

Enzymic and Non-Enzymic Oxidation in the Brewhouse: A Theoretical Consideration

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Received 1st February 1999

Comparison of the available kinetic information for the lipoxygenase- and peroxidase-catalysed reactions with that for the non-enzymic activation of oxygen suggests that it is the availability of oxygen which is likely to limit enzymic action in a mash rather than the availability of enzymes, even a heat-labile one such as lipoxygenase.

Key Words: *Oxygen, lipoxygenase, peroxidase, free radical.*

INTRODUCTION

Whereas it has long since been recognised that the presence of oxygen in final package is detrimental to the shelf-life of beer, it is only in the relatively recent past that the proposal has been made that oxygen consumption earlier in the process, notably in the brewhouse, is also disadvantageous to the flavour life of beer.

However, most of the studies which have linked oxygen uptake in the brewhouse to flavour destabilisation downstream have been made on a small scale. In such conditions, there is usually a far higher surface:mash (or wort) ratio than is the case in commercial scale operations. Consequently the opportunity for oxygen to pass into wort are considerably greater. Accepting that all mashes conducted in the presence of air do have the capacity for oxygen uptake, there is nevertheless no certainty as to whether the substances which are principally involved in utilising the oxygen are relevant to staling downstream.

An additional complication concerns the assessment of oxygen consumption at this stage in the process. Direct measurement of oxygen in mash is fraught with difficulty and most people have reverted to redox measurements (using redox probes or colorimetric techniques) to gain an indication of the extent of oxidative damage. The interpretation and relevance of such measurements is by no means straightforward.

Even if we assume at this point that oxygen consumption in the brewhouse is detrimental to flavour stability, there is no firm agreement amongst researchers about the reason why this should be so. It may be through the formation of substances which go forward directly (or after further modification) to beer as staling substances. Alternatively oxygen may be involved in lowering the level of antioxidant materials surviving into beer, thereby making it more susceptible to the effects of oxygen in final pack.

The complexity of the chemistry leading to the production of staling substances has been reviewed elsewhere³. Suffice here to say that the precursors of the carbonyl compounds (which are particularly associated with cardboard flavours in beer) may be multiple and include bitter compounds, higher alcohols and amino acids. However, the class of compounds most frequently cited as being those from which stale substances originate are the unsaturated fatty acids, notably linoleic acid. Even so, it is uncertain whether oxidative damage at this stage is principally non-enzymic or whether the principle cause of oxidative damage is mediated by enzymes, notably lipoxygenase. This paper presents a theoretical analysis of the likely significance of non-enzymic as opposed to enzymic pathways for the oxidation of a single possible precursor, linoleic acid.

OXYGEN CONSUMPTION IN MASHING

The solubility of oxygen in pure water decreases as temperature increases (Table I).

TABLE I. Dissolution of oxygen in pure water in equilibrium with air at 1 atm.

Temperature (°C)	Dissolved oxygen (ppm)
50	5.4
60	3.8
75	2.8

For simple solutions, the amount of gas dissolved (in equilibrium) is determined by Henry's Law:

$$x = k p \quad (1)$$

where x = the mole fraction of gas in solution*

p = the vapour pressure of gas above the liquid

k = Henry's constant, which depends on the gas and the solvent

Noting that Henry's law concerns itself with mole fractions it is apparent why the presence of other solutes in water diminishes its capacity to dissolve oxygen. Making the gross approximation that the sugar content of a 10° Plato wort is 3mM, then the oxygen concentration in such a wort will be some 20% lower than for pure water at a given temperature and pressure. Of course this gravity isn't instantly achieved in mashing, meaning that oxygen concentration is potentially higher at the start rather than at the end of the process.

In static systems, the concentration of oxygen per unit volume of liquid is primarily determined by the temperature and the amount of oxygen in the headspace above the wort. If the headspace is devoid of oxygen (e.g. a nitrogen blanket) then any oxygen entrained in solution will migrate into the headspace, also according to Henry's Law, which in these circumstances would be transformed to

$$p = x/k \quad (2)$$

Consider two static mashes of 10°P, one commencing with a glucanolytic stand at 50°C and the other with mashing-in at 65°C. In neither mash is any attempt made to adjust the headspace gas composition, which we will assume to be air at 1 atm.

The initial oxygen content of the mash at 50°C (assuming it to be homogeneous) will be of the order of 5.4 ppm (0.17 mM), i.e. the same as for pure water c.f. Table I, because the extent of dissolution of materials at this temperature will be much less than it is at conversion temperature. At 65°C, the oxygen concentration will be approximately 3 ppm (0.1 mM). In fact the oxygen will be introduced into the mash

during the mashing-in process. For the present purposes I have made the simplification that the amount of oxygen dissolved once mashing-in is complete is the equivalent to what it would be in a thin solution in contact with the atmosphere.

Of course, it is well accepted that oxygen is consumed in mashes, and so a dynamic situation will operate in which oxygen lost from solution through reaction with constituents of the mash will be replaced from any atmosphere to which it has access. In a given brewing vessel the equilibrium (or quasi-equilibrium) between oxygen consumption and replacement will be restricted to air-mash interfaces, either at the surface of the mash or at the myriad of new surfaces generated during agitation events, such as a mashing-in and rousing. According to Henry's Law, at no stage can the concentration of oxygen in wort be higher than the levels given above, although the absolute amounts of oxygen consumed in a mash may be considerable (see later).

In respect of the consumption of oxygen, we can differentiate between:

- reactions which have a damaging effect
- reactions which protect against this damaging effect
- reactions which are irrelevant because they have no influence on the quality of the wort as it relates to the beer.

Concerning type a reactions, we might consider the lipoxygenase reaction, the reaction of oxygen with sulphhydryl groups in proteins (through which cross-linking of proteins and reduced rates of mash filtration are said to result) and the production of free radical species.

In respect of type b reactions, we might consider the scavenging of free radicals by protective entities such as superoxide dismutase and non-enzymic antioxidants. Insofar as preferential consumption of oxygen in type c reactions would "block" type a reactions, then type b and type c reactions will both be beneficial.

HOW IMPORTANT IS LIPOXYGENASE?

Lipoxygenase catalyses the oxidation of unsaturated fatty acids, and it is claimed that the products of this reaction can subsequently break down to yield carbonyl substances that afford undesirable cardboard characters to beer. Whilst the potency of lipoxygenase is undisputed, there is some disagreement concerning the extent to which it will be able to act in a mash.

Two factors are primarily relevant:

- i. two substrates, unsaturated fatty acid and oxygen, need to be present simultaneously, and in concentrations adequate to support enzyme action
- ii. enough enzyme must be present, its level depending on the amount of lipoxygenase in the grist and the rate at which it is destroyed in the mash.

Of course, the substrate for lipoxygenase is a lipid, and is accordingly insoluble. The lipoxygenase reaction is likely to take place at surfaces of grist particles in a mash, in regions where conditions would conceptually be different to those pertaining in a homogeneously mixed system. Once again, though, it is necessary to simplify the situation, and to assume that oxygen levels are homogenous through the mash and, furthermore, that the enzyme and its other substrate are also free in solution.

There have been astonishingly few reports of the K_m value for O_2 of any lipoxygenase and nobody has quoted this value for the barley enzyme. Tappel reports values for the soybean enzyme ranging from 0.03 mM to 0.3 mM, depending on the available concentration of linoleic acid¹³. Whittaker suggests K_m values for O_2 in soybean lipoxygenase of 0.18 mM and in the green pea enzyme of 0.36 mM¹⁴.

Let us assume the lowest of these figures (highest affinity) to be the relevant value for malt lipoxygenase. An enzyme acts at its maximum rate when a substrate is present at a concentration 10x higher than the K_m . Thus at 0.1 mM and 0.17 mM O_2 respectively in mashes (see earlier), the amount of available oxygen is not enough to saturate the enzyme. Relatively small changes in oxygen concentration will have a substantial effect on the rate of enzyme action, which would be considerably favoured at the lower mashing temperature. If the K_m for O_2 for the barley lipoxygenase was as high as 0.36 mM, then clearly the enzyme will tend to be substantially limited for oxygen, certainly at 65°C.

It should be realised that it is the *concentration* of oxygen which is important in determining the lipoxygenase reaction. Although we have referred above to the opportunity for continuing replenishment of oxygen through agitation process, it is only if the oxygen concentration reaches a level which can sustain lipoxygenase action that the latter will become a problem.

The total linoleic acid content of a malt is of order of 15-20 mg per g dry weight and a wort of 10° Plato may contain 10-20 mg linoleic acid per litre¹. This value will depend greatly on the clarity of the wort: such figures

are typical of worts from a lauter tun. Levels may be 10-fold higher from a mash filter. It is the unesterified linoleic acid in wort which is susceptible to oxidation in the lipoxygenase-mediated reaction, so the concentration of linoleic acid will be of the order 0.003 to 0.005 mM. K_m values of barley lipoxygenase for linoleic acid of 0.015 mM have been reported⁹. Just as for oxygen, it seems that there is insufficient linoleic acid to saturate the enzyme (in fact, it is very much limiting) and, therefore, that relatively small changes in linoleic acid content of worts will have a sizeable impact on the extent of lipoxygenase action.

Apart from the influence of the concentration of its substrates, the rate of oxidation also depends, of course, on the amount of lipoxygenase present in the mash. Boivin *et al* quote levels for lipoxygenase in malt ranging from 59 to 148 nkat/g⁶. Let us take the lower value. 1 nkat is the amount of enzyme which converts 1 mol of substrate per second (at 30°C in this instance). Thus 59 nkat/g equates to 59 nmol per second per g malt, or 1.0 mg linoleic acid oxidised per minute per g malt.

We are considering mashes at 50°C and 65°C. The effect of increasing the temperature is on the one hand to inactivate the enzyme at a progressively faster rate, but on the other hand to accelerate the rate of the lipoxygenase reaction. Probably no more than 50% of the lipoxygenase is destroyed after 30 minutes of mashing at 50°C, whilst (applying the rule of thumb that a 10°C rise in temperature leads to doubling of reaction rate) the initial rate of the lipoxygenase reaction will be four times faster than when performed at 30°C. Assuming equivalence in the extractibility of lipoxygenase and linoleic acid from the malt, then it is clear that there is ample enzyme to deal with all of the fatty acid within about 10 minutes of mashing. (However, it will be remembered that the levels of the two substrates are not saturating the enzyme, which accordingly will not be operating at maximum rate: *in vitro* enzymes are usually assayed with saturating levels of substrates.)

The enzyme is rapidly lost during mashing at 65°C, but equally the enzyme will act more than ten-times faster at 65° than at 30°C. Thus although perhaps only 10% of the lipoxygenase will survive 15 minutes at 65°C, it will still have been able to convert all of the available lipid.

It is recognised that there are a number of phenolic inhibitors of lipoxygenase in malt⁷ and these are likely to reduce enzyme activity directly (and also by acting as an alternative "outlet" for oxygen – see later).

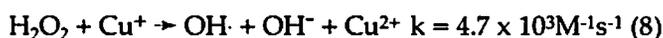
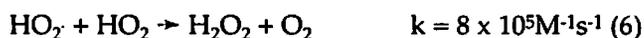
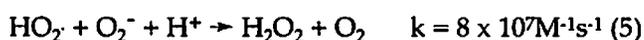
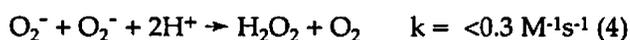
However, it is apparent that the extent of lipoxygenase activity in mashing is much more likely to be determined by substrate availability than enzyme level.

The key opportunity to limit the effects of this enzyme is to deplete it of its key substrate oxygen. We have already seen that there must be considerable doubt about whether the concentration of oxygen developed in wort during mashing is sufficient to sustain lipoxygenase action, certainly at 65°C and even at 50°C. This is particularly so when it is recognised that there are several ways in which oxygen may be consumed non-enzymically.

NON-ENZYMIC REACTIONS LEADING TO A CONSUMPTION OF OXYGEN

It has been suggested that autoxidation of unsaturated fatty acids by activated forms of oxygen is a more important cause of staling than is the lipoxygenase reaction⁴. This certainly would be the case at all points downstream of the mash tun where there is no surviving lipoxygenase. However, equally it is worth comparing the rate of lipoxygenase and non-enzymic oxidative reactions likely to be occurring in a mash in order to ascertain which is likely to happen the more rapidly.

Groundstate oxygen has very low reactivity and it needs to be activated *inter alia* by light, or enzymes, or traces of metal ions before it can enter into most of its reactions. It is converted to superoxide, peroxide and hydroxyl, each of which has greater reactivity. What is the rate of such activation reactions¹⁰?



No indication was given of the temperatures at which these rate constants were measured. Frequently they are calculated at ambient temperature, and therefore for the present purposes we are assuming that they were all measured at 20°C.

Let us say that in a wort there is an adventitious level of 0.1 ppm iron ($1.8 \times 10^{-6}\text{M}$). Then with the level of oxygen we assumed to be in the mash at 50°C ($1.7 \times 10^{-4}\text{M}$) the rate of superoxide formation (if the iron and oxygen come into contact) will be derived from equation 3 (and I have introduced a factor of 8 to account for the increase in reaction rate due to the temperature increase from 20°C to 50°C):

$$r = k[\text{Fe}^{2+}] [\text{O}_2] = 8 \times 1.3 \times 10^6 \times 1.8 \times 10^{-6} \times 1.7 \times 10^{-4} \\ = 3.2 \text{ mmoles per litre per second.}$$

In other words, this reaction alone would be capable of consuming all of the oxygen in wort almost instantaneously in the formation of superoxide. (Even if only a tenth of this level of iron was present the reaction would still be astonishingly fast and, of course, copper can catalyse the same reaction.) Such a phenomenal rate of oxygen consumption obviously does not happen (although oxygen take-up into wort is certainly known not to be sluggish), not least because it depends on both substrates being instantly in complete contact. However, this serves to remind us that the lipoxygenase reaction, which is admittedly fast, is in competition with some other potentially rapid "sinks" for oxygen. *The potential for oxygen consumption in non-enzymic reactions is vastly greater than that for consumption by lipoxygenase.*

Superoxide, O_2^- , exists in equilibrium with its acidic form perhydroxyl, $\text{O}_2\text{H}\cdot$, an equilibrium with a pKa of 4.8. $\text{O}_2\text{H}\cdot$ is capable of reacting directly with unsaturated fatty acids to initiate the autoxidation reaction, whereas O_2^- isn't. Accordingly, the lower the pH of a system, the more reactive (and potentially damaging) will be superoxide. At wort pH's, the unprotonated form would predominate, whereas the opposite obtains for the majority of beers. That is, lipid oxidation promoted by superoxide is unlikely to occur to any significant extent during wort production, the superoxide 'discharging' itself in other directions.

A beer of pH 4.8 (rather higher than is the case for most beers) would have equal quantities of the two forms of superoxide, whereas one of pH 4.1 would feature them in the proportions 5 parts perhydroxyl: 1 part superoxide anion, according to the Henderson-Hasselbalch equation written in the form:

$$\log (\text{O}_2^-) = \text{pH} - \text{pK}_a \quad (9)$$

$$\log \text{O}_2\text{H}\cdot$$

The rate constant for the reaction of linoleic acid with $\text{O}_2\text{H}\cdot$ is $1.18 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$

Taking therefore a concentration of linoleic acid in beer of no more than 0.15 μM , then the rate of oxidation for a beer containing 0.3 ppm (9.4 μM) oxygen, such oxygen being converted into superoxide of which 83% (7.8 μM) was protonated, then the rate of lipid oxidation would be given by:

$$0.15 \times 10^{-6} \times 7.8 \times 10^{-6} \times 1.18 \times 10^3 = 1.38 \times 10^{-9} \text{ M}^{-1}\text{s}^{-1}$$

That is approximately 1 nM linoleic acid oxidised per second – i.e. all of the linoleic acid would be oxidised in

150 seconds - just 2.5 minutes at ambient temperature.

However, the perhydroxyl radical will also be available for other reactions:

a) it will be able to participate in the dismutation reaction given in equation 6 above. There are two arguments in favour of this being more significant than the oxidation of unsaturated fatty acid: first, it is much more likely that a perhydroxyl will encounter another perhydroxyl radical rather than a molecule of linoleic acid (because these radicals are assumed to be in much greater concentration than linoleic acid and also because they will tend to be produced in proximity to one another); second, the rate constant for the dismutation reaction is much greater; in fact dismutation is 678-fold faster than lipid autoxidation.

b) it will react with other species, including endogenous and exogenous antioxidants. The rate constant for its reaction with ascorbic acid is $1.25 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ whilst the rate constant for the reaction of superoxide with quinones such as catechol (c.f. polyphenols) is of the order of 1×10^9 and with glutathione $7.7 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ respectively¹⁰, both values being considerably greater than that for the oxidation of unsaturated fatty acid.

We must not lose sight, however, of the fact that autoxidation of unsaturated fatty acids is a chain reaction which is self-propagating. In other words, once a perhydroxyl radical has initiated the formation of peroxy radicals from unsaturated fatty acids, the latter are capable of effecting further oxidation of unsaturated fatty acids. Note, too, that the extent of linoleic acid oxidation needed to generate sufficient nonenal to be detected as a cardboard flavour is only about $2 \times 10^{-5}\%$ ³.

HOW MUCH OXYGEN IS CONSUMED IN A MASH AND WHERE DOES IT GO TO?

The above calculations clearly indicate that, at least in theory, there is enormous potential for the consumption of oxygen in static mashes within very short time intervals. For the most part, however, brewhouse operations are not static. During the filling of vessels, transfers between vessels, 'cutting' of contents with impellers etc., there is much opportunity for the production of new surfaces across which gas transfer can occur. Furthermore, if there are chemical and enzymic reactions occurring in a mash, then the thermodynamic equilibrium will be such as to have oxygen moving on a gradient into the mash.

It is notoriously difficult to measure oxygen levels, or indeed oxygen consumption, in a mash. Problems include scale effects and the fact that many of the

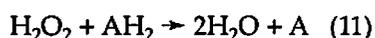
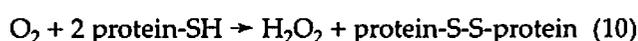
conclusions have been drawn using sulphite mashes, because of the complexity of mash composition and the tendency of oxygen to be consumed, preventing steady state assessment of oxygen concentration.

Based on the available literature covering small-scale mashes it would seem that total oxygen consumption in a brewhouse could conceivably be between 50 and 200 ppm (see³). At the start of this discourse we deduced a concentration of oxygen in a static 10° Plato wort of some 3-6 ppm: clearly we must invoke considerable gas exchange taking place in a brewhouse operation.

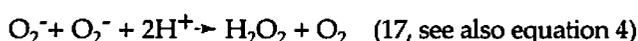
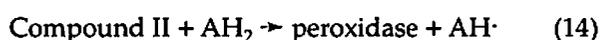
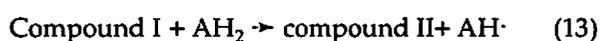
It is important at this stage to stress that the oxygen consumption levels reported in the literature refer to the entire brewhouse operation, including the kettle boil. Thus, for example, Ohtsu claimed a take-up of 20 ppm oxygen during mashing, but twice as much in boiling and wort clarification¹². Enzymic reactions cannot take place during boiling, whereas non-enzymic 'activation' reactions of the type referred to above decidedly can.

Reaction of oxygen with sulphhydryl proteins and polyphenols

It has long been recognised that sulphhydryl-containing compounds in mashes, including some proteins (notably the gel proteins), can react with oxygen. Most recently Muller¹¹ showed that hydrogen peroxide rather than water is the product of this reaction (reaction 10), indicating that this is a ready source of substrate for the peroxidases of malt (reaction 11), which are abundant and of relatively high stability⁸. Immediately one can recognise a potent pathway for the oxidation of mash components (AH₂):



Peroxidase, however, may actually only need catalytic quantities of hydrogen peroxide to enable it to perform and, in its own right, to mop up oxygen. In its reaction it makes a radical of its substrate and some of these radicals are powerful enough to generate superoxide from oxygen. The superoxide dismutates to form hydrogen peroxide:



The net effect of this is to have a consumption of oxygen by the substrate AH_2 . Reflection of the rate constant for the dismutation reaction (equation 17 = equation 4, see earlier) would suggest that this would limit this series of reactions. However, mashes will contain the enzyme superoxide dismutase, which will survive long enough in mashes at both temperatures in order to accelerate this reaction².

Peroxidase has diverse substrates, but it is generally assumed that the most significant of these in mashing are polyphenols.

In the context of polyphenols and sulphhydryl groups, let us consider the data of Bamforth *et al*, in which the levels of -SH compounds and polyphenols in small scale mashes were compared⁵.

Concerning measurable -SH groups, these were vastly lower under aerobic as opposed to anaerobic conditions. For a 1h mash at 65°C, taking the difference between the levels under high and low oxygen conditions respectively, we arrive at an -SH 'consumption' of approximately 25 $\mu\text{mol/litre}$. This would be equivalent to an oxygen consumption of 12.5 $\mu\text{mol/litre}$ (0.5 mg per litre), as each oxygen molecule cross-links two sulphhydryl groups.

It is important to note that the amount of oxygen introduced into the mash by Bamforth *et al* was high, amounting to a bubbling of 1 litre oxygen per minute through a mash of 150 g malt plus 450 ml water. That is, using the value of 22.4 litres per mole of gas, we are introducing 1.3 g of oxygen per minute.

It seems that only a relatively small proportion of oxygen is consumed through -SH oxidation.

Comparing the extent of polyphenol loss for high versus low O_2 mashes we find a difference of approximately 60 mg/litre. Let us make the enormous simplification that the polyphenol is 100% catechin (molecular weight 290). Then 60 mg/litre is equivalent to 200 μmol per litre. Taking a 1:1 stoichiometry for oxygen consumption by polyphenols, then it is clear that the extent of oxygen take up by the polyphenol fraction (approximately 6.4 mg per litre) is substantially more than that which reacts with the sulphhydryl groups.

It is worth noting that twice as much polyphenol was lost when treatment with high air levels was replaced by one with hydrogen peroxide, i.e. approximately 0.4 μmol per ml. Muller has calculated that the yield of peroxide in a mash of the order of 1 μg per g malt, i.e. 0.03 μmol per g malt¹¹. For a liquor-grist ratio of 3:1, this equates roughly to 0.01 $\mu\text{mol/ml}$. The K_m of peroxidase for hydrogen peroxide varies between isozymes, but for the major isozyme is approximately 0.13-0.15 mM. Thus it would certainly appear that the level of peroxide available in a mash is limiting, whereas the level of peroxidase is not: a typical peroxidase activity for a mash at 65°C is of the order of 1 unit/ml, where 1 unit is

the amount which catalyses the conversion of 1 μmol substrate per minute. Thus whereas there is 0.01 μmol peroxide per ml of mash, there is enough peroxidase to consume 100x that level of peroxide every minute. Just as for lipoxygenase, we are substrate limited: there is little point in worrying about the level of the enzyme, but rather the extent to which its substrate (hydrogen peroxide) is developed.

CONCLUSION

Based on the theoretical calculations made in this paper, which in turn are based on published experimental data, it is suggested that oxygen consumption during wort production is more likely to be channelled into non-enzymic rather than enzyme-driven reactions. In particular, it is proposed that any strategy seeking to minimise lipoxygenase action during mashing should focus on limiting the availability of oxygen rather than attending to the level of the enzyme *per se* which is likely to be present in ample levels, even in mashes where it is rapidly destroyed.

If unsaturated fatty acids are one source of staling aldehydes in beer, then autoxidation due to the perhydroxyl radical in the finished product is likely to be more significant than upstream oxidation.

REFERENCES

1. Anness, B. J. & Reed, R. J. R., *Journal of the Institute of Brewing*, 1985, 91, 82.
2. Bamforth, C. W., *Journal of the Institute of Brewing*, 1983, 89, 420.
3. Bamforth, C. W., *Brauwelt International*, 1999, 17, 98.
4. Bamforth, C. W., Muller, R. E. & Walker, M. D., *Journal of the American Society of Brewing Chemists*, 1993, 51, 79.
5. Bamforth, C. W., Hughes, P. S., Muller, R. E., Walters, M. T., Antrobus, C. J & Large, P. J., *Proceedings of the Sixth International Brewing Technology Conference*, Harrogate, 1996, 267.
6. Billaud, C., Garcia, R., Boivin, P. & Nicolas, J., *Proceedings of the European Brewery Convention Congress*, Maastricht, 1997, 159.
7. Boivin, P., Allain, d., Clamagirant, V., Mailard, M. N., Cuvelier, M. E., Berset, C., Richard, H., Nicolas J. & Forget-Richard, F., *Proceedings of the European Brewery Convention Congress*, Oslo, 1993, 397.
8. Clarkson, S. P., Large, P. J. & Bamforth, C. W., *Journal of the Institute of Brewing*, 1991, 98, 111.
9. Doderer, A., Kokkelink, I., van der Veen, S., Valk, B., Schram, A. W. & Douma, A. C., *Biochimica et Biophysica Acta*, 1992, 1120, 97.
10. Halliwell, B. & Gutteridge, J. M. C., *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford, 1985.
11. Muller, R. E., *Journal of the Institute of Brewing*, 1997, 104, 307.
12. Ohtsu, K., Hashimoto, N., Inoue, K. & Miyaki, S., *Brewers Digest*, 1986, 61(6), 18.
13. Tappel, A. L., *Methods in Enzymology*, 1962, 5, 539.
14. Whittaker, J. R. in *Oxidative Enzymes in Food*, Robinson, D. S., Eskin, N. A. M., Eds., Elsevier Applied Science: London, 1991, 175.

*Footnote: In a system with two components, A and B, then the mole fraction of A is given by

$$\frac{n_A}{n_A + n_B}$$

where n_A is the number of moles of A and n_B the number of moles of B.