M. Moll

Determination of antioxidants in brewing

Part 1 Chemical methods

During the last ten years many new methods for the determination of antioxidant activity in the malting and brewing process have been described. Several new methods of analyses of wort and beer antioxidants were simply transfered from other food industries, e.g. oil, fat, meat, dairy or from the medical/pharmaceutical research. Brewing raw materials such as barley, malt, special malts, hop products, caramel, Farbebeer, wort and beer are complex in their composition, type and quantity of oxidisable substances. Several authors have published syntheses on this subject: *Bamforth* et al. 1993, *Dadic* 1984, *Huige* 1993, *Kaneda* et al. 1999, *Liegeois* and *Collin* 2000, *Moll* and *Moll* 1985, 1990, *Pflugfelder* 1992, *Walters* et al. 1996. There are no analytical methods for antioxidants in the malting and brewing industry proposed by ASBC, EBC, IOB, but MEBAK, 1993, vol. II, described two methods (2. 20. 1 and 2). This paper describes methods of analysis of antioxidants which have been applied in the malting and brewing industry. All references which are not directly linked to brewing are mentioned in the text. The methods of analyses described below are separated into chemical/biochemical methods and physical methods.

BC 30 General (analysis methods)

(Descriptors: Antioxidant activity.

Deskriptoren: Antioxidative Aktivität).

1 2,6-Dichlorophenol-indophenol (DCI) (Table 1, 1)

DCI or Tillmans reagent (*Tillmans* et al. Z. Unt. Lebens. 1928, **63**, 1 – 21) is a compound for the determination of ascorbic acid, or more specifically for the enediol group which reduces the dye to a colourless hydroxy compound. DCI is used as a Na salt with a m.w. of 290.08. DCI has an oxidation potential of 0.668 volts. The following autors have worked on and improved this method:

- *Hartong* 1934 (Entfärbungszahl, decoloration figure using a comparator);
- *Gray* & *Stone* 1939 (Indicator Time Test, ITT, which is the time in seconds to reduce the DCI at 80%, degree of reduction measured using a comparotor);
- *De Clerck* & *Van Cauwenberge* 1956 (Reducing substances determined by spectrophotometry at 520 nm and divided into three groups:

  < 15 seconds: very strongly reducing substances: sugar reductones, ascorbic acid, SH-groups;

  < 5 minutes: strongly reducing substances: bisulphite, melanoidins;

  5 – 150 minutes: weakly reducing substances: tannoids, hop resins.
- *Klopper* 1956 (Redox-titration value using a spectrophotometer);
- *Hartong* 1956 simplified the Klopper method;
- *Schilfarth* and *Appel* 1958 (modification of the DCI method by adding ether after 20 seconds for the extraction of the uncoloured DCI);
- *Reich* and *Bock* 1960 (DCI-reducing substances);
- *Case* and *Thompson* 1961 compared different DCI methods;
- *Steiner* 1964 (Reducing capacity which is the method used by MEBAK 1993 method 2. 20. 1 in Vol. II.);
- *Karakus* and *Scriban* 1974 (Reductones, application of the method of Mitsuda and Shikanai, Vitamins, Japan,1957, **13**, 394 – 401, using DCI dissolved in butanol).

2 1,1-Diphenyl-2-picrylhydrazyl (DPH or DPPH) (Table 1, 2)

DPPH is a stable free radical which, because of its structure, can accept a hydrogen radical to become a stable, diamagnetic molecule. DPPH can be oxidised with difficulty and then irreversibly. The intense violet colour at 520 nm is produced by the odd electron on the hydrazyl nitrogen. DPPH has a m.w. of 394.32 with an oxidation potential of 1.2 volts. This reagent was used for the determination of reducing substances after 5, 10 and 30 minutes in wort and beer by *Owades* and *Zientara* 1960 (adapted from *Blois*, Nature 1958, **181**, Nr. 4617, 1199, 1200). DPPH does not react with bisulphite, glucose, other sugars, purines, pyrimidines, amino acids, monohydroxyphenols. The following authors have used this technique :

- *Owades* and *Zientara* 1960 (Oxidisable substances);
- *Kaneda* et al. 1995 (Reducing activity);
- *Goupy* et al. 1999 (Antioxidant activity).

3 Ceric sulphate $Ce(SO_4)_2$

Ceric sulphate, m.w. 332.24, is a strong oxidant and its reduction is accompagned by a simple one-valence change from the oxidised to the reduced state. The change in potential with time is measured with a platinum electrode, a saturated calomel reference electrode with a pH-millivolt meter. This oxidation/reduction

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titration curve gives a ceric-number for each type of beer. Ceric sulphate is less affected by rapidly reducing substances (e.g. ascorbic acid) than DCI and measures slowly reducing compounds in beer. This method was applied by:

- ❑ *Williams* et al. 1967 (Ceric-number).

4 Silver nitrate with ammonium hydroxide (Reducing power)

Silver nitrate, m.w. 169.88, with ammonia has been applied to barley and malt extract for the colorimetric determination of "rapid" reductones (2 h) and "slow" reductones (4 h). The yellow / brown colour produced is due to the formation of a colloidal silver sol (*Chapon* et al. 1971 in Discussion). The colour is measured in a comparator against Brand coloured glasses. This method was developed at Beck's Brewery in Bremen and described by:

- ❑ *Kretschmer* 1970 (Reducing power).

5 2,2' Dipyridyl (Reducing power) (Table 1, 3)

2,2' Dipyridyl, m.w. 156.18 is a reagent for the determination of iron. The complex iron(3)- 2,2 dipyridyl is a strong oxidant. It turns from colourless DP Fe^{3+}, to red as it is reduced toDP Fe^{2+} and the latter is measured at 510 nm at 25 °C. The following reducing substances may be characterised after 3 minutes:

- ❑ instanteneous: ascorbic acid (2 equiv. / mol) and part of reductones;
- ❑ steadely : melanoidins, sulphite, sulphydryl compounds;
- ❑ slowly: polyphenols, tannoids;
- ❑ without effect: alcohol, sugar, polysaccharides, proteins, bitter substances.

This method, when used for beer reducing substances, gives similar results to that of DCI and was developed by:

- ❑ *Chapon* et al. 1971;
- ❑ *Louis*, 1972.

6 Ferricyanide

Potassium ferricyanide, $K_3 Fe (CN)_6$, m.w. 329.25 is widely used for the determination of reducing power in the food industry. Karakus and Scriban, 1974 adapted the method of Adachi, (J. Agr. Chem. Soc. Japan, 1958, **32**, 313 – 316) for malt exracts. The reduction of potassium ferricyanide is measured at 700 nm after 20 minutes at 50 °C and applied by:

- ❑ *Karakus* and *Scriban* 1974 (Reducing power).

7 2-Thiobarbituric acid (TBA) (Table 1, 4)

TBA, m.w. 144.15, is an analytical reagent for measuring tissue lipids, unsaturated fatty acids and esters of unsaturated fatty acid. TBA is widely applied for the determination of rancidity in meat products, oxidation in milk fat, oil,and other food products. Grigsby and Palamand 1976 used this technique to measure the degree of beer staling during storage :

- ❑ *Grigsby* and *Palamand* 1976.

8 Sulphite oxidation value

Sulphite oxidation value was suggested by *Lie* et al. 1977 for the determination of oxygen absorption in the brewhouse. The diffusion of oxygen into "simulated mash" containing liquor and sulphite is determined by following the oxidation of sulphite to sulphate by iodometric titration:

$$SO_3^{2-} + I_2 + H_2O \rightarrow SO_4^{2-} + 2I^- + 2H^+$$

$$2S_2O_3^{2-} + I_2 \rightarrow S_4O_6^{2-} + 2I^-$$

- ❑ *Lie* et al. 1977 (Sulphite oxidation value in the brewhouse);
- ❑ *Zürcher* and *Gruss* 1989.

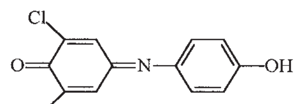
9 Iodine reducing activity

Iodine reducing activity was measured by potentiometric titration using 0.005 N I_2 solution. The contents of reducing agents in the sample are calculated as ppm of sodium ascorbate and applied by:

- ❑ *Ohtsu* et al. 1986.

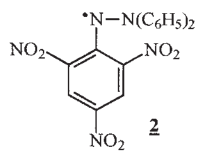
10 Reducing power: reduction of iodine to iodide

Reducing powers are measured after 2.5 minutes and corrected for initial conductivity. A fraction >300 000 NMW from ultrafiltration of malt extract has been found to be characteristic of malt, by ultrafiltration of worts and beers. Reducing power can be monito-



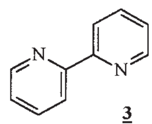
1

DCI
2,6-dichlorophenol-indophenol



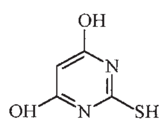
2

DPPH
1,1-diphenyl-2-picrylhydrazyl (Free Radical)



3

2,2'-Dipyridyl



4

TBA
4,6-dihydroxy-2-mercaptopyrimidine
2-Tiobarbituric acid

$[-SC_6H_3(NO_2)CO_2H]_2$

5

DTNB
5,5'-dithiobis-(2-nitrobenzoic acid)
Ellman's reagent

Table 1 Five reagents applied for the determination of antioxidants in brewing (1 – 5)

red through the processing and is also a marker for oxidative damage to worts and beers. The technique has been used by:

- *Brown* 1989;
- *Pflugfelder* 1992.

11 Accelerated activity of Fe^{2+} oxidation

Oxidation of Fe^{2+} ion by H_2O_2 is accelerated in the presence of beer. The acceleration power of the beer sample has been compared to that of water and used by:

- *Ohtsu* et al.1986.

12 Oxygen consuming activity

Oxygen consuming activity is determined by the oxygen absorbed during 46 hours at 50 °C by the beer sample, as applied by:

- *Ohtsu* et al. 1986.

13 H_2O_2 – Forming activity

H_2O_2 – forming activity is measured colorimetrically by the H_2O_2 formed in beer after 30 minutes of irradiation with light and described by:

- *Ohtsu* et al. 1986.

14 Auto-oxidation of methyl linoleate

The antioxidant activity is determined according to the method of *Cuvellier* et al., (Science des Aliments, 1990, **10**, 797 – 806), based on the accelerated autoxidation of methyl linoleate (methyl 9,12 octadecadienoate, m.w. 294.48) in anhydrous dodecane under strong oxidising conditions (110 °C + intensive oxygenation for several hours). Gas chromatography was applied to monitor the disappearence of methyl linoleate from the reaction medium (initial concentration of methyl linoleat: 40 mg/l). The antioxidant activity is determined by the percentage increase in the half-life time of the control oxidation without any antioxidant. This method was used by:

- *Boivin* et al. 1993;
- *Maillard* and *Berset* 1995.

15 Lipoxygenase inhibition

Lipoxygenase inhibition is applied to estimate the potent antioxidant activity in barley and malt extracts. Lipoxygenase activity is expressed as nanomols of oxygen consumed per second (nkat). The antioxidant activity is expressed as the percentage of inhibition of lipoxygenase activity compared with the reference. The larger the lipoxygenase activity the more efficient is the inhibition.

- *Boivin* et al. 1993;
- Richard *Forget* et al. 1995;
- *Goupy* et al. 1999.

16 Ellman's reagent: 5,5'-Dithiobis (2-Nitrobenzoic acid) or DTNB (Table 1, 5)

DTNB was applied by *Muller* 1995 as a measure of oxidation (a loss of free thiol groups) during mashing. DTNB is a colourless compound which reacts with thiols in neutral solutions to liberate 2-nitro-5-thiobenzoate, which is intensely yellow and can be measured at 412 nm.

- *Muller* 1995.

17 Superoxide radical assay

Superoxide radical quenching assay, described by *Scott* et al., (Free Radical Research Communications 1993, **19**, 241 – 253), is based on the hypoxanthine / xanthine oxidase system in the presence of potassium phosphate buffer, EDTA and cytochrome C. The rate of cytochrome C reduction is followed spectrophotometrically at 550 nm. The inhibition of cytochrome C reduction is used to measure the superoxide radical scavenging ability of potential antioxidants.

- *Walters* et al. 1996.

18 Hydroxyl radical assay

Hydroxyl radical assay is a method for measuring both antioxidant and pro-oxidant activity described by *Scott* et al. (Free Radical Research Communications, 1993, **19**, 241 – 253).

$OH^{\cdot}$ radicals are generated via a Fenton type reaction:

Fe^{3+} - EDTA + Ascorbate	→	Fe^{2+} - EDTA + Ascorbate ox
Fe^{2+} - EDTA + H_2O_2	→	OH^- + OH + Fe^{3+} - EDTA
OH + Deoxyribose	→	Fragments heat + Thiobarbituric acid (TBA) + Trichloracetic acid (TCA) Malonaldehyde (MDA)
MDA + 2 TBA	→	Pink chromagen measured at 532 nm

For the pro-oxidant assay, ascorbate is removed from the reaction mixture.

- *Walters* et al. 1996.

19 Lipid peroxidation assay

The nonenzymic auto-oxidation of linoleic acid is measured by the formation of conjugated diene (absorption at 234 nm). The reaction mixture is composed of linoleic acid and varying concentrations of antioxidants in sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate buffer, Tween 20. The absorbance is measured immediately at 234 nm (initial time) and then again after 161 hours of storage in the dark at 25 °C. The rate of linoleic acid autoxidation is calculated by the subtraction of initial time absorbance from final time absorbance.

- *Walters* et al 1997.

20 ABTS-TROLOX : 2,2'-Azinobis (3-ethyl-benzothiozoline-6-sulfonate) (Table 2, 6 , 7)

The basis of the method is the generation of long lived $ABTS^{\cdot +}$ cation, 2,2'-Azinobis (3-ethylbenzothiazoline 6 sulfonate), radical cation chromophore absorbing at 630, 734 and 812 nm and the relative ability of antioxidants to quench the radical to that of TROLOX, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, a water-soluble vitamin E analogue. Thus the TROLOX equivalent antioxidant activity is defined as the concentration of

TROLOX with the same antioxidant activity as 1 mM concentration of the substance under investigation. The method, adapted from *Miller* et al. (Clinical Science, 1993, **84**, 407 – 412), was first described in brewing uses by *Rice-Evans* and *Bourne* 1998. *Araki* et al. 1999 determined antioxidant activity in wort and beer using the TRAP assay. AAPH 2,2'-azobis (2-amidinopropane) dihydrochloride was used as a peroxy radical generator. The final reaction mixture for the assay contained ABTS and AAPH in a pH 4.3 acetate buffer incubated at 45 °C during 60 minutes and then cooled to room temperature. The decrease in absorbance at 734 nm after 15 minutes at 25 °C by the additve sample is measured in comparison to an ascorbate standard solution. The TRAP assay is in brewing a good indicator of antioxidative activity essentially derived from polyphenols.

❑ *Rice-Evans* and *Bourn*e 1998;

❑ *Araki* et al. 1999.

21 AAPH : 2,2'-Azobis(2-amidinopropane-dihydrochloride) (Table 2, 8)

AAPH, a water soluble azo compound, has been first applied in studies by *Fantozzi* et al. 1998, based on the method of *Niki*, (Methods Enzymol. 1990, **186**, 100 – 108) and *Ghisella* et al., (Free Radical Biology and Medecine, 1995, **18**, 29 – 36). A fluorescent probe (protein R-Phycoerythrin, R-PE) loses its fluorescence when exposed to a constant flow of peroxyl radicals, generated by the thermal decomposition of AAPH. Solutions containing antioxidants are able to inhibit R –RE oxidation in a dose-dependent manner. By standardising with a known antioxidant (Trolox, a water soluble analogue of vitamin E) it is possible to quantify the antioxidant capacity of the sample and expressed as Trolox equivalents, defined as the antioxidant capacity of 1.0 mM Trolox. *Liegeois* et al.1999, 2000, applied the AAPH compound to assess the oxidisability of linoleic acid in an aqueous dispersion (resembling wort) in the presence of antioxidants from malt, hops, wort and beer. The method is based on the inhibition of lipid oxidation and gives a measure of how efficiently natural antioxidants protect against lipid oxidation in vitro. The oxidation of exogenous linoleic acid by a thermal free radical producer, AAPH, is followed by a spectrophotometer at 234 nm.

❑ *Fantozzi* et al., 1998;

❑ *Ghisella* et al., 2000;

❑ *Liegeois* et al., 1999, 2000.

22 AOXP (Antioxidant potential of speciality malts) (Table 2, 9)

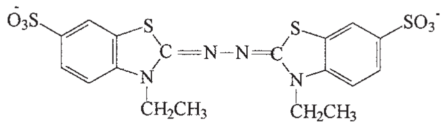
The AOXP method of *Bright* et al. 1999 is adapted from *Auerbach* et al., Anal. Biochem. 1992, **201**, 375 – 380 who measured the hydroperoxides formed from lipoxigenase activity in serum samples. The assay, as modified by *Bright* et al., 1999, is based on the ability of lipid hydroperoxides formed in a Fenton-type reaction to oxidise the N-benzoyl leucomethylene blue (LMB) (9) (colourless), in the presence of hemoglobine, releasing methylene blue, measured et 666 nm. Polyphenols were eliminated using ionic strength, PVPP, low pH and cold extraction temperature. The antioxidant potential (AOXP) is calculated as the inverse of the amount of malt required for 50 % inhibition of the reaction versus a reaction blank containing no antioxidants. This method is useful for measuring the antioxidant potential of speciality malts.

❑ *Bright* et al., 1999.

23 Co-oxidation of beta-carotene in a linoleate model system

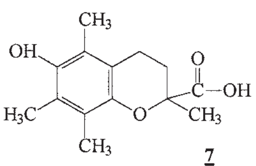
A method using cooxidation of beta-carotene in a linoleate model system was developed by *Pratt* and *Hudson*, (In : Food Antioxidants, Elsevier,1990, pp 171 – 191) and adapted by Goupy et al. 1999 for barley and malt extracts. Beta-carotene is a scavenger of free radicals on the account of the diene system of the molecule. The bleaching of beta-carotene was followed by spectrophotometry at 470 nm in presence or absence of antioxidants. The antioxidant activity was expressed as the percentage of inhibition of co-oxidation of beta-carotene (AOP) as compared with the reference ; the larger the AOP, the more efficient is the antioxidant.

❑ *Goupy* et al. 1999.



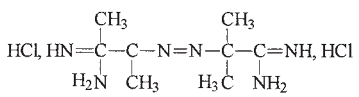
6

ABTS
2,2'-azino-bis(3-ethylbenzothiozoline-6-sulfonic acid)



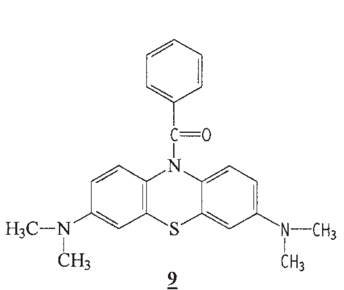
7

TROLOX
6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (hydrosoluble analog of Vitamin E)



8

AAPH
2,2'-azobis(2-amidinopropane)dihydrochloride



9

LMB
N-benzoyl-leucomethylene blue

Table 2 Four reagents applied for the determination of antioxidants in brewing (6 – 9)

Moll, M.: Bestimmung von Antioxidantien in der Brauerei, Teil 1 Chemische Methoden — Monatsschrift für Brauwissenschaft 54, Nr. 1/2, 28 – 32, 2001

BC 30 Allgemeines (Brauereibetriebskontrolle)

Während der letzten Jahre wurden viele neue analytische Methoden für die Bestimmung von Antioxidantien in der Brauerei entwickelt. Mehrere Methoden wurden aus anderen Industriezweigen oder aus der medizinisch/pharmazeutischen Forschung übernommen und ihre Anwendung in der Brauerei erfordert eine Anpassung. Die vielfältige Auswahl der Methoden erschwert das Leben der Qualitätssicherung in der Brauerei. In Anbetracht des komplexen Mediums (Würze und Bier), in welchem der Gehalt von Antioxidantien so oft als möglich während der Bierbereitung bestimmt werden sollte, müsste der Brauer seinerseits in der Lage sein, mit vielen Prozessvariablen zu intervenieren. Mehrere der beschriebenen chemischen und biochemischen Methoden sind schwierig zu interpretieren, was auf die Komplexität des Mediums zurückzuführen ist. Neue physikalische Methoden sind vielversprechend und finden eine praktische Anwendung in der Brauerei.

Moll, M.: Détermination d'antioxydants en brasserie, 1ère partie Méthodes chimiques — Monatsschrift für Brauwissenschaft 54, No 1/2, 28 – 32, 2001

BC 30 Généralités (Contrôle de fabrication en brasserie)

Au cours des dernières années de nombreuses méthodes analytiques ont été développées pour la détermination des antioxydants en brasserie. Plusieurs techniques ont été utilisées provenant d'autres industries ou de la recherche médicale/pharmaceutique. Leur application en brasserie nécessite quelque adaptation. Le choix multiple de méthodes rend la vie difficile à l'assurance qualité en brasserie. En considérant la complexité du milieu (moût et bière) dans lequel on doit déterminer le taux d'antioxydants aussi souvent que possible pendant la durée de fabrication, il faut que le brasseur soit en mesure d'intervenir sur de nombreuses variables d'action. Plusieurs méthodes chimiques et biochimiques décrites sont difficilement interprétables en raison de la complexité du milieu. De nouvelles méthodes physiques sont promises à des applications pratiques en brasserie.

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Der 2. Teil des Artikels folgt mit Literaturangabe in Heft 3/4, 2001.