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Antioxidant Pool in Beer and Kinetics of EPR Spin-Trapping

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The kinetics of spin-trap adduct formation in beer oxidation exhibits an induction period if the reaction is carried out at elevated temperatures and in the presence of air. This lag period lasts until the endogenous antioxidants are almost completely depleted, and its duration is used as an indicator of the flavor stability and shelf life of beer. This paper demonstrates that the total kinetics of the process can be characterized by three parameters—the lag period, the rate of spin-trap adduct formation, and, finally, the steady-state spin-adduct concentration. A steady-state chain reaction mechanism is described, and quantitative estimates of the main kinetic parameters such as the initiation rate, antioxidant pool, effective content of organic molecules participating in the chain reactions, and the rate constant of the 1-hydroxyethyl radical EtOH• spin-adduct disappearance are given. An additional new dimensionless parameter is suggested to characterize the antioxidant pool—the product of the lag time and the rate of spin-trap radical formation immediately after the lag time, normalized by the steady-state concentration of the adducts. The results of spin-tapping EPR experiments are compared with the nitroxide reduction kinetics measured in the same beer samples. It is shown that although the kinetics of nitroxide reduction in beer can be used to evaluate the reducing power of beer, the latter parameter does not correlate with the antioxidant pool. The relationship of free radical processes, antioxidant pool, reducing power, and beer staling is discussed.

KEYWORDS: Beer; EPR; nitroxides; reducing power; spin traps; lag period; antioxidant pool

INTRODUCTION

It is now well established that the shelf life of beer can be estimated from measurements of spin-trap adduct formation by electron paramagnetic resonance (EPR) (*1*). Typically, a spin-trapping compound, such as phenyl-*N*-*tert*-butylnitrone (the usual abbreviation is PBN), is added to a beer, which is then subjected to an accelerated oxidative stress carried out at 60 °C in the presence of air. Although PBN is not paramagnetic, upon reaction with intermediate short-lived free radicals relatively stable PBN spin-adducts are formed. These adducts can be monitored by EPR. Typically, for a beer undergoing an accelerated oxidation, the characteristic spin-adduct EPR signal appears not immediately, but after a period of time. In chemical kinetics this time interval is called an induction period, but the term lag period is more common in brewing. The lag period can be used as an indicator of beer flavor stability and was found to correlate with the beer age (*1*−*3*). The end of the lag period indicates the moment when the main endogenous antioxidants are almost completely depleted. After the lag period, the EPR signal increases and then reaches a steady-state level. In this paper we discuss kinetic aspects of free radical reactions in beer and demonstrate that another composite experimental parameter—the product of the lag time and the rate of spin trap radical formation immediately after the lag time, normalized by the steady-state level of the spin-trapped adducts—may be used for more accurate and simple characterization of the antioxidant pool in beers.

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METHODS

Fresh Miller Genuine Draft (MGD) beer in cans was provided by Miller Brewing Co. (Milwaukee, WI). Spin-trap PBN was purchased from Sigma (St. Louis, MO). This batch of PBN had a negligible concentration of paramagnetic impurities as was verified by control EPR experiments. Therefore, PBN was used without any further purification.

A 1 M stock solution of PBN was prepared in ethanol and added to beer to a 50 mM final concentration. After incubation of the mixture in small open flasks at 60 °C, the samples were drawn with a syringe into gas-permeable Teflon capillaries (PTFE, 0.81 mm i.d., 0.86 mm o.d.; Zeus Industrial Products, Orangeburg, SC). The ends of capillaries (length ∼ 3 cm) were closed by crimping. Each sample capillary was inserted into a standard EPR quartz tube one after another. The oxygen content in the sample was regulated by flux of nitrogen, air, or oxygen bathing the gas-permeable capillary in this tube.

EPR spectra were taken with a Varian (Palo Alto, CA) Century Series E-112 X-band (8.8−9.5 GHz) spectrometer. Typically, the spectra were recorded at 9.0517 GHz microwave frequency. The center of the

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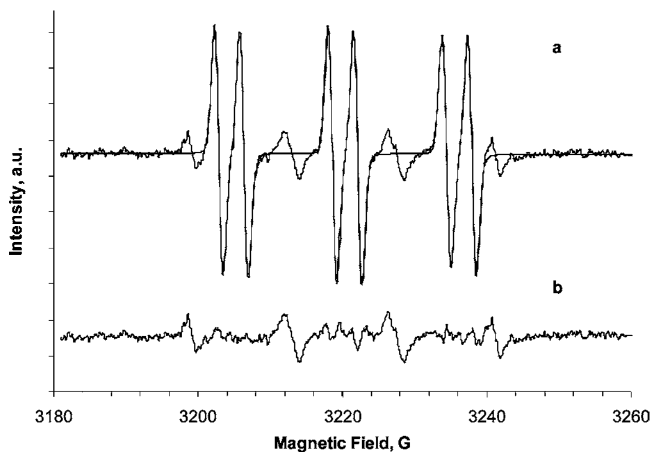


Figure 1. (**a**) Typical experimental EPR spectrum of PBN spin-adducts in beer; (**b**) four-line spectrum after least-squares subtraction of the main spin-adduct signal.

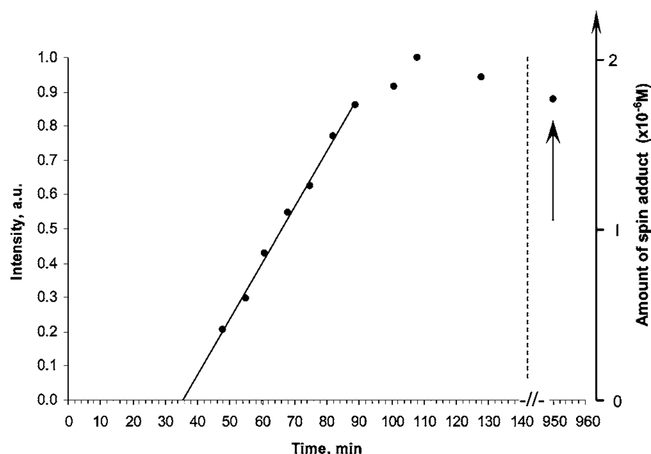


Figure 2. Kinetics of spin-adduct formation in fresh MGD beer at 60 °C. The last measurement was taken the next morning.

magnetic field was set at 3222 G, and the sweep width was 80 G. Incident microwave power was 0.5 mW, which was found to be an optimal value as it does not saturate the EPR line but gives a strong signal. The modulation amplitude was ≤1.0 G at a 100 kHz frequency. This value of modulation was chosen to optimize the signal-to-noise ratio of the spin-adduct EPR spectra and caused only moderate broadening of the EPR lines.

RESULTS

EPR Spectra. The EPR spectra of PBN adducts observed upon incubation with MGD beer are shown in **Figure 1**. Typically, a six-line PBN spin-adduct EPR spectrum with isotropic hyperfine coupling parameters $a_N = 15.8$ G and $a_H = 3.5$ G, which are similar to ones described in refs *5* and *6,* was observed. In some experiments we observed an additional weaker four-line signal with parameters $a_N = a_H = 14.4$ G. Although the exact nature of the latter signal is not important for the purpose of this study, it is most likely to originate from the hydrolysis of PBN adducts (*7*). Here we will analyze only the kinetics of the dominant primary six-line component. The notations EtOH$^•$ and PBN-EtOH$^•$ for 1-hydroxyethyl radical and its spin-adducts will be used, respectively.

Typically, EPR spin-trapping experiments with beer are carried out in an ambient air atmosphere and at elevated temperature of 40−60 °C—the conditions that accelerate the exhaustion of natural antioxidants in beer. However, little attention has been paid to the oxygen-induced broadening of spin-adduct EPR spectra. To demonstrate the role of this effect, a sample of MGD beer initially incubated with PBN in an open flask was placed into a thin gas-permeable PTFE capillary, allowing change in the oxygen content in beer by switching the gas bathing the capillary. It was observed that the peak-to-peak amplitude of the spin-adduct EPR spectrum increased ~5-fold when the gas bathing the capillary was switched from oxygen to nitrogen. On the contrary, the presence of paramagnetic oxygen broadens the PBN spin adduct EPR spectrum in solution, and the line width increases from ~150 mG in the absence of oxygen to ~800 mG for solutions equilibrated with 100% oxygen at atmospheric pressure. Although the second integral of the spectrum, which is proportional to the number of spin-adduct molecules in the sample, was approximately constant upon switching the gas from oxygen to nitrogen, the oxygen broadening leads to an undesirable decrease in the peak-to-peak amplitude of the observed EPR spectra, thus decreasing the method sensitivity. It is important to note that although MGD beer was incubated with the spin-trap in a small open flask maintained at 60 °C, the EPR measurements were carried out at room temperature and, to increase the sensitivity of the method, the samples were deoxygenated using PTFE capillaries as described above.

Experimental Kinetics. During the first 0.5 h of the experiment no EPR signals from the spin-trap incubated with beer were observed. Typically, the kinetics of formation of the six-component spectra for the fresh beer had a lag period of ~36 min (**Figure 2**), which is similar to the values reported earlier (*3*). After the lag period, the line intensity increased at an approximately constant rate until it finally reached a plateau. This steady-state level is probably determined by competition of the rates of formation and degradation of the spin adducts. Usually the experiment with the forced beer oxidation is stopped before reaching this steady state (*2*−*4*).

Additional heat treatment carried out by storing the beer in cans over several days at 32 °C before the PBN experiments resulted in a decrease of the lag period. Specifically, the lag period decreased to one-third of the initial value after 12 weeks of the heat treatment.

DISCUSSION

Kinetic Mechanism. Beer staling is thought to be related to an oxidation process. When oxygen concentration is low, the oxidation could be initiated by production of HO$^•$ free radicals in Fenton's reaction with participation of iron and/or copper ions. Alternatively, hydroxyl radicals could be produced during thermally or photochemically induced homolysis of some weak bonds of organic molecules present in beer. The hydroxyl radicals are among the most reactive radicals, and their life is determined by the diffusion and collision rates with organic molecules that are abundant in solution. In beer, the most abundant organic compound is ethanol, which at ~1 M concentration surpasses carbohydrates, proteins, and amino acids that are present in typically at <50 mM concentrations. On the basis of these considerations it was concluded that in lager beers the hydroxyl radical is almost immediately converted into 1-hydroxyethyl radicals with a pseudo-first-order rate constant of 1.9×10^9 s^{-1} (*8*). The 1-hydroxyethyl radicals participate in a chain propagation of free radical reactions yielding a number of products. Dominant elementary steps of the chain termination in beer are not clear at this moment because of the complexity of this multicomponent system. We anticipate that for a fresh

beer antioxidants play an important role in reducing the concentration of active chains by forming much less reactive radicals.

The free radical reactive intermediates in beer are typically detected and characterized by spin-trapping EPR. Specifically, in EPR spin-trapping experiments with PBN the spin-adducts yield characteristic six-line EPR spectra (*1*). Although PBN has a high rate constant for trapping hydroxyl radicals [(6−9) × 10^9 M^1 s^{-1} (*8*, *9*)], again its 50 mM initial concentration results in a pseudo-first-order rate constant for the capture of hydroxyl radicals of only (3−4) × 10^8 s^{-1}. Therefore, as in the case with the main components of beer, hydroxyl radicals are mainly reacting with ethanol and converted into 1-hydroxyethyl radicals before reacting with PBN (*8*). Thus, the six-line EPR signal observed in such experiments is mainly originating from the PBN 1-hydroxyethyl adduct, although it could be overlapped with that of the $HO^\bullet$−PBN adduct (*5*, *6*). On the basis of the first-order rate constants, the ratio is near 80% for the first and 20% for the second adduct. The similarity of the spectral parameters does not allow differentiation of these components based on EPR spectra. The values of a_N and a_H for the first adduct are a_N = 15.94 G and a_H = 3.34 G (*5*), and for the second one they are a_N = 15.7 G and a_H = 3.2 G (*6*). Adducts formed by the secondary radicals generated in the chain propagation steps can be neglected because these radicals are less reactive than $HO^\bullet$ and are present in a much lower concentration. PBN itself is relatively stable in solution and is not appreciably destroyed in nonradical reactions during the experiment (*10*, *11*).

Previous EPR studies of forced oxidation processes in beer have focused on correlating the lag period for the appearance of the spin-adduct EPR signal with the beer flavor stability (*1*−*4*, *8*). All of those experiments were conducted at elevated temperatures and in the presence of oxygen from air. We can assume that under the latter conditions molecular oxygen is directly reduced by organic components of beer, consequently forming superoxide radical, then hydrogen peroxide, and finally $HO^\bullet$. Although some antioxidants may react with oxygen directly, we anticipate that the main process will be the reaction with 1-hydroxyethyl radicals formed in the chain process. When the natural antioxidant pool is exhausted at the moment of time τ, beer oxidation is accelerated and the chain propagates further, leading to the appearance of EPR signals from spin-adducts.

Steady-State Analysis. A simple model can be put forward to explain this phenomenon. The rate V of formation of the primary $HO^\bullet$ radicals is determined by several initiation steps in the radical chain reaction, possibly with the participation of peroxides and Fenton's reaction:

$$^1/_2\ \text{oxygen, } H^+ \rightarrow HO^\bullet \qquad \text{(initiation, rate } V) \qquad (1)$$

Because of the catalytic nature of this reaction, we can assume that V is practically constant in the beginning and does not change significantly during an EPR spin-trapping experiment. $HO^\bullet$ radicals can react with different components of beer, but first producing 1-hydroxyethyl radicals because of the H-atom transfer from the ethanol C−H bond in the chain propagation step:

$$HO^\bullet + EtOH \rightarrow H_2O + EtOH^\bullet \qquad (k_2) \qquad (2)$$

Some other parallel elementary reactions are

$$HO^\bullet + A \rightarrow HOA^\bullet \quad \text{(reaction with antioxidant, } k_3) \quad (3)$$

$$HO^\bullet + PBN \rightarrow HOPBN^\bullet \quad \text{(formation of EPR-active compound with spin-trap, } k_4) \quad (4)$$

$$HO^\bullet + R_2^\bullet \rightarrow HOR_2 \quad \text{(recombination; } R_2 \text{ is a secondary radical, } k_5) \quad (5)$$

Similar to reactions 3−5, $EtOH^\bullet$ participates in the following reactions:

$$EtOH^\bullet + A \rightarrow EtOHA^\bullet \quad \text{(reaction with antioxidant, } k_6) \quad (6)$$

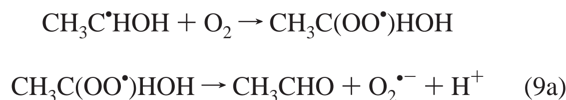
$$EtOH^\bullet + PBN \rightarrow HOEtPBN^\bullet \quad \text{(formation of EPR-active adduct with spin-trap, } k_7) \quad (7)$$

$$EtOH^\bullet + R_2^\bullet \rightarrow HOEtR_2 \quad \text{(recombination; } R_2 \text{ is the same or another radical, } k_8) \quad (8)$$

One should also consider reactions of $EtOH^\bullet$ with various organic compounds present in beer. These reactions are regenerating ethanol molecules and can be described by effective parameters k_9 and C

$$EtOH^\bullet + HC \rightarrow C^\bullet + EtOH \qquad (k_9) \qquad (9)$$

and subsequent chain termination with participation of these organic radical species $C^\bullet$, including those formed by thio groups, polyphenols, and aldehydes. Note that in this case C does not mean carbon, but an organic molecule, so that the H atom is transferred from the bond which has lower bond dissociation energy. It is known that the bond dissociation energy of ArO−H in phenols is relatively low because the radicals are conjugatively stabilized by the aromatic nucleus. As the result, aryloxyl radicals $ArO^\bullet$ are easily formed, thus inhibiting chain reactions. This is well-known for tocopherols (vitamin E). It is also possible that 1-hydroxyethyl radicals could react with oxygen to form intermediate peroxy radicals that can then rearrange and release acetaldehyde and less active superoxide radicals. Instead of the abbreviation $EtOH^\bullet$ to show the process more clearly in this case we use molecular formula



$$CH_3C^\bullet HOH + O_2 \rightarrow CH_3C(OO^\bullet)HOH$$

$$CH_3C(OO^\bullet)HOH \rightarrow CH_3CHO + O_2^{\bullet -} + H^+ \qquad (9a)$$

If a steady-state approximation is fulfilled for hydroxyl radicals, then

$$HO^\bullet = \frac{V}{k_3A + k_2EtOH + k_4PBN + k_5R_2^\bullet} \qquad (10)$$

where EtOH, PBN, and $R_2^\bullet$ are concentrations of correspondent reagents. For simplicity here and further, we omit the square brackets in the concentration notations.

Because the steady-state concentration of the secondary radicals is small compared with the concentrations of the antioxidants and spin-traps and all of the reaction constants are diffusion limited, the contribution from the termination reactions 5 and 8 can be ruled out as insignificant.

Then for the steady-state concentration of $EtOH^\bullet$ we have

$$EtOH^\bullet = \frac{k_2 EtOH}{k_6 A + k_7 PBN + k_9 C} \times \frac{V}{k_3 A + k_2 EtOH + k_4 PBN} \quad (11)$$

The experimentally measured rate of formation of EPR-active spin adducts is

$$r = \frac{d}{dt}(HOPBN^\bullet + HOEtPBN^\bullet) = (k_4 OH^\bullet + k_7 EtOH^\bullet)PBN \quad (12)$$

It is useful to consider several limiting cases detailed below.

A. The rate of $HO^\bullet$ adduct formation, r_1, is (1) very small in the beginning, when the antioxidant concentration is high, and (2) $(k_4/k_2)(PBN/EtOH)V$ after all of the antioxidant is used up and the reaction with ethanol is dominant. The fraction of PBN-captured $HO^\bullet$ depends on competing reactions and is proportional to the concentration of the spin-trap, PBN, with a coefficient α (M^{-1}):

$$\alpha = \frac{k_4}{k_2} \frac{1}{EtOH} \quad (13)$$

On the basis of the values of the pseudo-first-order rate constants given above, we have that α is ~4−5 M^{-1}. Then the rate of $OHPBN^\bullet$ formation is determined by a competition of reactions with PBN and EtOH. On the basis of the rate constants and concentrations mentioned earlier, we estimate this rate as ~0.2V.

(3) The rate of $HO^\bullet$ adduct formation, r_1, is V if the ethanol concentration is low. The main termination step is the spin-adduct formation.

B. The rate of $EtOH^\bullet$ adduct formation, r_2, is (4) again very small in the beginning of the oxidation process, when the antioxidant concentration is high, and (5) $(k_7/k_9)(PBN/C)V$, after all of the antioxidant is used up, and the reaction with other organic components (k_9C) is dominant. After the lag period, the rate of EPR signal should initially grow linear with time. This agrees well with the experiment (**Figure 2**).

(6) Finally, if the ethanol concentration is low, as in a nonalcoholic beer or a lemonade, the rate of $EtOH^\bullet$ formation should be small and equal to $(k_2/k_4)(EtOH/PBN)V$.

Evidently, in cases 5 and 6 r_2 is lower than V.

The rate of the antioxidant consumption is

$$\frac{dA}{dt} = -\left[k_3 + \frac{k_2 k_6 EtOH}{k_6 A + k_7 PBN + k_9 C}\right] \times \frac{AV}{k_3 A + k_2 EtOH + k_4 PBN} \quad (14)$$

and during the lag time (a lot of active antioxidant A), we have a natural result that the antioxidant is consumed with the constant rate V:

$$\frac{dA}{dt} = -V \quad (15)$$

The total antioxidant content A in beer can be approximated simply as $V\tau$, where τ is the lag period.

The usual way to characterize A is simply to use τ, which has the units of time and is acceptable only if V is not affected by the experimental conditions (or when the experimental conditions are identical). Clearly, the initiation rate V could be affected by many factors such as the concentration of iron and/or copper and the intensity and wavelength of light, as well as temperature. Indeed, the main idea of using an elevated temperature of 60 °C in EPR experiments with spin-traps is to increase V to the level when τ, which is inversely proportional to V, could be determined in a reasonable period of time.

One could argue that the presence of spin-traps in beer could affect the course of the chain reactions. However, a small (micromolar range) concentration of spin-adducts observed in such experiments despite the high (50 mM) PBN spin-trap concentration indicates that the effects of PBN on the overall course of beer oxidation are rather negligible.

The chemical kinetic model we described here may be useful for the analysis and comparison of spin-trapping EPR experiments with beer under different experimental conditions. According to the patent disclosure (*2*), the lag period for a certain type of beer was close to 60 min, and then the rate of spin-adduct formation was 1.7×10^{-2} μM/min. Forster et al. (*3*) reported the lag time of 82 min and the rate of spin-adduct formation of 1.25×10^{-2} μM/min. In our experiments with fresh MGD beer at 60 °C the lag time was 34 min and the rate of spin-adduct formation was 3.3×10^{-2} μM/min. Assuming case B5 from above, on the basis of eq 15 this means that the concentration of antioxidants was >1.0 μM in all of these three different experiments.

$$\tau r_2 = \frac{k_7}{k_9} \frac{PBN}{C} A \quad (16)$$

To verify these considerations, we used detailed EPR spin-trapping data presented by Uchida and co-workers in ref *12*, Figure 5. We have found that the product of the rate of spin-adduct formation and the lag period τ increase only moderately (by 30%) when the incubation temperature is increased from 60 to 80 °C. This very weak temperature dependence should be expected because the r_2 is proportional to the ratio of two rate constants for the diffusion-limited reactions which have low activation energies.

The product τr_2 could vary from beer to beer because of different conditions for competing reactions. In the future it would be interesting to compare its value for different beers and its dependence on light intensity, ethanol, and SO_2 and also the concentration and type of phenolic compounds.

Additional Dimensionless Parameter: A New Way To Avoid EPR Spectrometer Calibration. Earlier we have already discussed the lag period and the rate of spin-adduct and EPR spectra formation. However, the whole kinetics of PBN spin-adduct formation appears to be more complex. **Figure 2** demonstrates that over the period of an EPR experiment the intensity of the six-component spin-trap spectra actually reaches a maximum and then decreases slowly over time. Evidently the whole kinetics should be characterized by three parameters, which are the lag time, τ, the rate of the radical formation, and the steady-state level of the radicals. We are not aware of any attempts to do this before.

The main problem with quantitative EPR kinetic measurements and determination of the absolute quantity of the radicals in the sample is determined by the necessity to calibrate the EPR spectrometer and to employ some internal standards because the intensity of the EPR spectra even for samples of similar nature and geometry can be affected by tuning of the instrument, position of the sample, etc. These day-to-day uncertainties in experimental parameters may account for >20% variation in the signal intensity. To obtain quantitative values of the antioxidants and adducts molar concentration, it is necessary to use internal standards that are usually based on

Mn^{2+}, MgO, or stable free radicals such as nitroxides (*2*, *3*). Typically, only relative changes in the radical concentration are measured in an EPR experiment (*4*, *12*).

To minimize this experimental calibration problem and to make the analysis simpler for practical use in a brewery, we can suggest a new parameter, Ω:

$$\Omega = \frac{\tau r}{[\mathrm{HOEtPBN}^\bullet]_S} \tag{17}$$

This dimensionless parameter includes all three important characteristics of the whole kinetics, that is, the lag period, the rate of adduct formation, and the steady-state content of adducts (subscript). Simultaneously, it gives the relative value of the antioxidants content, A, used during the lag time, normalized with respect to the steady-state content of spin-adducts. Here we assume that the EPR spectra are determined mainly by the $HOEt^\bullet$ adduct and that the formation of the $HO^\bullet$ adduct could be neglected. It also means that $r_2 = r$, where r is the rate of the EPR-active adduct formation.

Dimensionless parameters are very popular in engineering, and usually they demonstrate the ratio of two different but related processes. For example, one of the so-called Damkohler numbers characterizes the ratio of reactions and diffusion rates (*13*).

Calculations of the parameter Ω from experimental data are not affected by calibration of the EPR spectrometer and day-to-day changes of experimental conditions because this parameter is proportional to the ratio of the spin-adduct formation rate r (M/s) to the steady-state adduct concentration (M), and, therefore, the latter two parameters can be measured in relative units. Evidently, the parameter Ω makes it possible to compare the results obtained from different types of beer and measured with different EPR instruments without additional calibration. Such a comparison is important for practical purposes and could involve everyday tests for beer quality control or comparison of results measured with the interval of 1 month or more, important for characterization of beer shelf life.

Recently, several other empirical parameters for the characterization of antioxidants in beverages were described in the literature. It should be noted that some of these parameters could be misleading. For instance, the antioxidative potential (stability index), suggested in refs *14* and *15*, is equal to the lag period plus antiradical characteristics (area under the EPR kinetic curve), plus antiradical potential, measured with 2,2-diphenyl-1-picrylhydrazyl, plus reducing power, measured with 2,6-dichlorophenol-indophenol. All of these values have different units and, even when normalized in percent, have very different physical meanings.

Now we consider conditions following the formation of the steady-state concentration of spin-adducts. After the kinetics has reached a plateau, we have observed a slow decrease in PBN spin-adduct concentration (**Figure 2**) that is due to the well-known limited stability of the PBN spin-adducts (*8*, *10*). The latter reaction is of first order with respect to the spin-adduct (*16*) and is characterized by a rate constanr, k (min^{-1}), so that the steady-state condition for the spin-adduct in the realistic case B5 can be written as

$$k[\mathrm{HOEtPBN}^\bullet]_S = \frac{k_7}{k_9}\frac{\mathrm{PBN}}{\mathrm{C}}V \tag{18}$$

where the steady-state concentration has the subscript S.

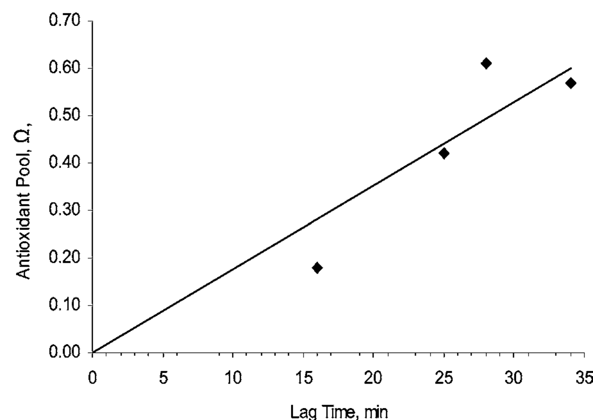


Figure 3. Dependence of the dimensionless parameter Ω on the lag period τ measured for MGD beer samples with different storage times at 32 °C. The storage times for decreasing τ were 0, 4, 8, and 12 weeks, respectively.

The physical meaning of Ω is easier to see after further substitution of eq 18 into eq 16:

$$\Omega = \frac{k_9\mathrm{C}}{k_7\mathrm{PBN}}k\tau = \beta\tau \tag{19}$$

As long as τ is proportional to the antioxidant pool A, Ω is proportional to the product of concentrations of antioxidants and other organic components C, reacting with the main free radicals and deactivating them. For the fresh MGD beer the value of Ω in our experiments was near 0.55. Furthermore, knowing τ and Ω, we can calculate the value of the coefficient β. As expected, this coefficient was approximately constant and equal to $0.017 \pm 0.005\ \mathrm{min}^{-1}$ for beer stored for 0, 4, 8, and 12 weeks.

If r and the steady-state concentrations are the same for a set of different beer samples, Ω has to be proportional to τ and therefore to the antioxidant pool. Experimental dependence of Ω versus τ for the samples of the same beer stored at 32 °C for various periods of time demonstrates that Ω increases with τ (**Figure 3**). Changes of experimental conditions can change r and the steady-state adduct concentrations, and it can be better to compare all of these results using Ω and not τ.

$HOEtPBN^\bullet$ Is the Main Radical Adduct in Beer. If we assume that the main radical adduct in beer is $OH^\bullet$ and the ethanol concentration is low (case A3), then

$$k[\mathrm{HOPBN}^\bullet]_S = V = r_1 \tag{20}$$

and

$$\Omega = k\tau \tag{21}$$

Parameter β in this case is simply equal to k, and it means that the characteristic time ($1/k$) for adduct disappearance is near 1 h. The experimental value of the characteristic time for the hydroxyl radical spin-adduct of PBN is near 1.5 min (*16*). This again demonstrates that the main radical adduct in beer is the $HOEt^\bullet$ adduct. This adduct is much more stable. It has been shown that in vivo this radical adduct could be reduced by liver microsomes, but even in this case it can be observed and analyzed by HPLC after 10 min (*17*).

If the main PBN adduct is that of $OH^\bullet$ after all of the antioxidant is used up and the reaction with ethanol, present in

relatively high concentration, is dominant, we have (case A2)

$$\tau r_1 = \frac{A}{\alpha PBN} \quad (22)$$

Using experimental values of τ, r, and $\alpha = 4\ M^{-1}$, we have that the antioxidant concentration A is only 2×10^{-7} M, which seems to be unreasonably low.

On the basis of this analysis the most realistic condition for the rate of EtOH$^\bullet$ adduct formation is case B5. Then we can carry out several simple estimates. On the basis of the value of the characteristic lifetime of the HOEt$^\bullet$ adduct (~10−60 min) we estimate that

$$\frac{k_9}{k_7}\frac{C}{PBN} \sim 1 \div 6$$

As long as both diffusion-limited rate constants are of the same order, $k_9 \sim k_7$, we have that C is of the order 50 mM and, based on eq 16, we have that A is 1−6 μM.

It is interesting to mention that the concentration of SO_2 and sulfites in beer can reach 100 μM, which is probably too high to explain the antioxidant pool value. This conclusion is supported by the fact that the content of SO_2 in dry wines is an order of magnitude higher but the lag time is still near 100 min, which is comparable to that of the best beers (*14*). The role of SO_2 can be in the reduction and regeneration of the antioxidants used up in the aging process. The total polyphenol content is near 100−150 mg/L (*15*), that is, 100−200 μM, assuming molecular weights 500−1000, and, probably, the main part of them is acting as organic molecules reacting with HOEt$^\bullet$, although some of them can act as antioxidants.

Antioxidant Pool and Reducing Power. We can compare the parameters of EPR spin-trapping experiments with some other kinetic parameters used for beer characterization. Recently we have described the kinetics of the stable nitroxide radical's reduction in beer. (*18*). It was demonstrated that the reaction could be described as the first-order process with respect to both nitroxide and just one reducing agent in beer (*18*). Very often beer is characterized by its so-called "reducing power", measured with the redox indicator 2,6-dichlorophenol-indophenol (*14*). The concentration of the reducing agent calculated from nitroxide reduction kinetics is probably similar to the reducing power, but it gives the answer in the units of concentration and not arbitrarily chosen changes of color after a fixed time. From the nitroxide reduction experiments, the concentration of the reducing agent varied from 60 μM for a fresh beer to 100 μM for a beer stored for 1 week at an elevated temperature of 32 °C. After 3 months of such storage, this concentration decreased to 50 μM (Figure 6 in ref *18*). Relative changes of Ω as a function of beer storage time at the same 32 °C (**Figure 4**) show that the antioxidant pool stays practically constant during the first week and then monotonically decreases with the storage time. Final changes of Ω are much more evident than the decrease of reducing power. Evidently, the kinetics of nitroxide reduction in beer and the lag period observed in spin-trapping experiments are determined by different factors and reflect different components and processes in beer. We have demonstrated that the kinetics of nitroxide reduction is determined by the content and the oxidation (or accessibility) state of cysteine groups in proteins (*18*, *19*). Mild and selective reaction of nitroxides with protein SH groups can be used to characterize the initial state of the proteins in the system, which could be partially hydrolyzed during the first week, thus increasing the access to the cysteine groups. This parameter would be also

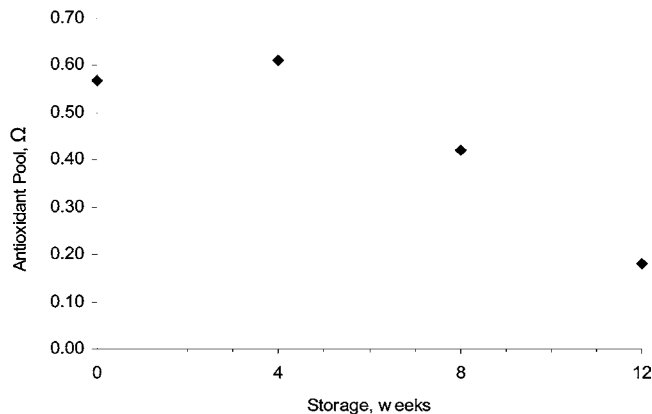


Figure 4. Changes of the dimensionless parameter Ω as a function of storage time at 32 °C.

extremely important for all subsequent steps of redox and radical reactions, where cysteines can participate as the redox and antioxidant buffer in the oxidative stress and storage of beer.

Combination of both methods—kinetics of nitroxide reduction, sensitive to the state of the proteins and especially cysteine groups, and modified lag period method with spin traps, sensitive to the antioxidants—will provide a more comprehensive description of factors, components, and radical-mediated processes in beer and other beverages upon aging. It is important to note here that both nitroxide-based kinetic parameters as described in refs *18* and *19* and the parameter Ω suggested in this paper do not require tiresome quantitative calibration of EPR spectrometers, which is normally used in spin-trapping to get quantitative results. Combination of these parameters is important because they reflect different elementary chemical reactions. The first one is the characteristics of the redox properties of beer, which means the ability to donate electrons. This type of reaction is often coupled with H^+ transfer (*11*) and may be relatively slow, as it is with nitroxides. The main component determining the reducing power of beer can be attributed to cysteines present in protein molecules (*18*, *19*). The second type of reaction is based on the ability of antioxidants to capture reactive radical species, such as HO$^\bullet$ or rather EtOH$^\bullet$, forming much more stable radicals. Antioxidants are present in relatively low concentrations, but are very active and, with time, are used up in irreversible processes. We can think of reducing power as a powerful factor, helping regeneration of antioxidants. When the antioxidant pool becomes depleted, one could expect that cysteines will be oxidized into thiyl radicals, which can serve as pro-oxidants in beer that upon reacting with isohumolones would result in development of the light-struck flavor (*20*). It is also known that mashing results in hydrogen peroxide formation and loss of thiol groups (*21*). Clearly, these studies have to be continued to fully understand the complex relationship of reducing power, oxidation in chain reactions, and flavor changes in beer.

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