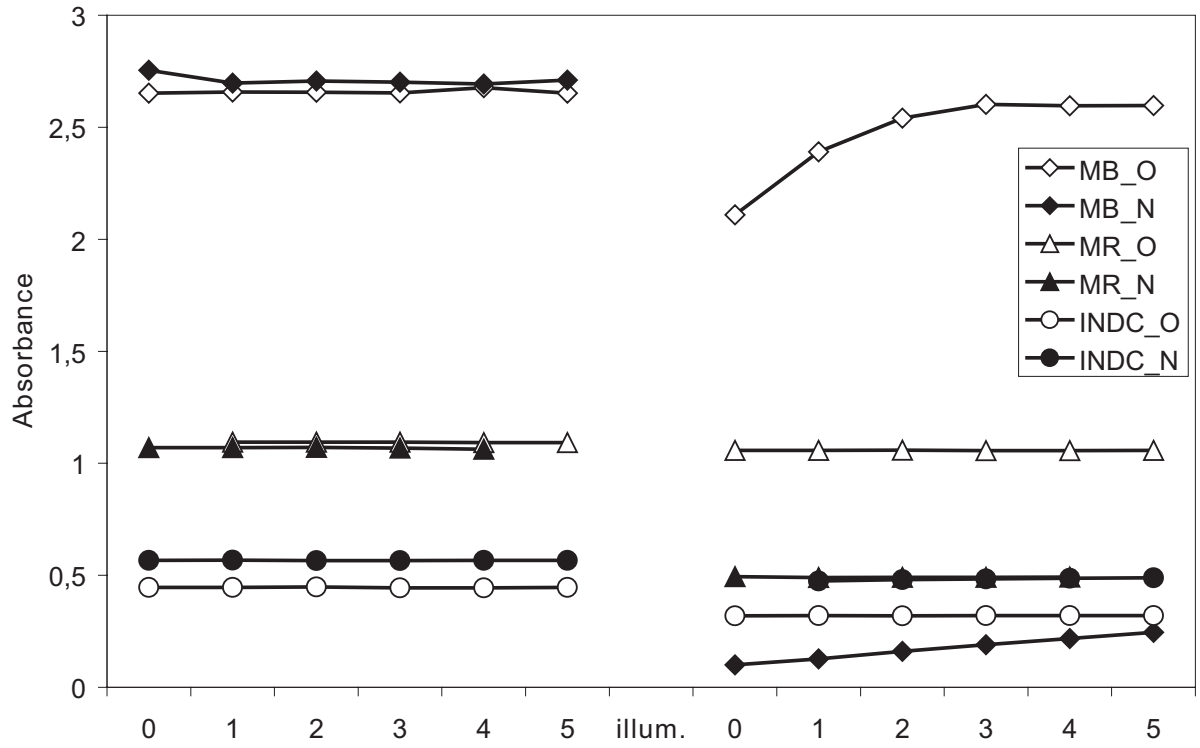

Fig. 4 **Absorbances of various indicators dissolved in beer (10 mg/L) taken in 1 min intervals before and after illumination (5 min). MB – methylene blue (666 nm), MR - methyl red (520 nm), INDC – indigocarmine (610 nm) in sealed cuvettes after initial aeration (index _O) or nitrogenation (index _N)**

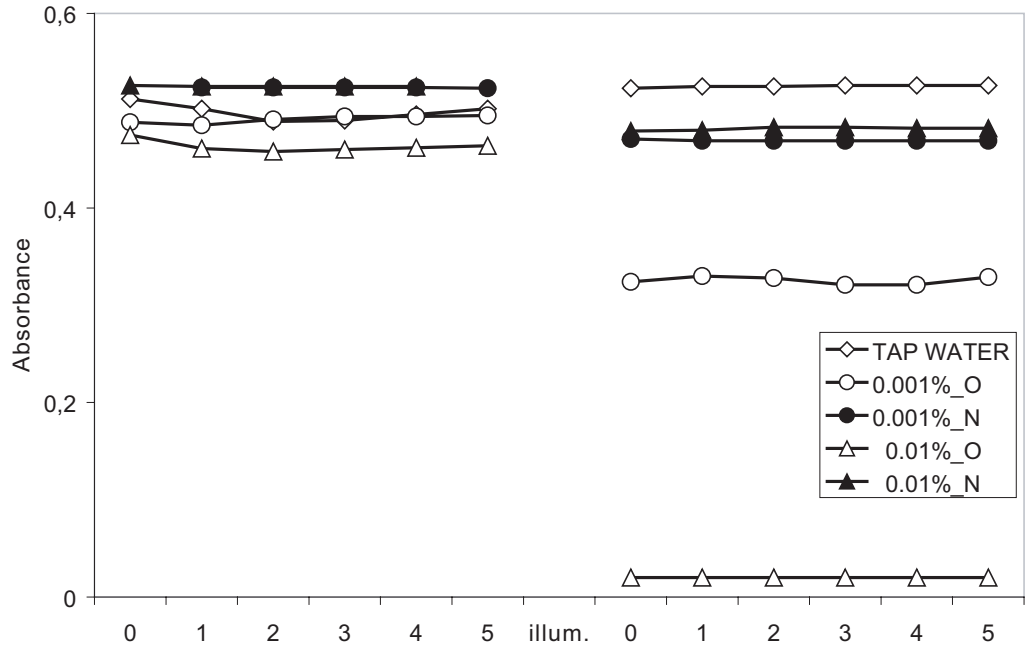

Fig. 5 **Absorbance (610 nm) of indigocarmine (10 mg/L) in disodium disulfite (0.001 and 0.01 % m/m) solutions taken in 1 min intervals before and after illumination (5 min) in sealed cuvettes after initial aeration (index _O) or nitrogenation (index _N)**

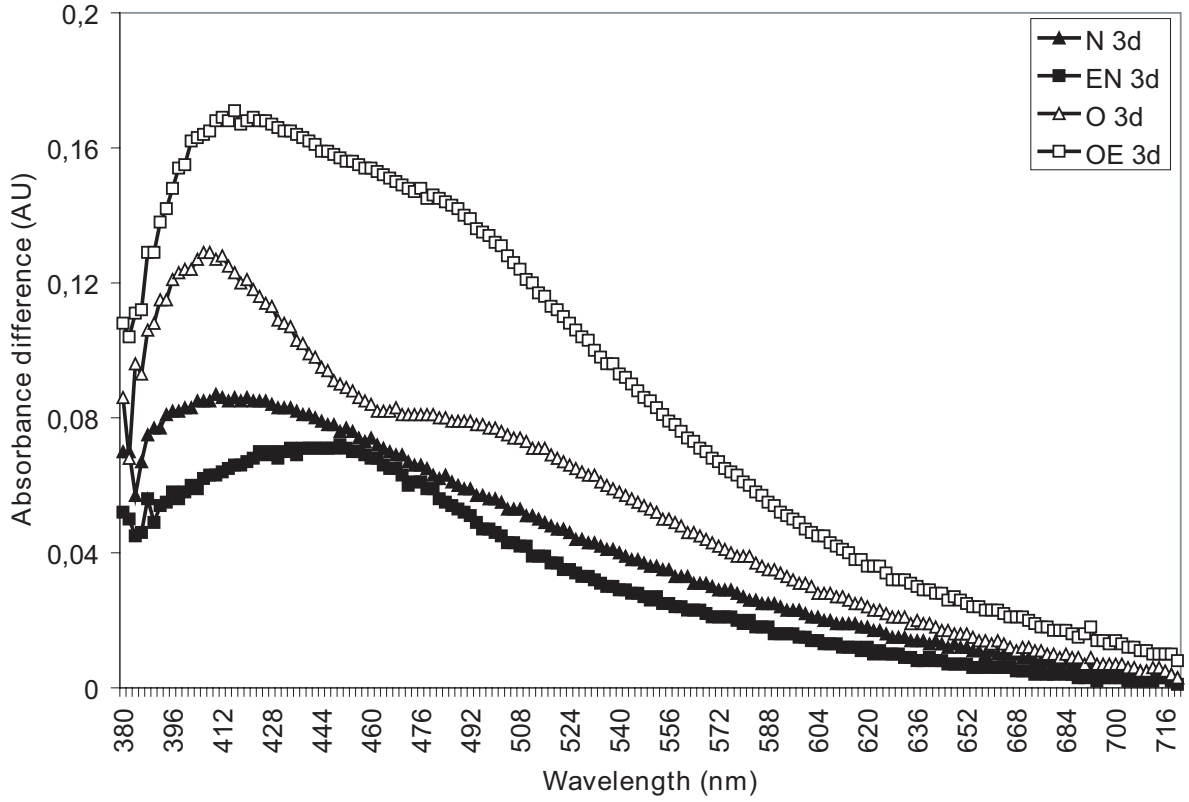

Fig. 6 **Absorption spectra of beer without and with epicatechin (with E) addition (100 mg/L) heated for 3 days at 45 ºC in sealed cuvettes after initial aeration (index O) or nitrogenation (index N)**

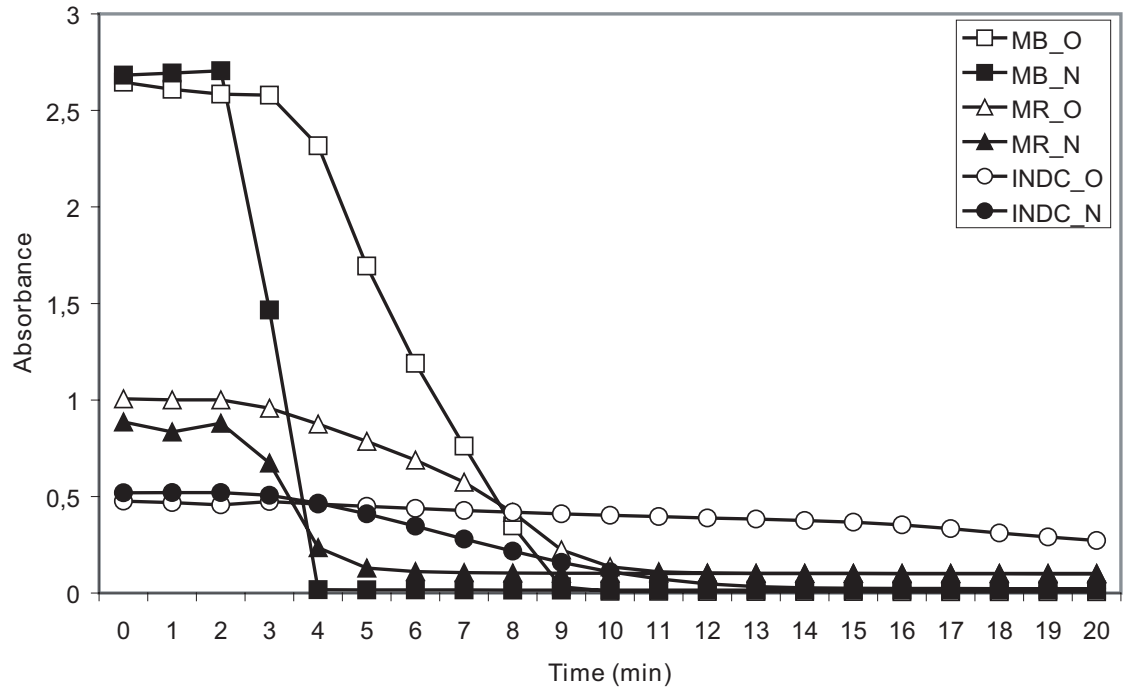

Fig. 7 **Absorbance of various indicators in beer (MB – methylene blue, MR – methyl red, INDC – indigocarmine) dissolved in beer (each at 10 mg/L) before and after addition of DAB (at 2nd min) in sealed cuvettes after initial aeration (index _O) or nitrogenation (index _N)**

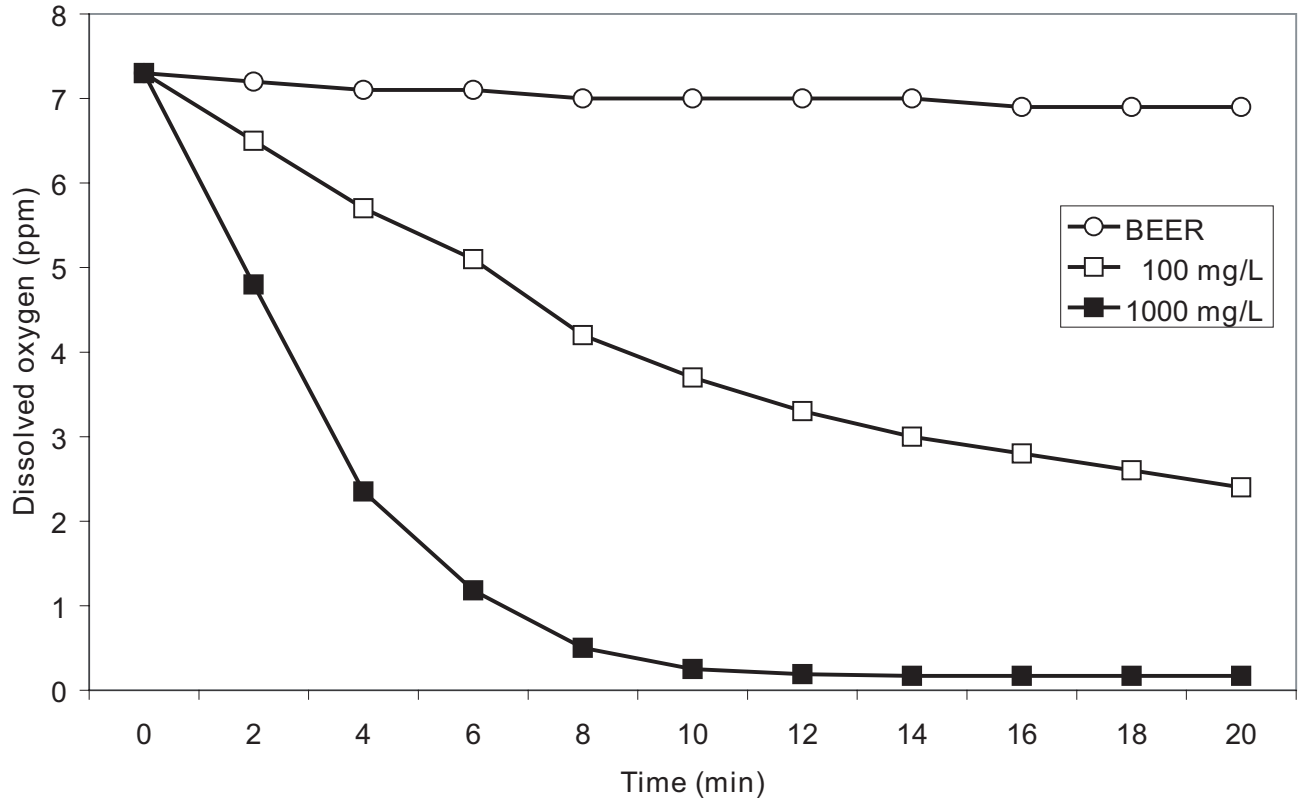

Fig. 8 Concentration of dissolved oxygen (ppm) in beer after DAB (1,2-diaminobenzene) addition (100 a 1000 mg/L)