M. Moll

Determination of antioxidants in brewing

Second part – Physical methods

In the first part a description of 23 chemical and biochemical methods of antioxidant determination in brewing was presented. In this second part the physical methods are developed.

BC 30 General (analysis methods)

(Descriptors: Antioxidant activity, survey, procedures.

Deskriptoren: Antioxidative Aktivität, Übersicht, Verfahren).

1 rH, Redox potential (Table 3, 10, 11)

De Clerck in 1934 first described the use of the oxido-reduction potential (redox potential) in brewing scientific studies. This is the negative logarithm of the pressure of reducing hydrogen existing in the liquid and expresses the degree of oxidation just as pH indicates the degree of acidity. *De Clerck* proposed two methods:

- the electrometric method using a platinum electrode and a reference electrode (hydrogen electrode),
- the colorimetric method using methylene blue and indigo which are colored in the oxidised state and colourless in the reduced state. Methylene blue, m.w. 320, changes from blue to colourless between rH 15.5 and 13.5. Potassium indigo tetrasulphonate, m.w. 734, changes colour between rH 12.0 and 11.0. Potassium indigo trisulphonate, m.w. 616, changes colour between rH 11.5 and 9.5.

Van Gheluwe and *Valyi*, 1974, described the change of rH before and after fermentation by means of a spring analogy. If the rH is high prior to fermentation, the spring will be compressed greatly during fermentation and has a potential to expand to a high rH level again after fermentation. Wort should have a low rH to improve the flavour stability of beer.

Drawert et al., 1977, indicated that the redox potential of beer is only weakly transmitted to the indicating platinum electrode. There are essentially three redox couples involved in potential formation:

- The dissolved oxygen (in the + 280 to + 180 mV versus standard hydrogen electrode range, E varying linearely with log [O_2]),
- The heavy metals and their complexes (+ 200 to + 150 mV, mainly iron complexes and copper), and
- The DCI (Dichlorophenolindophenol) substrates (+ 150 to + 120 mV); reductones.
- The measured potentials decrease with time for up to five days and represent the momentary oxido-reduction-state of the mentioned compounds in a mathematically complicated way. For this reason the redoxpotential of beer cannot give any quantitative information on its oxidation state, because its electrochemically inactive constituents have no influence on it.

Van Strien and *de Jong*, 1982, described the "redox value determination" which is based on the measurement of the reaction rate when given reducing substances from beer or wort are oxidised by an oxidising agent (e.g. DCI).

Steiner and *Länzlinger*, 1984, tested five electrodes for the determination of rH in wort and beer and found no relevant informations for quality control in brewing.

Van Strien, 1987, mentioned an equation which is derived from the redox coefficient (rH) that is not pH dependent and described an instrument suitable for rH measurement. The rH scale is limited by the potential difference between hydrogen and the oxygen electrode which is 1.23 V. The rH scale range is from 0 (strongly reducing) to 42 (strongly oxidising).

Following authors have publishid on rH determination in brewing:

- *De Clerck*, 1934;
- *Mendlik*, 1934;
- *Chapon* and *Urion*, 1953;
- *Raible*, 1955;
- *Van Gheluwe* and *Valyi*, 1974;
- *Sipos*, 1976;
- *Drawert* et al. 1977;
- *Van Strien* and *de Jong* 1982;
- *Steiner* and *Länzlinger*, 1984;
- *Van Strien*, 1987;
- *Bamforth* et al., 1996;
- *Buckee* et al., 1997.

2 Oxidation change using ^{18}O

In 1966 *Owades* and *Jakovac* injected a non-radioactive oxygen isotope ^{18}O, into the headspace of bottled beer. During eight months of storage all ^{18}O was incorporated into beer components. Various fractions were analysed by proton bombardment in a Van de Graaf accelerator. Headspace gaz analysis, using a mass spectrometer, showed that all of the original headspace oxygen had reacted with the beer. The percent of tannin molecules which contained an atom of ^{18}O was 65%. 5% of the isohumulone

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contained an ^{18}O atom. 30% of the carbonyls that reacted with 2,4-dinitrophenyl-hydrazine contained an ^{18}O atom.

The team at Louvain la Neuve: *Collin* et al. 1997, *Noel* et al. 1999, *Lermusieau* et al. 1999, has undertaken a similar study, injecting in a beer bottle 96 ppm with $^{18}O_2$ in the headspace just before ageing (3 months at room temperature). Despite the large quantity of oxygen present in the headspace, GC-MS revealed no significant difference in trans-2-nonenal concentration between oxygen receiving, 0.23 ppb, and oxygen free, 0.21 ppb, sample. In fresh beer the trans-2-nonenal level was 0.1 ppb and the sulphite concentration 2 ppm. Proton bombardment analysis of carbonyl extracts prepared from beer aged (3 months at room temperature) in presence of oxygen headspace (96 ppm $^{18}O_2$) gave 1 ppt of carbonyl compounds having bound an $^{18}O_2$ atom. This result shows that the cardboard flavour is not due to the oxidation of lipids in the final beer. Although oxygen caused considerable oxidation of sulphite, polyphenols and isohumulones, it was not incorporated into the carbonyl fraction.

The use of this technique is described in the following publications:

- *Owades* and *Jakovak,* 1966;
- *Collin* et al., 1997;
- *Noel* et al., 1999;
- *Lermusieau* et al., 1999.

3 Red colour index

Van Gheluwe et al. 1969 proposed the "red colour index" which is the spectrophotometric absorbance of a sample measured in 1 cm cell at 465 nm (A_{465}) and 550 nm (A_{550}). The "red colour index" is = A_{550} / A_{465} x 1000. Oxidation of beer increases the "red colour index" which is due to browning reactions.

- *Van Gheluwe* et al., 1969.

4 Kinetics of beer oxidation determined by oxidising beer at constant pressure

The concentration of antioxidants in beer expressed in equivalents of ascorbic acid was determined by *André* and *Moll*, 1986, by oxidising beer at constant pressure. Kinetic studies of the oxidation of beers at constant pressure in presence of copper ions as catalyst demonstrated that two temporal stages can be observed:

- one over a short time, linked to a rapid rate of oxidation and dependent on the concentration of antioxidant and on time,
- and the other characteristics of long time, at a slow rate that is almost independent of time and the quantity of antioxidants in beer.

The phase of rapid oxidation involves chain reaction in which the primary antioxidants act as both initiators and inhibitors of the oxidation reaction.

- *André* and *Moll*, 1986.

5 Electrochemical detection (ED)

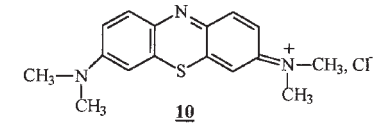
Reversed phase (RP) HPLC-ED (high performance liquid chromatography with electrochemical detection) is a suitable and a sensitive method for the determination of ascorbic acid at low levels in beer. *Moll* et al. 1989 have determined endogenous antioxidants electrochemically active compounds such as phenolic acids (e.g. caffeic, vanillic, protocatechuic and syringic acids), mixture of three synthetic Amadori compounds solutions, Ala-Fru, Val-Fru and Pro-Fru. A pure synthetic Amadori compound does not posses any functional group in its molecule that would confer on its reactivity in redox changes, but after one hour of reflux, electroactive compounds were formed in the medium. The following papers described this analytical technique:

- *Moll* et al., 1989;
- *Boivin* et al., 1993;
- *Peyrat-Maillard* et al., 2000 (Coulometric detection).

6 Chemiluminescence (CL) (Table 3, 12, 13)

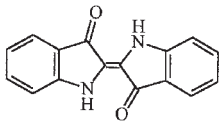
Kaneda et al. 1990a,b, 1991 described chemiluminescence (CL) produced in beer which was detected by a single photoelectron counting system, adapted from *Usuki* et al., J. of Food Science, 1979, 44, 1573. The incubation of beer during 120 minutes at 60 °C was followed by CL and the CL intensity showed a good relationship with staling degree in mean panel score.

CL-HPLC was applied by *Kobayashi* et al., 1993, using isoluminol-microperoxidase solution as luminescing reagent. The hydroperoxides started to increase just after mashing-in, reached a maximum at 65 °C, then decreased and disappeared prior to mash-separation. Mashing is the most important process for lipid oxidation during wort production.



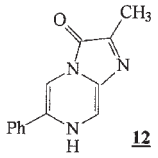
Methylene blue

10



Indigo blue

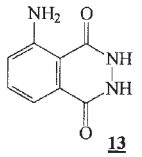
11



CLA

2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]-pyrazine-3-one
(*Cypridina luciferin* analog)

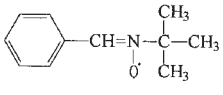
12



Luminol

3-aminophtalhydrazide-5-amino-2,3-dihydro-1,4-phtalazinedione

13



PBN

N-ter-butyl-α-phenylnitrone

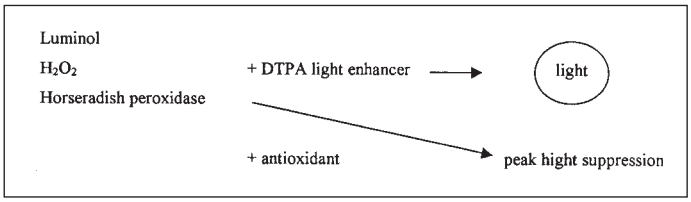
14

Table 3 Five reagents applied for the determination of antioxidants in brewing (10 – 14)

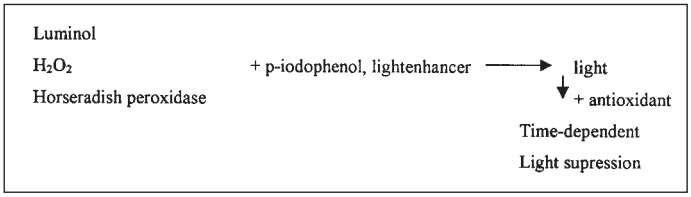
The role of active oxygen during flavour staling in beer was studied by luminescence analysis using 2-methyl-6-phenyl-3,7-dihydroimidazo [1,2-a] pyrazine-3-one, the *Cypridina* luciferin analog (CLA) (**12**) was introduced by *Kaneda* et al.1991, 1994 adapted from *Nakano* et al. (Anal. Biochem. 1986, 169, 363 – 366). CLA can only react with singlet oxygen ($^1O_2$) and superoxide (O_2^-) and shows a marked luminescence.

Walters et al., 1996, applied the enhanced chemiluminescence assay adapted from *Ashida* et al., (Anal. Sci. 1991, 7, 93 – 96) and *Whitehead* et al., (Anal. Chem. Acta, 1992, 266, 265 – 277).

The peak height procedure is based on the reaction of luminol (**13**) with hydrogen peroxide in presence of a light enhancer, diethyl-etriamine pentaacetic acid (DTPA), which produces a flash output of light lasting 30 seconds. The superoxide radical has been implicated in this chemiluminescent pathway. Antioxidant activity is measured from a dose-response curve as the concentration of antioxidant giving 50% suppression of the signal output in absence of antioxidant : The schematic diagram below describes the peak height suppression method:



Walters et al., 1996, applied a second chemiluminescent method: time delay adapted from assay kit from Amersham plc, UK. In this assay p-iodophenol is used as a light enhancer which results in a more intense, prolonged and stable light emission. The schematic diagram describes the time delay method:



Uchida and *Ono*, 1999, developed a CLA-FIA method for the determination of H_2O_2 in beer. H_2O_2 was generated after a definite lag period in the early stage of oxidative forcing test. The principle of the method for H_2O_2 determination in beer is to measure the chemiluminescence emission, which is produced by the enzymic peroxidase reaction of luminol with H_2O_2 under alkaline conditions. H_2O_2 generation significantly correlated with the « lag - time » (see § 7 ESR) of OH- radical generation (EA value) of beer.

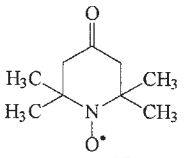
- *Kaneda* et al., 1990a,b, 1991;
- *Walters* et al., 1996;
- *Uchida* and *Ono*, 1999.

7 Electron Spin Resonance (ESR) (Table 3, **14**, Table 4, **15**–**18**))

Kaneda et al., 1988 applied ESR for the free radical detection in beer using N-ter-butyl-α phenylnitrone (PBN) (**14**) as spin trapping reagent, adapted from Evans, (Aldrichimica Acta, 1979, 12, 23). Beer was incubated at 60 °C during 8 hours to 5 days and hydrogen peroxide was generated and examined by ESR with PBN.

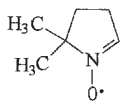
Uchida and *Ono*, 1996a,b, developed a method for determining an endogenous antioxidant activity of beers. This technique allows prediction of the oxidative flavour stability. Free radicals in beer were detected during an oxidative forcing test at 60 °C with 9.5 ml air in head space using a spin trapping method with ESR. The detected free radicals were identified as hydroxyl radical by analysing hyperfine structures of spin reagents of PBN and 5,5'-dimethyl-1-pyrroline-N-oxide and 4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl (4-oxo-Tempo) (**15**) was used as standard of OH^- radical. The OH^- radical was not always generated immediately after starting the forcing test but was generated after a definite time period "lag-time" of OH^- radical generation. The "lag-time" was considered to be related to the endogenous antioxidant activity (EA value) and could be used as an indicator for the prediction of flavour stability. Another index was defined by *Uchida* and *Ono*, 2000a: the OH^- radical generation activity, which is expressed as the quantity of OH^- radical generation in wort at a 120 minutes forcing test.

Andersen and *Skibsted*, 1998, used the following four spin traps: 2-methyl-2-nitrosopropane (MNP) (**16**), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (**17**) , phenyl-*N*-*tert*-butylnitrone (PBN), 4-pyridyl-1-oxide-*N*-*tert*-butylnitrone, (POBN) (**18**). Spin adducts of free radicals formed in beer under aerobic conditions have been characterised by ESR spectroscopy. The 1-hydroxyethyl radical is found to be the quantitatively most important radical. The hydroxyl radical is trapped only by DMPO at high concentration (0.5). Fenton-like oxidation processes in beer involve reaction of the 1-hydroxyethyl radical in an important reaction with oxygen converting to hydroxyperoxyl radicals. *Andersen* et al., 1999,

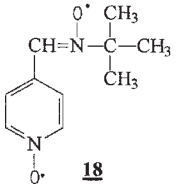


15 **4-OXO-TEMPO**
4-oxo-2,2,6,6-tetramethyl-piperidine-1-oxyl

$[(CH_3)_3CNO]_2$ **MNP**
16 2-methyl-2-nitrosopropane



17 **DMPO**
5,5-dimethyl-1-pyrroline-*N*-oxide



18 **POBN**
4-pyridyl-1-oxide-*N*-ter-butylnitrone

Table 4 Four reagents applied for the determination of antioxidants in brewing (15 – 18)

studied the formation of radicals in sweet wort using ESR. The effect of flavanoids as radical scavengers in sweet wort was examined by comparing the formation of stable spin adducts with POBN in sweet worts of two barley varieties: Maud (contains flavanoids) and Caminant (with negligible content of flavanoids). The rate of formation was found to be identical in the two worts, suggesting that flavanoids exhibit no effect, neither antioxidative nor pro-oxidative, on formation of radicals. Long-term storage of beer was carried out to study the role of flavanoids on the stability of beer using the bottled pilot beers from Maud and Caminant barley. A similar trend was observed regarding the length of the ESR lag-time as defined by *Uchida* and *Ono* 1996a. ESR pattern in flavour stability of beer is rather defined by SO_2 content than by concentration of phenolic species. This result was confirmed by *Andersen* et al., 2000. Sulphite was found to be the only compound that was able to delay the formation of radicals, whereas phenolic compounds such as phenolic acids, catechin, epicatechin and proanthocyanidins dimers had no effect on the formation of radicals in beer. Ascorbate, cysteine and cysteamine were found to be pro-oxidants.

- *Kaneda* et al., 1988, 1999;
- *Uchida* and *Ono*, 1996a,b, 2000a,b;
- *Laane* et al., 1999;
- *Andersen* and *Skibsted,* 1998;
- *Andersen* et al., 1999, 2000;
- *Forster* and *Back*, 1999.

8 Summary

During the last years many new analytical methods have been developed for the determination of antioxidants in brewing. Several techniques were transfered from other industries or medical/pharmaceutical research and their application in brewing need some practical applications. The multiple choice of methods makes life of the quality assurance in brewing difficult. Considering the complex medium (wort and beer) in which the antioxidant level should be determined as often as possible during the process, the brewer on his side should be able to act on many process variables. Several chemical and biochemical methods described are difficult to interpret due to the complexity of the medium. New physical methods are promising to have practical applications in brewing. The second part of this article describes pysical methods.

9 Zusammenfassung

Moll, M.: Bestimmung von Antioxidantien in der Brauerei, Teil 2 Physikalische Methoden — Monatsschrift für Brauwissenschaft 54, Nr. 3/4, 64 – 69, 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. Im zweiten Teil dieses Beitrags werden die physikalischen Methoden vorgestellt.

Moll, M.: Détermination d'antioxydants en brasserie, Deuxième partie Méthodes physiques — Monatsschrift für Brauwissenschaft 54, No. 3/4, 64 – 69, 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. Dans le deuxième part de cet article les méthods physique sont presentéés.

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