J. Savel

Negative role of oxidised polyphenols and reductones in beer

The oxidation-reduction reactions of the oxidised pyrogallol as an example of oxidised polyphenol with ascorbic acid were studied using methylene blue reoxidation reaction. This technique uses methylene blue photobleaching followed by its colourless leucoform reoxidation. Oxidation activity of oxidised pyrogallol increased with the time of pyrogallol oxidation. Polyphenols can act as an carrier of oxygen to reductones which is associated with the formation of degradation products accelerating electron exchange. The illumination also accelerated the redox reaction between polyphenols and reductones providing various radical and non-radical oxidation species. Beer ageing comprises polyphenol or reductone oxidation providing reactive species under aerobic or anaerobic condition

Descriptors: beer ageing, photooxidation, oscillating reaction, polyphenol, reductone.

1 Introduction

Beer undergoes oxidation changes caused by a complicated oxidation mechanism. Important beer compounds are damaged or destroyed during this process giving simple reactive products such as sugar aldehydes, polymerized polyphenols or volatile aldehydes formed from aminoacids and alcohols (1, 2).

This process is irreversible and it can't be completely reversed but only slowed by reducing agents such as ascorbic acid, sulphite or dithionite. Neither yeast reductases can convert sugar and aminoacid degradation products into initial compounds although the reduction of some aldehydes into former alcohols can occur. The mixture of reversible and irreversible reactions is the main feature of beer ageing, which is supported by temperature, light and various oxidation agents such as oxygen in the presence of catalysers.

Beer reductones and the Maillard reaction products play an important role in beer ageing to be natural electron donors. The degradation products of sugars contribute to non-oxidative browning of aminoacids and proteins by rearrangement and elimination pathways which generate deoxydicarbonyl compounds, such as deoxyglucosone and methylglyoxal. Paradoxically they can act as prooxidants as well as antioxidants enabling electron exchange (3, 4).

On the other hand polyphenols can also take part in ageing beer via higher alcohols oxidation in the presence of Cu^{2+} and oxygen (5). Some of them have an amine oxidase-like activity which is similar to oxidative ability of the Maillard reaction products (6). Polyphenols easily undergo oxidation generating reactive oxidised products which reacts with beer proteins forming haze (7).

It has commonly been believed, that beer colour reading at 430 nm responds mostly to melanoidins while polyphenols absorb at long wavelengths and the beer colour increases during ageing.

Differential spectroscopy helped to recognise gentle colour changes attesting to electron transport during oxidation of compounds that are naturally present in beer and absorb light in the visible region. Another technique has been developed based on beer oxidation by methylene blue which was accelerated by light. Photobleached methylene blue reoxidation in the presence of ascorbic acid determined the range of the oxygen reactive species formation.

Recently we have concentrated on polyphenol role in electron transport. Quinones were added to beer that was pasteurized in the presence or absence of oxygen. Simple quinones could act as catalysers accelerating ascorbic acid oxidation. Oxygen was consumed by beer in a short time but beer ageing continued in the absence of oxygen. Various mechanisms of ageing such as oxidative action of quinones, peroxides or anaerobic Fenton reaction were expected to play an important role in this process (8).

The aim of this work is to elucidate the mechanism of electron transport between beer reductones and polyphenols.

2 Experimental procedures

2.1 Chemicals

Stock solutions: Methylene blue (1000 mg.l^{-1}), cupric chloride dihydrate $CuCl^2.2\ H2O$ (0.0268 g.l^{-1} = 10 mg.l^{-1} Cu^{2+}), ferrous chloride tetrahydrate $FeCl^2.4\ H2O$ (0.0356 g.l^{-1} = 10 mg.l^{-1} Fe^{2+}), ferric chloride hexahydrate (0.0484 g.l^{-1} = 10 mg.l^{-1} Fe^{3+}), ascorbic acid (1.0 % w/w), potassium bichromate (1000 mg.l^{-1}) and 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 1000 mg.l^{-1}) were prepared by dissolving of the components in deionised water. All chemicals were obtained from Sigma Aldrich.

2.2 Pyrogallol oxidation by air

Pyrogallol solutions (100 ml, 200 mg.l^{-1}) in deionised water in 150 ml Erlenmeyer flasks with and without metal ions (0.1 mg.l^{-1} in the flask) or beer were stored under air at 45 ºC in the dark. Periodically taken samples (4.9 ml) were pipetted into a Dr.Lange round glass cuvettes and absorbance at 430 nm was measured (Fig. 1).

Methylene blue (50 µl) and ascorbic acid (50 µl) stock solutions were added and the cuvettes were illuminated with a halogenous bulb (50 W) from the distance of 1 cm for 5 - 10 s. The absorbance at 666 nm was recorded in 1 min time intervals (Fig. 2, 3).

2.3 Reoxidation of the methylene blue reduced by ascorbic acid

The methylene blue stock solution (50 µl) was pipetted into a Dr.Lange round glass cuvette (diameter = 1 cm) containing

Jan Savel, Budejovicky Budvar, n.p., K. Svetle 4, CZ – 37021, C.Budejovice, Czech Republic

Figures see Appendix

4.9 ml of the deionised water (DI) with and without Cu^{2+} (0.1 $mg.l^{-1}$ in the cuvette) or potassium bichromate (10 $mg.l^{-1}$) all at final concentration in the cuvette). The samples were bubbled by nitrogen for 30 min or by air for 5 min, 50 µl of the ascorbic acid solution (1.0 % w/w) was added (0.01 % w/w in the cuvette) and sealed with rubber stoppers. The cuvettes were illuminated with a halogenous bulb (50 W) from the distance of 1 cm for 5 – 10 s and some of them were covered with aluminium foil while other were exposed to spring daily light in the laboratory. The foils were removed only for the measurement (15 – 20 s) and then the cuvettes were recovered again. The absorbance at 666 nm was recorded in suitable time intervals (Fig. 4, 5).

2.4 Methylene blue degradation

The methylene blue stock solution solution (50 µl) was pipetted into a Dr.Lange round glass cuvette (diameter = 1 cm) containing 4.9 ml of the deionised water (DI). The samples were bubbled by nitrogen for 30 min or by air for 5 min, 50 µl of the ABTS stock solution (in the cuvette) was added (10 $mg.l^{-1}$ in the cuvette) and sealed with rubber stoppers. Half of them were covered with aluminium foil while others were exposed to spring daily light in the laboratory. The foils were removed only for the measurement (15 –20 s) and then the cuvettes were covered again. The absorbance at 666 nm was recorded in suitable time intervals (Fig. 6).

2.5 Absorbance differences during photooxidation of sweet wort, hopped wort and beer

Absorbances of laboratory sweet wort, hopped wort or beer were measured in 1 cm glass cuvettes in five 0.5 min intervals followed by 1 min illumination with a halogenous bulb (50 W) from the distance of 1 cm and the procedure was repeated (Fig. 7).

2.6 Instruments

CADAS 200 spectrophotometer (Dr. Lange, Germany) was used with 1 cm round cuvette using distilled water as a blank.

3 Results and discussion

Pyrogallol is an example of simple colourless model polyphenol with reducing power that undergoes oxidation by air especially in the presence of hydroxyl ions, which is associated with polymerisation and the formation of yellow to brown reaction products. It was used as a model polyphenol in several studies (5,6,9,10,11). Dihydroxybenze and pyrogallol are present in the products of sugar oxidation as well as in fruits and trees (12, 13, 14).

There are two interpretation of the uncertain term "polyphenol": (i) naturally occurring phenols, that contain more than one aromatic hydroxyl group, (ii) molecules with two or more phenol rings (11, 15, 16). In the sense of the definitions the some simple phenolic acids found in beer are sometimes classified as polyphenols.

Although pyrogallol has not been proved in beer its application usually simulates the effect of 1,2,3 trihydroxybenze ring of simple phenolic acids e.g. gallic acid or 3',4',5' trihydroxy B-ring of flavanoids such as (+) gallocatechin and epigallocatechin which are present in beer. This strategy uses high reactivity of such compounds towards to oxygen (5,6,10). The most efficient combination of hydroxyl groups was that of 1,2,4 trihydroxybenzene (5, 6).

Oxidation of the pyrogallol occurs even at slightly acid medium such as deionized water. The presence of cuprum or iron ions at low level (0.1 $mg.l^{-1}$) didn't increase the intensity of the colour substantially after oxidation. Under the same condition the beer colour increased which was probably caused by the polyphenol oxidation because the velocity of Maillard reaction was expected to be too slow (Fig. 1). Increasing colour witness the irreversible changes leading to the polymeric products formation. It is interesting that such changes didn't need any oxidative agent except oxygen even in the absence of metal ions. Similar reaction resulting into cocktail of various products was described for the hydroquinone photooxidation (17).

The oxidation or oxygen carrier activity of the oxidised pyrogallol was tested by the methylene blue reoxidation reaction (8). This technique uses the methylene blue photobleaching connected with the ascorbic acid photooxidation by methylene blue. After reduction of the methylene blue the leucoform reoxidation continues due to reactive oxygen species formation during oxygen reduction.

Ascorbate radical, oxygen-free radicals and hydrogen peroxide are formed during this process (18). The reduction of oxygen provides oxygen reactive species that can degrade other organic compounds. Similar procedure might provide other reactive oxidation species derived from other oxidation agents different from oxygen e.g. quinones even under the condition of anaerobic oxidation.

A lot of degradation products were found after peroxodisulfate oxidation of the monosaccharides. In total 147 compounds were identified in the investigated reaction mixtures. They comprised α-hydroxycarbonyl, α-dicarbonyl and dicarbonyls compounds, diuloses, formaldehyde, acetaldehyde, various derivates of furan and pyrans, aromatic compounds and others. Some of them had strong reducing features (14).

Pyrogallol oxidation provided reaction products which accelerated ascorbic acid oxidation by the methylene blue and oxygen. Beer oxidation showed similar effect including colour increase (Fig. 1, 2, 3).

The redox reaction between methylene blue and ascorbic acid can be accelerated by illumination with artificial source of light or daily ambient light. Methylene blue is supposed to be a sensitizer transferring energy to overcome energetic barrier between both redox pairs.

During illumination the methylene blue is transferred into its colourless leucoform, which is again reoxidised by oxygen. The further methylene blue reduction catalysed by ambient light showed an oscillating pattern between oxidised and reduced form of the methylene blue (Fig. 4).

Non-linear chemical oscillation in various chemical reactions in which inorganic as well as organic oxidation-reduction reactions play an important role. Alkaline sugar degradation in the presence of methylene blue provided oscillating reactions dependent on the kind of sugar, polyphenols caused perturbation of chemical oscillation. Oscillating reactions are to a handful of elements that posses multiple stable oxidation states although oscillation reaction derived from the core reaction was also observed (19, 20).

The course of the oxidation strongly depended on the light intensity, time of illumination and the presence of catalyser or an oxidation agent, which could be oxygen or another oxidation agent such as potassium bichromate (Fig. 4, 5).

Chapon used the methylene blue technique in his fundamental study on electron transport between ascorbic acid and quinone but he preferred the enzymatic oxidation to photobleaching (21). Methylene blue exists in two stable forms, MB^+ and MBH although radical intermediate (MB●) can also occur. Radical intermediates

are responsible for the acceleration of some oxidation-reduction reactions.

Methylene blue photobleaching enables the visualization of this mechanism. At first the reaction is catalysed by light (bleaching) then the generated radicals reoxide methylene blue again. A pseudo-equilibrium state exists between oxidised and reduced form of the methylene blue during ascorbic acid oxidation by oxygen from air.

First the reaction is catalysed by the illumination that causes methylene blue bleaching followed by radical reoxidation of its leucoform (growing blue). Then the bleaching continues but after some time both ascorbic and methylene blue become completely oxidised by oxygen.

The explanation of the electron transport mechanism is based on the fact that many slow reactions can be accelerated by the formation of reactive intermediates e.g. radicals. During this process the number of the oxidation-reduction pairs increases.

The electron transport process depends on both kinetic and thermodynamic reactions. For instance oxygen can spontaneously react with hydrogen (thermodynamic) but the reaction rate is very slow because of high resistance against electron transport. An initiation caused by high temperature or a spark provides radicals which enable the rapid course of hydrogen oxidation. A similar mechanism occurs during oxidation of the organic compounds associated with oxygen reduction, e.g.:

$$O_2 + e^- \rightarrow O_2^{\bullet -}$$
$$O_2^{\bullet -} + e^- + 2\,H^+ \rightarrow H_2O_2$$
$$H_2O_2 + e^- \rightarrow OH^- + HO^{\bullet}$$

Various radicals can be also formed by the oxidation of neutral molecules such as dehydroascorbic acid, quinones, sugars or alcohols.

In the course of the ascorbic acid (an example of a reductone) oxidation two main oxidation products occur (22):

	$-e^- - H^+$		$-e^-$		
$AscH^-$	$\leftrightarrow$	$Asc^{\bullet -}$	$\leftrightarrow$	DHA	
	$+e^- + H^+$		$+e^-$		oxidation or/and
ascorbate	ascorbate radical		dehydroascorbate		degradation (splitting) into
reductone	reductone radical		oxidation product		redox pairs, polymerisation
	reversible process				irreversible process

The presence of oxygen especially with a transient metal accelerates the process although oxygen can be substituted by another oxidation agent e.g. quinone, methylene blue which can also act as an oxygen carrier.

In an analogous reaction a simple phenol or polyphenol is oxidised by an oxidative species:

Poly(phenol)	$\leftrightarrow$semiquinone radical	$\leftrightarrow$quinone	oxidation or/and
			degradation (splitting) into
	reversible process		redox pairs, polymerisation
			irreversible process

In the presence of air oxygen free radicals (OFRs) and hydrogen peroxide are formed in both cases. During beer oxidation quinones can play role of the oxygen carrier or an oxidant. Beer ageing can be considered as a general oxidation reaction with the reactive species formation. Oxidation process is usually associated with the simple catechin dimers generation followed by the polymerisation reaction (23).

There is a certain similarity between the role of the reductons or polyphenols in the course of the oxidation by air: higher oxidation rate at higher pH, high reactivity to oxygen, the formation of the polymerised products, oxygen radicals and hydrogen peroxide. The enol-like groups are present in both types of the reducing agents.

Both polyphenols and reductones (melanoidins) are expected to be involved in electron transport chain transferring electrons from reductones through polyphenols to oxygen or to an oxidative agent. It has been found that also o-quinone compounds which had an α-dicarbonyl group were known to catalyse the oxidation deamination known from the Maillard reaction (24).

Beer photooxidation has been studied for a long time being usually linked to sunlight-struck beer flavour. We have also studied the colour changes in the case of peroxodisulfate oxidation or photooxidation of beer and its intermediates in our previous work.

The starting decrease of melanoidin colour was followed by the polyphenol oxidation, which provided the colour increase. Similar changes occurred during initial phase of beer ageing. The photooxidation of hopped wort and beer provided the absorbance decreases at 380 nm in comparison to the absorbance increase in the sweet wort (8).

Riboflavin is often considered to be the only photosensitizer starting beer oxidation in beer although the participation of photosensitive melanoidins or polyphenols may also be expected.

The initial colour decrease during beer photooxidation, heating or chemical oxidation changes are probably caused by the reductive reaction of reductones with coloured polyphenols. After partial reductone degradation the oxidation prevails and the beer colour increases steadily. During beer ageing oxygen can be continuously delivered through the crown into beer in the bottle (25).

Prooxidant and antioxidant mechanisms of the polyphenol oxidation comprise three different types: (i) the formation of semiquinones and their disproportionation into reactive methides acting as prooxidants (ii) the enhancement of the antioxidant potential after coupling quinones with parent flavanoids (ii) the antioxidant effect linked to parallel attack at various sites of flavanoids (26).

The oxidation-reduction theory was confirmed in the course of beer oxidation by methylene blue in the light and dark. Beer reduces methylene blue even in the absence of ascorbic acid because reductive action of beer reductones is sufficient for the partial methylene blue decolourisation. The initial decolourisation of the methylene blue was followed by its colourisation as a consequence of further oxidation process. Further illumination increased the range of reduction but the changes were less evident using aged beer. It might be caused by both reductone degradation and polyphenol oxidation.

It is reasonable to expect that any acceleration of the oxidation reaction in beer is associated with irreversible changes such as irreversible degradation of natural beer compounds. Beer ageing comprises polyphenol or reductone oxidation providing reactive oxidising species.

Non-oxygen radicals can occur during thermal degradation of organic compounds. An action spectrum from 320 to 400 nm was determined for both ascorbate oxidation and the photobleaching of the advanced glycation end products at anaerobic condition (27).

An example of anaerobic degradation reaction is methylene blue degradation by ABTS. Reactive radical of ABTS was able to degrade methylene blue although strong oxidation using Fenton reaction was less efficient. The rate velocity of oxidation-reduction reactions depends on both kinetic and thermodynamic attributes and the presence of suitable redox pairs can accelerate this process when kinetic point prevails.

The typical photochemical oscillating anaerobic degradation reaction which can be realised in beer is the degradation of the methyl red which accelerates with increasing reduction power or hydrogen peroxide addition.

The methylene blue degradation by ABTS is an example of non-oscillating anaerobic reaction (Fig. 6). Carbon centered radicals were formed by oxidative decomposition of beer bitter acids (28).

Recently the new experimental results seem to confirm our expectation. Polyphenols and Maillard reaction products implied in beer ageing have been identified in the organic extract. The initial absorbance decrease of $ABTS^{\bullet+}$ radical was observed followed by slow increasing (29).

4 Conclusion

- Oxidised pyrogallol acceleretes ascorbic acid oxidation by the methylene blue.
- Oxidised pyrogallol is a model oxidised polyphenol causing ascorbic acid or reductone degradation.
- Both pyrogallol and ascorbic acid undergoe degradation, which is associated with formation of other reactive radicals or non-radical oxidation agent. By the combination of the reaction products the polymerisation can occur.
- Ageing beer is based on the redox reaction between reductones and polyphenols providing reactive species.
- This process is strongly speeded up in the presence of oxygen which is caused by OFR and ROS formation.
- The final effect of this reaction is the accumulation of the oxidised polyphenol and reductone products of their degradation, which is the basis of the irreversible process.
- The remaining reductones can't reduce the oxidised polyphenols completely which is the basis of the irreversible process.
- This process can comprise both aerobic and anaerobic electron transport. In the aerobic process the polyphenols serve as an oxygen carrier.
- Oscillating reactions are expected to take part in ageing beer.

5 Literature

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Appendix

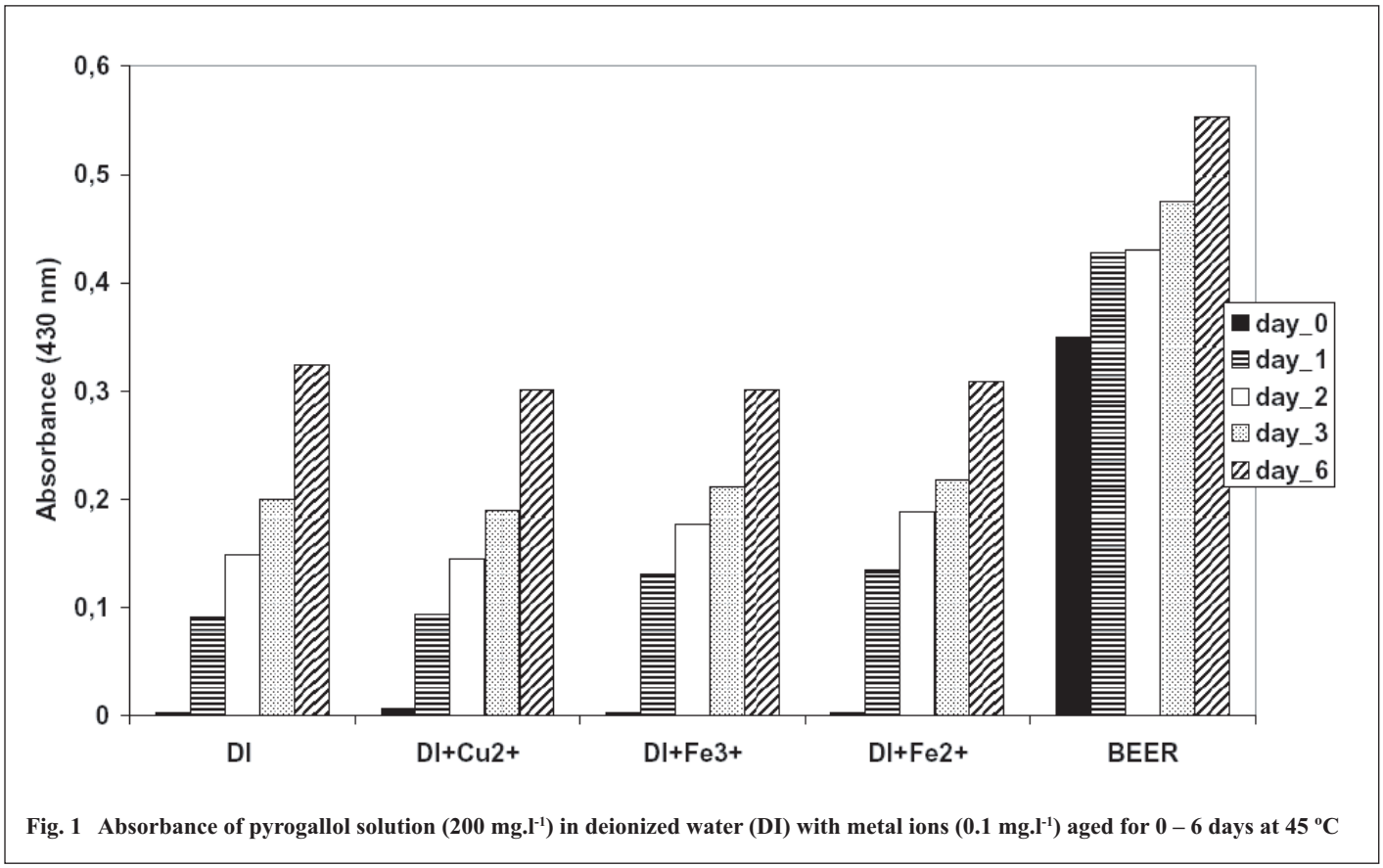

Fig. 1 Absorbance of pyrogallol solution (200 mg.l^{-1}) in deionized water (DI) with metal ions (0.1 mg.l^{-1}) aged for 0 – 6 days at 45 °C

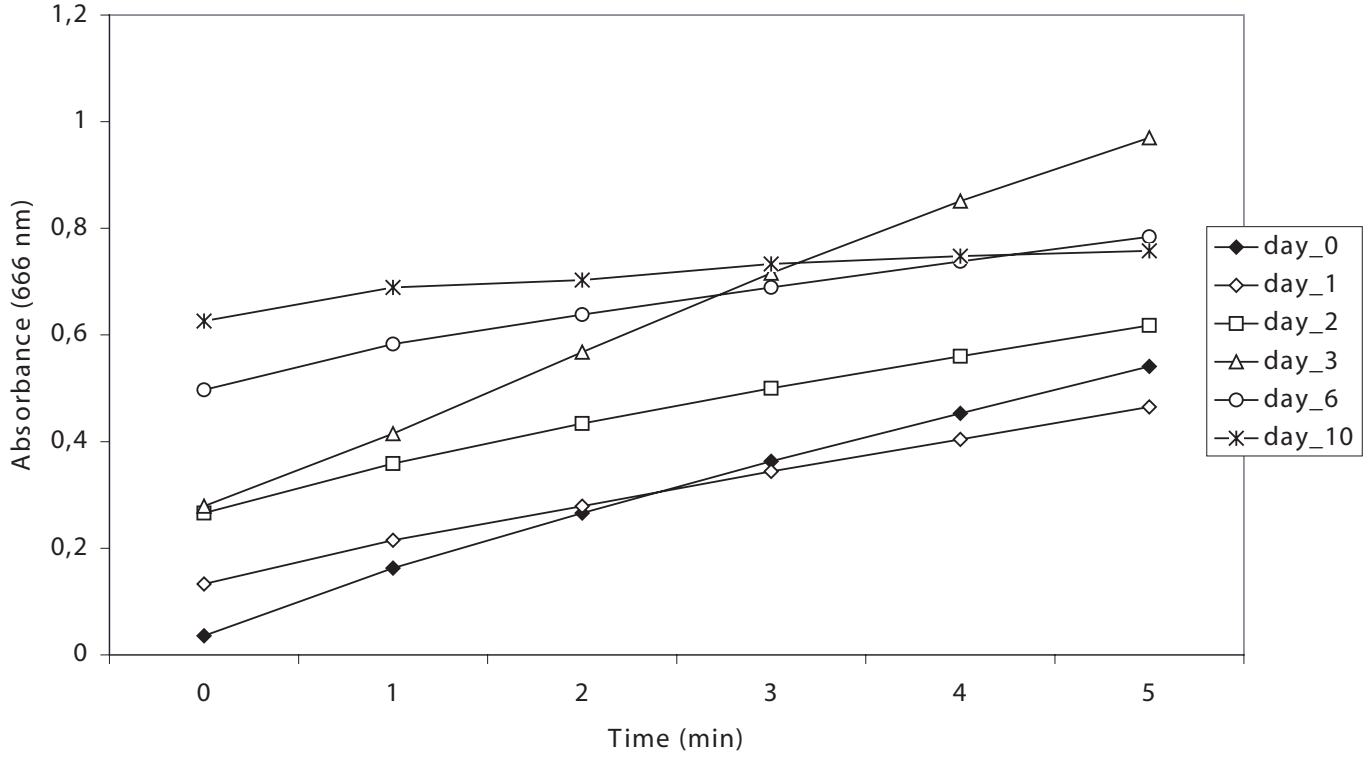

Fig.2 Reoxidation of methylene blue (10 mg.l^{-1}) reduced by ascorbic acid (0,01 % w/w) in pyrogallol solution (200 mg.l^{-1}) aged for 0 – 10 days at 45 °C

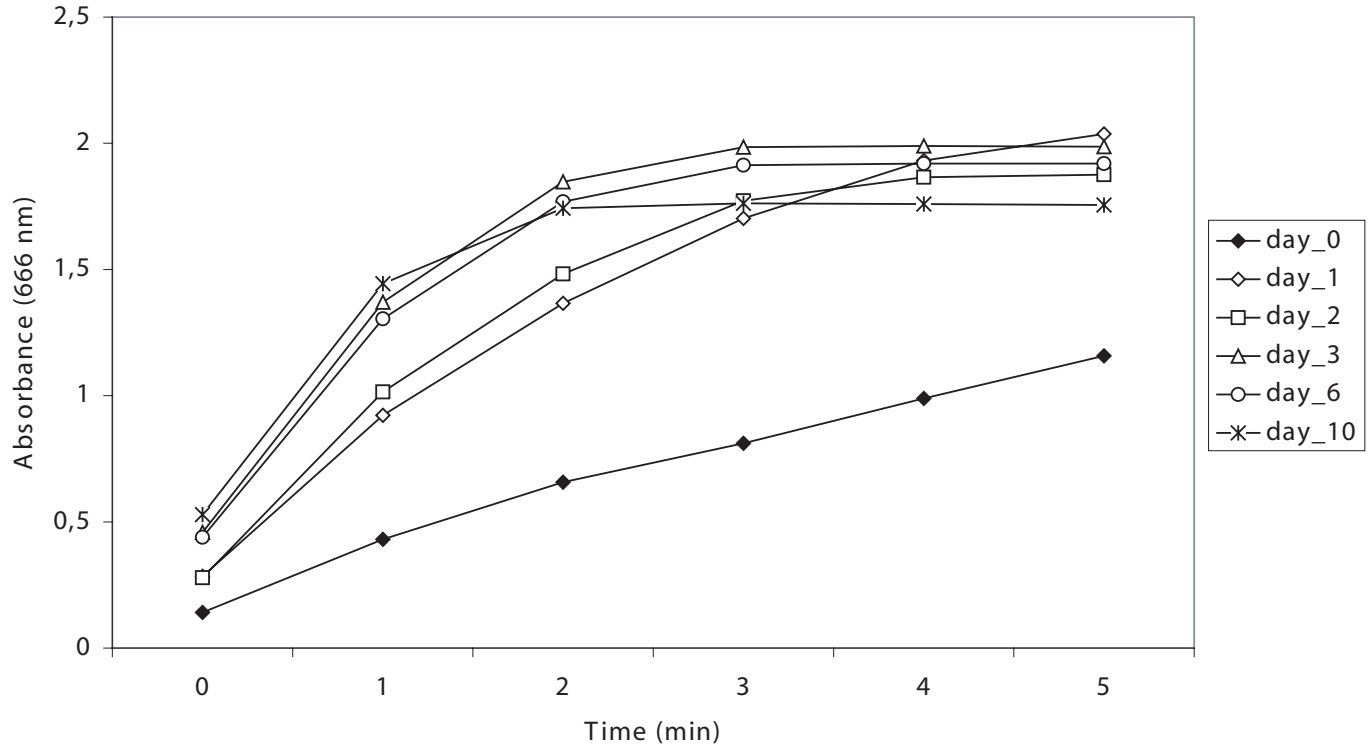

Fig.3 Reoxidation of methylene blue (10 mg.l^{-1}) reduced by ascorbic acid (0,01 % w/w) in beer aged for 0 –10 days at 45 °C

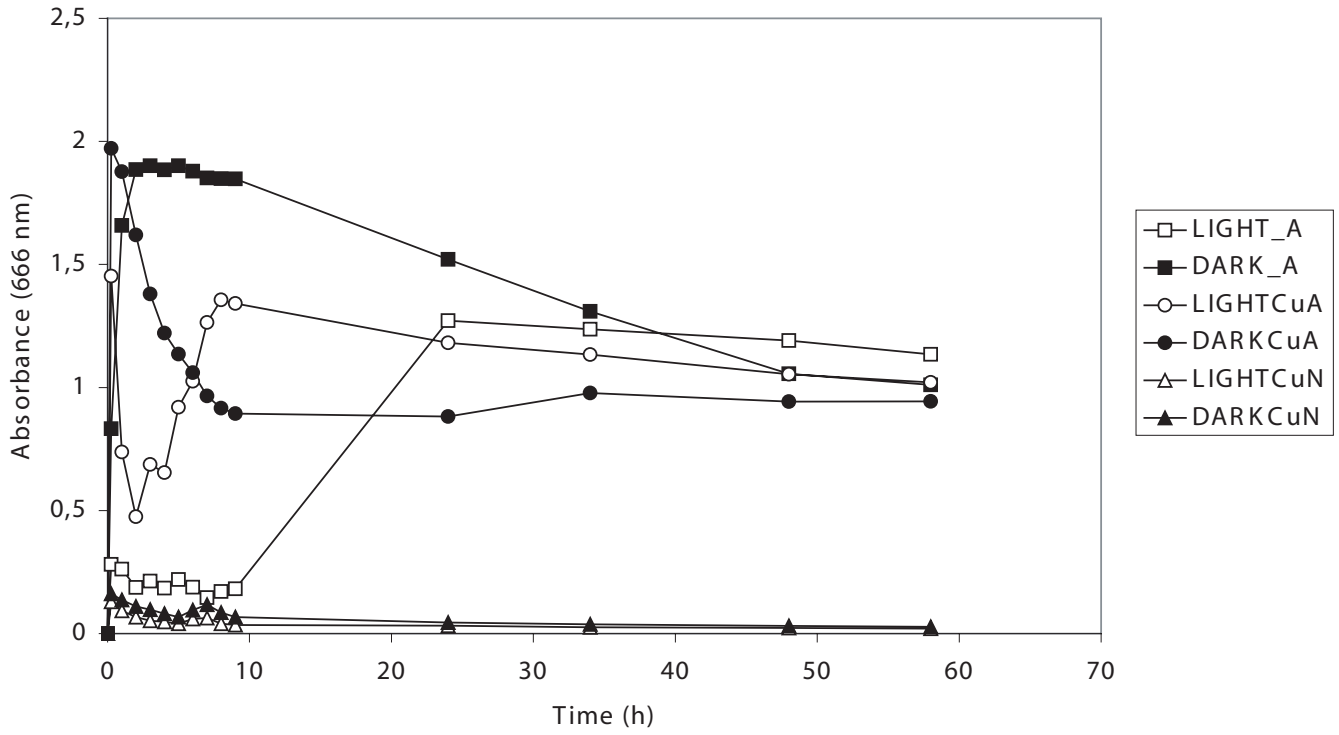

Fig.4 Reoxidation of methylene blue (10 mg.l^{-1}) reduced by ascorbic acid (0,01 % w/w) in aerated (A) or nitrogenated deionized (DI) water) with and without Cu^{2+} (0.1 mg.l^{-1}) in the light and dark.

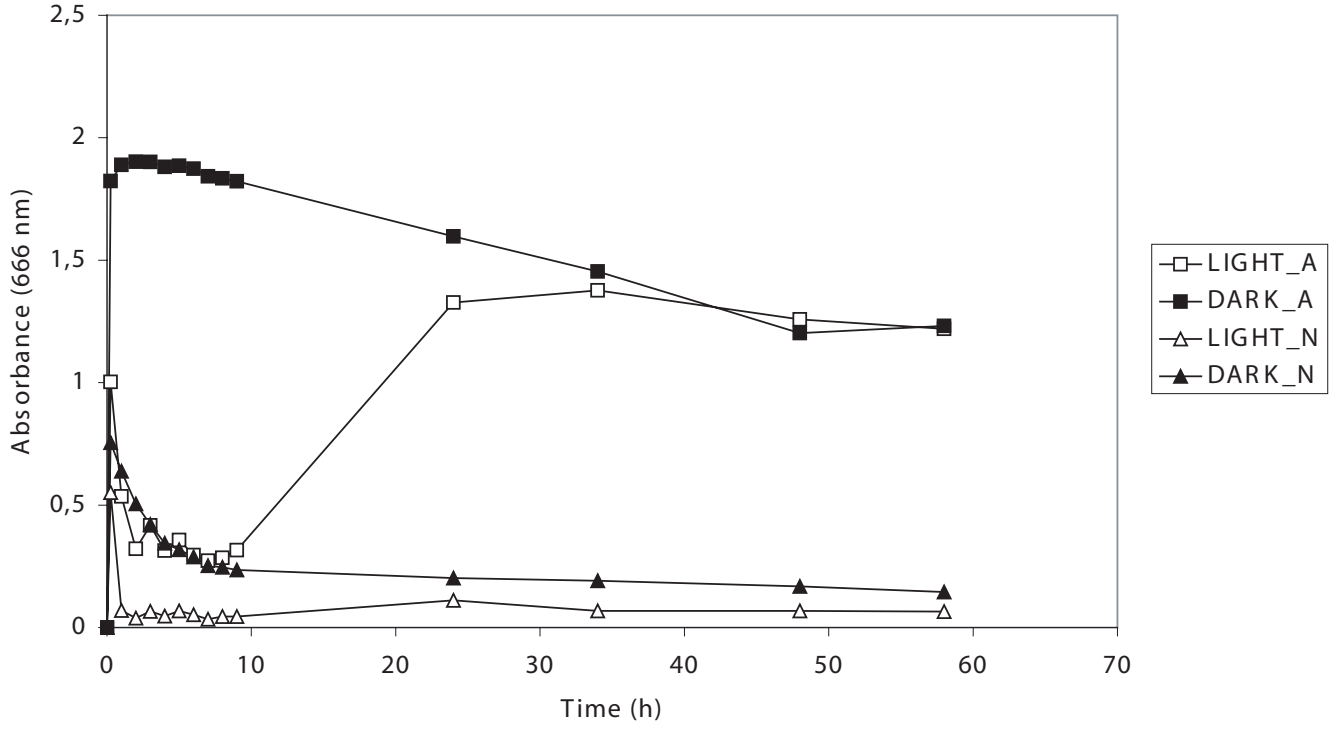

Fig.5 Reoxidation of methylene blue (10 mg.l^{-1}) reduced by ascorbic acid (0,01 % w/w) in aerated (A) or nitrogenated (N) deionised water) with potassium bichromate (10 mg.l^{-1}) in the light and dark.

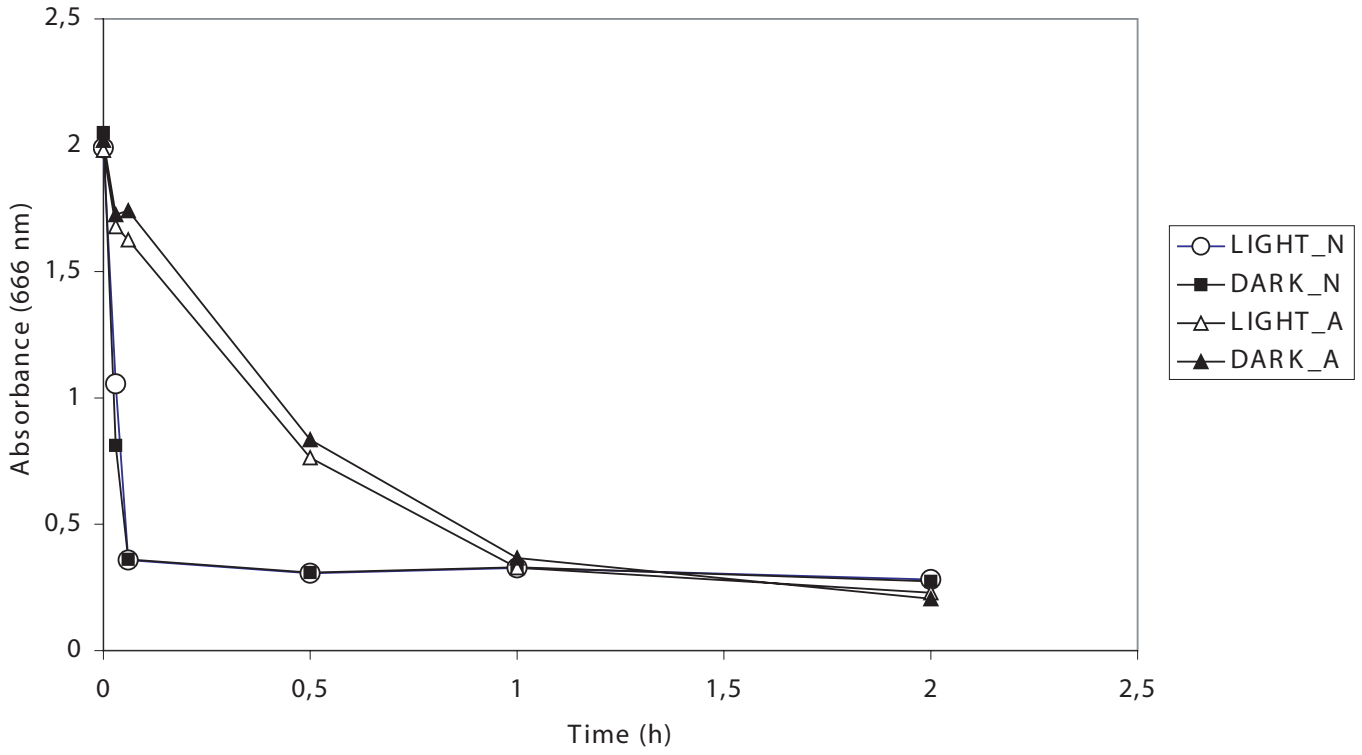

Fig.6 Methylene blue degradation by ABTS (10 mg.l^{-1}) in aerated (A) or nitrogenated (N) water in the light and dark

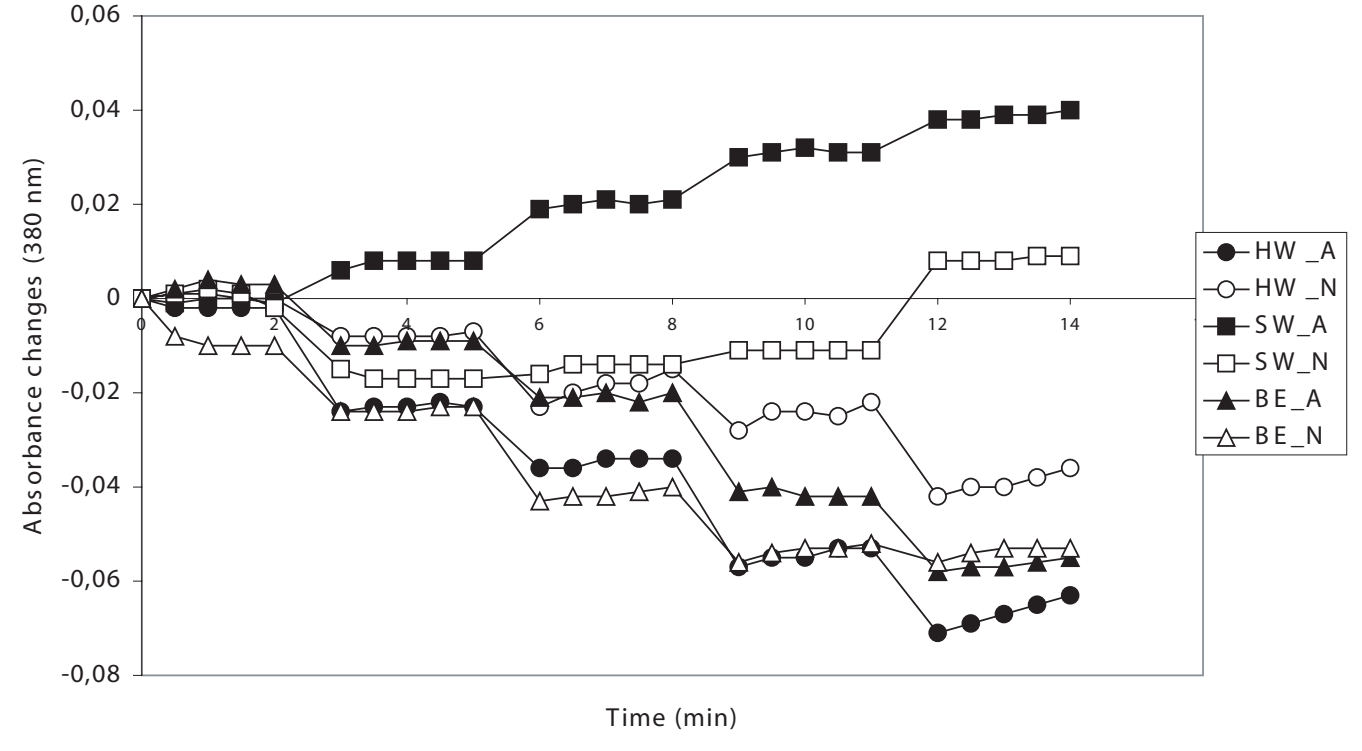

Fig.7 Photooxidation of laboratory sweet wort (LW), hopped wort (HW) and beer (BE) in aerated (A) or nitrogenated (N) medium. Absorbance (380 nm) in 0.5 min intervals interrupted by 1 min illumination.