The Role of Copper, Oxygen, and Polyphenols in Beer Flavor Instability

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ABSTRACT

The rate of flavor staling in beer is significantly increased by traces of Cu(II), even at levels below 100 μg/L. The Cu(II) catalyzes oxidation reactions that require prooxidants such as cysteine and 1,2,3-trihydroxypolyphenols to recycle the copper through its reduced state. Primary alcohols can be coupled to the oxidation process to yield aldehydes among the products. However, 2-nonen-1-ol is present in beer at concentrations that are too low for it to be a significant precursor of *trans*-2-nonenal. Model studies suggest that either partially oxidized fatty acids or bisulfite complexes may serve as precursors of the stale-flavored, unsaturated aldehydes that are produced in beer as a result of oxidation processes during aging. The oxidation process in model reactions is inhibited by ethylenediaminetetraacetic acid disodium calcium salt (EDTA), lysine, metabisulfite and 1,2-dihydroxypolyphenol species. Lysine and EDTA also inhibit the formation of aldehydes during beer aging.

Keywords: Flavor stability, Copper, Iron, Polyphenol, Oxidation, Fatty acids, Bisulfite complex

The instability of beer flavor is known to be exacerbated by oxygen, and the stale flavor compounds formed during aging are generally believed to be aldehydes. However, the identity of the aldehyde precursors and the mechanism by which molecular oxygen reacts with them remain uncertain. Because oxygen is a triplet species, it is fundamentally slow to react with organic compounds in their singlet ground states. Consequently, transition metal ions in beer have been proposed as catalysts that activate molecular oxygen (1,2,7,8).

The reaction sequence is thought to proceed as shown below and to involve electron transfer to oxygen from a metal ion in its lower oxidation state. The resulting superoxide is subsequently reduced to peroxide by an electron from an additional metal ion in its reduced state.

$$M^{n+} + O_2 \rightarrow M^{(n+1)+} + O_2^{\cdot}$$
$$M^{n+} + 2H^+ + O_2^{\cdot} \rightarrow M^{(n+1)} + H_2O_2$$
$$2M^{n+} + 2H^+ + O_2 \rightarrow 2M^{(n+1)+} + H_2O_2$$

The sequence may not necessarily involve a simple one-electron transfer from a single metal ion to molecular oxygen as the initial step. The latter is thermodynamically unfavorable for both Cu(I) and Fe(II) and, at least in the case of Fe(II), the actual intermediate apparently contains two metal ions bridged by a dioxygen molecule (24).

The identity of the oxygen species that actually reacts with the stale flavor precursors is not known with certainty. Hydrogen peroxide, for example, can oxidize certain organic compounds directly and can initiate the oxidation of others indirectly via the Fenton reaction. The latter generates the extremely reactive hydroxyl radical, which has been proposed as the main causative agent of oxidative damage to beer flavor (1,2). The Fenton reaction (shown below) also requires the metal ion to be in its lower oxidation state.

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^- + OH^{\bullet}$$

Reducing agents must be coupled to the oxidation process in beer in order to recycle the transition metal ions through their lower, oxygen-active redox states. Ascorbic acid has the ability to perform this role during the Fe-catalyzed oxidation of ethanol in model reactions (7,8), but the actual reducing agents in beer have never been identified.

Therefore, a series of model oxidation studies was undertaken to identify 1) coupled reducing agents in beer, 2) aldehyde precursors, and 3) inhibitors of metal-catalyzed oxidation reactions in beer. A direct, organoleptic confirmation of the effect of transition metal ions on beer flavor staling was also sought.

EXPERIMENTAL

Materials and Supplies

All chemicals used were reagent grade or better. Specialty chemicals were obtained as follows: 1-butanol and 1-octanol from J. T. Baker Chemical Co., Canada; *trans*-2-nonenal from Pfaltz and Bauer Inc., United States; d-catechin from Fluka A. G. Chemische Fabrik, Switzerland; ethylenediaminetetraacetic acid disodium calcium salt (EDTA), lysine monohydrochloride, cysteine, purpurogallin, and ascorbic acid from Sigma Chemical Co., United States; gallic acid from Aldrich Chemical Co., United States; 12-hydroxy-9-octadecenoic acid (ricinoleic acid) from Anachemia Chemical Co., Canada; pyrogallol from Matheson Coleman and Bell, Canada; Merck Extrelut tubes, catechol, and hydroxylamine hydrochloride from BDH Ltd., Canada; ferric chloride and cupric chloride from Fisher Scientific Co., Canada; 2-nonen-1-ol from Johnson Matthey/Alfa Products, United States.

Hexanal bisulfite was prepared using a published method (19).

Analysis of Copper and Iron in Beer

Copper and iron were determined by graphite furnace atomic absorption with a Varian GTA 95 (H. Wagner, *unpublished method*).

Effect of Added Cu(II) on Stale Flavor Development

A stock solution of copper(II) chloride was used to spike bottles of a production lager with 25–200 mg/L added Cu(II). The bottles were fobbed before recapping and were then heated at 50°C for four days in a warm air incubator. The bottles were cooled to 8°C, and the stale flavor intensity of each beer was evaluated by a taste panel.

Effect of Endogenous Copper on Stale Flavor Development

Random samples of a production lager were taken over a four-month period and analyzed for copper and iron. The beers were stored at 23±2°C for five months, cooled to 8°C, and then evaluated by a taste panel.

Taste Panel Evaluation of Aged Beer

Evaluations were made by a panel trained in stale flavor attributes. The panelists scored the stale flavor intensity on a scale of 1–10, with 1 representing fresh, unoxidized beer and 10 representing an extremely stale, oxidized beer. In the case of the force-aged products, the panelists' scores were summed to give a total stale flavor intensity score for each beer. In the case of the products stored for five months, the total intensity score of each beer was

divided by the number of the panelists to give an average stale flavor intensity.

Model Oxidation Studies

The alcohol, usually 0.6m*M* 1-butanol, was added to a 1-L round bottom flask containing 0.6m*M* copper(II) chloride, or iron(III) chloride, and 0.6m*M* reducing agent dissolved in 500 ml of distilled water. The flask was fitted with an air condenser. The reaction solution was magnetically stirred at ambient temperature (23 ± 2°C) for two days, and 100 ml was then transferred to a 500-ml separatory funnel. The equivalent of 1 mg of 1-octanol per liter was added as an internal standard, and sufficient sodium chloride was added to saturate the solution. The resulting solution was shaken for 2 min with 8 ml of methylene chloride. The separated organic phase was dried over sodium sulfate and concentrated to 1 ml under a stream of nitrogen.

The concentrated extract was analyzed by gas chromatography (GC). The percent loss of 1-butanol was calculated by peak area comparison with that of a control mixture containing 0.6m*M* 1-butanol and 1-octanol (1 mg/L), which was extracted immediately after preparation. All of the 1-butanol oxidation reactions were performed in duplicate or triplicate, and average values were recorded for percent loss of 1-butanol.

Model oxidations of 1-hexanol, 2-nonen-1-ol, hexanal-bisulfite complex, 12-hydroxy-9-octadecenoic acid (ricinoleic acid), linoleic acid, and 12-hydroxyoctadecenoic acid with 0.6m*M* pyrogallol were performed and worked up as described for 1-butanol. The percent yields of products for the 1-hexanol, 2-nonen-1-ol, and hexanal bisulfite oxidations (as quoted in the results section) are semiquantitative. Product peaks were assumed to have the same response factor as the 1-octanol internal standard. The percent losses of 1-hexanol and 2-nonen-1-ol were derived in the same way as described for 1-butanol, that is, by peak area comparison with control extractions of 0.6m*M* solutions of the appropriate alcohol.

The dichloromethane extract of the 1-hexanol oxidation was further concentrated to 200 μl. The precipitated orange solid was dried, and its mass spectrum was obtained by solid probe insertion. The mass spectrum of the orange solid was identical with that of an authentic sample of purpurogallin.

GC

The concentrated extracts were quantitatively analyzed on a gas chromatograph (Hewlett-Packard 5890) fitted with a flame ionization detector at 250°C whose output was coupled to an integrator (Hewlett-Packard 3396A). Injections of 0.4 μl were made using an on-column injector (J and W, Chromatographic Specialties, Canada) onto a capillary column (30 m × 0.32 mm) (Supelcowax 10). The carrier gas was helium, and the oven was programmed from 50–240°C at 5°C/min, following an initial hold of 7 min.

Oxidation products were identified by injecting 0.5 μl of concentrated extract using an on-column injector (Hewlett-Packard) onto a capillary column (30 m × 0.32 mm) (Supelcowax 10), which was directly interfaced to a quadrupole mass spectrometer (VG 12-250). The GC oven (Hewlett-Packard 5790) was programmed 50–250°C from 4°C/min, and the carrier gas was helium. Eluted compounds were identified by comparing their mass spectra with authentic standards and/or with those contained in the National Bureau of Standards mass-spectral library.

Analysis of Carbonyls Produced in Force-Aged Beer

A bottle of production lager (341 ml) was opened and treated with 34 mg of EDTA dissolved in 1 ml of distilled water. The bottle was fobbed, recapped, and heated at 50°C for four days in a warm-air incubator. The beer was then cooled to 20°C, transferred to a 1-L separatory funnel, and spiked with 1-octanol (1 mg/L) as an internal standard. The solution was extracted with 2 × 100 ml of dichloromethane, and the combined extracts were transferred to a 1-L round bottom flask containing 10 g of sodium metabisulfite dissolved in 100 ml of distilled water. The mixture was stirred at ambient temperature for 24 hr. It was then transferred to a 500-ml separatory funnel, and the methylene chloride was discarded. The aqueous phase was cooled to 4°C, adjusted to pH 10 with 20% sodium hydroxide, saturated with sodium chloride, and extracted with 2 × 100 ml of diethyl ether in a 200-ml separatory funnel. An aqueous solution (50 ml) containing 0.5*M* sodium acetate and 0.5*M* hydroxylamine hydrochloride was added to the combined ether phases in a 500-ml round bottom flask fitted with a water condenser. The mixture was stirred under reflux for 2 hr using a water bath held at 40°C. The ether layer was separated, washed with 2 × 50 ml 8% sodium bicarbonate solution, dried over sodium sulfate, and concentrated to 4 ml in a Kuderna-Danish apparatus. The volume was further reduced under a nitrogen stream to 100 μl in a 5-ml sample vial.

A control beer and a beer spiked with lysine monohydrochloride (600 mg/L) were force-aged and worked up in the same way. An additional, untreated control beer was worked up without force-aging.

The extract of each beer was analyzed by GC-mass spectrometry. Individual oxime peaks were identified by mass-spectral comparison with the National Bureau of Standards mass-spectral library and with an in-house mass-spectral library assembled from authentic samples of aldehyde oximes synthesized previously (D. E. F. Gracey and R. L. Barker, *unpublished data*) (4). Relative peak areas were calculated for a series of oximes in each chromatogram by dividing the oxime peak area by the area of the internal standard peak. The stereoisomers of most oximes were resolved under the GC conditions used (see above), and in these cases, the peak areas were summed.

Preparation of Melanoidin Stock Solution

The melanoidin stock solution was prepared according to the method of Hashimoto (21) by refluxing a solution containing 72 g of maltose, 18 g of glucose, 150 mg of xylose, and 1.5 g of glycine in 1 L of 0.02*M* sodium phosphate buffer, pH 5.5, for 100 min. The solution was then cooled, and its pH was adjusted to 4.2 with 85% phosphoric acid.

Analysis of 2-Nonen-1-ol in Beer

Beer (100 ml) was degassed by stirring, and L-menthol (20 μg/L) was added. Aliquots (5 × 20 ml) of this solution were poured onto Extrelut tubes (5 × 20 ml), which were then allowed to stand for 1 min. The tubes were eluted under gravity by successive volumes of dichloromethane (2 × 20 and 1 × 10 ml), which were combined and concentrated to about 4 ml in a 250-ml Kuderna-Danish apparatus. The extract was further concentrated to 0.3 ml under a stream of nitrogen.

The extract (0.5 μl) was injected using an on-column injector (Hewlett-Packard) onto a capillary column (60 × 0.32 mm) (Supelcowax 10), which was directly interfaced to a quadrupole mass spectrometer (VG 12-250). The carrier gas was helium, and the GC oven (Hewlett-Packard 5790) was programmed from 40–140C° at 25°C/min, 140°C for 15 min, 140–250°C at 25°C/min, and 250°C for 5 min. Under these conditions, the internal standard and 2-nonen-1-ol eluted at 14.66 and 17.30 minutes, respectively.

The mass spectrometer was set up in the single-ion monitoring mode to monitor ions of mass 71 and 95 (L-menthol) and of mass 57 and 82 (2-nonen-1-ol). A calibration line was obtained daily using a method of standard additions applied to one of the beers being analyzed. Aliquots of an aqueous stock solution containing 20% methanol and 10 μg/L of 2-nonen-1-ol were used to spike aliquots of the degassed beer to concentrations of 5, 10, and 20 μg/L of 2-nonen-1-ol. The calibration line was obtained by plotting the area ratio of mass 57 to mass 71 against added concentration of analyte. The mass ratios of 71 to 95 and 57 to 82 were monitored to confirm the identities of the chroma-

tographic peaks as internal standard and analyte, respectively. The concentration of 2-nonen-1-ol in each unknown was calculated by dividing its mass ratio (57:71) by the slope of the calibration line.

RESULTS AND DISCUSSION

Effect of Cu(II) and Fe(III) on Beer Flavor Instability

Taking into account the reduction potentials of transition metal ion species and their typical concentrations in beer, copper, and iron are the most probable catalysts of oxidation by molecular oxygen. Other transition metals tend to occur at lower concentrations, and their generally higher reduction potentials make them less amenable to reduction by the reducing agents present in beer.

To confirm the effect of Cu(II) ions on beer flavor instability, a batch of production beer was force-aged after treatment with different levels of copper(II) chloride. A taste panel experienced in assessing stale flavor then scored the stale flavor intensity of each beer and found that higher levels of copper(II) did indeed lead to increased stale flavor intensity (Fig. 1) However, copper ions are known to bind strongly to species such as proteins, amino acids, and polyphenols, and it was not obvious whether endogenous copper in beer would have the same effect as that of added Cu(II). Accordingly, batches of production lager were taken over a four-month period, analyzed for copper and iron, and stored at 23°C for five months. The same taste panel then scored the stale flavor intensity of each batch. An obvious correlation was found to exist between the stale flavor intensity and the copper ion concentration, which varied from 40 to 95 μg/L (Fig. 2). No such correlation was observed with iron (Fig. 3), suggesting that copper has a more deleterious effect on beer flavor instability, at least over the concentration ranges reported. This organoleptic result is consistent with a recent analytical observation that adding Cu(II) increased the total aldehyde content of beer more than did adding Fe(III) (1).

Effect of Cu(II) and Fe(III) on Oxidation of Primary Alcohols

Since aldehydes are the immediate oxidation products of alcohols, and since the formation of aldehydes is concomitant with an oxidation process in aging beer, alcohols appeared to be the most logical aldehyde precursors. If this were the case, the overall, simplified sequence of reactions leading to the formation of aldehydes in beer could be as shown:

$$\begin{aligned} RH_2 + 2Cu^{2+} &\rightarrow 2Cu^{+} + R + 2H^{+} \\ 2Cu^{+} + O_2 &\rightarrow 2Cu^{2+} + 2[O]^{-} \\ 2[O]^{-} + \text{alcohol} &\rightarrow \text{aldehyde} + 2OH^{-} \\ \hline RH_2 + O_2 + \text{alcohol} &\rightarrow \text{aldehyde} + R + 2H_2O\,, \end{aligned}$$

where RH_2 = reducing agent and [O] = reactive oxygen species

The potential of copper and iron to catalyze such a couplec oxidation was investigated with a model system consisting of distilled water, 1-butanol, metal ion, and a coupled reducing agent. 1-Butanol was chosen as the substrate because higher molecular weight alcohols have limited solubilities in water, and a heterogeneous system would probably cause reproducibility problems. Although lower molecular weight alcohols also have satisfactory solubilities, their volatility would preclude accurate quantitation with a solvent extraction-concentration workup. Several potential reducing agents that were known to occur in beer or were chemically similar to known beer constituents were evaluated. A concentration of 0.6m*M* was chosen as being representative of the higher alcohols in beer. Control reactions without metal ion or coupled reducing agent consistently led to a 95 ± 1% recovery of 1-butanol; the 5% loss probably was due to evaporation (Table I). However, the combination of Cu(II) and cysteine, ascorbic acid, pyrogallol, or gallic acid resulted in significant losses of 1-butanol due to oxidation. The combination of Cu(II) with glucose, metabisulfite, catechol, or catechin had no effect on the 1-butanol. Fe(III) in combination with cysteine, ascorbic acid, or pyrogallol produced much less oxidative loss of 1-butanol than did the corresponding Cu(II) system. As was found for Cu(II), Fe(III) in combination with either glucose or metabisulfite had no effect on the 1-butanol (Table II).

Although the model reaction and the extraction technique were convenient for monitoring the residual 1-butanol, it seemed likely that oxidation products such as butanal would suffer considerable losses due to evaporation. Therefore, the products of primary alcohol oxidation in the model system were investigated with 1-hexanol. GC analysis subsequently revealed hexanal and pentanal as the major volatile products of 1-hexanol oxidation (Fig. 4). The volatile reaction products detected by GC accounted for only 17% of the lost 1-hexanol. The missing 23% probably evaporated as pentanal and hexanal or, alternatively, formed reactive intermediates that reacted with the pyrogallol to yield nonvolatile products. The major isolated oxidation product of the pyrogallol was purpurogallin (Fig. 5), which has been isolated from other pyrogallol oxidations (39).

The nature and the large number of the hexanol oxidation products are consistent with the intermediacy of a very reactive,

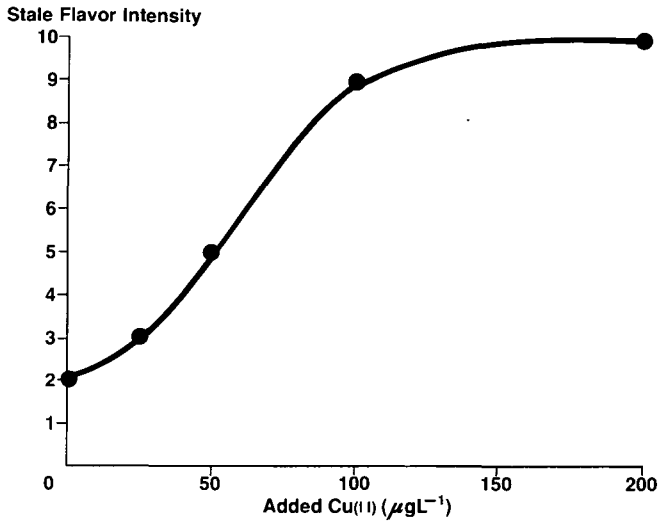

Fig. 1. Effect of added $CuCl_2$ on development of stale flavor in beer after force-aging at 50°C for four days.

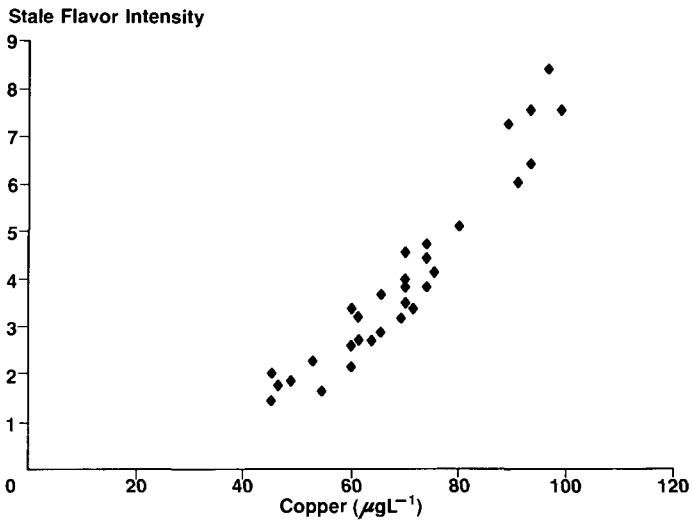

Fig. 2. Effect of endogenous copper concentration in beer on development of stale flavor after storage at 23°C for five months.

nonselective oxidizing species such as hydroxyl radical. A tentative reaction scheme is proposed in Figures 6–8 to explain the formation of the identified hexanol oxidation products. Assuming that hydroxyl radical is the oxidizing species, a series of radical chain propagation steps (Fig. 6, reactions 1, 4, and 7) can be envisaged, with alkyl radicals being formed by hydrogen radical abstraction at one of the six carbon atoms. Normally, hydrogen atom abstraction from hexanol would occur preferentially at C1 because of the stabilizing effect of the attached hydroxyl group. However, considering the reactivity of hydroxyl radical, it may show little selectivity during the abstraction step. The alkyl radicals so formed may participate in further chain propagation steps with molecular oxygen, leading to the formation of peroxides (reactions 2, 3, 5, and 6). The hexene-1-ol isomers and hexanediol may be the products of chain termination reactions (Fig. 7). Exact identification of the hexanediol isomer(s) was not possible by mass spectrometry. The C1 peroxide may be the precursor (Fig. 8) of both hexanoic acid (reaction 10) and hexanal (reaction 11). Pentanal is probably a rearrangement product of the C2 peroxide (reaction 12). Other peroxides (e.g., C3–5) may have led to the formation of even shorter chain aldehydes, although our analytical procedure would not necessarily have detected them.

It was expected that pyrogallol at high concentrations would compete with the alcohol for the reactive oxygen species. However, pyrogallol did not behave in this manner, as increasing its concentration by two orders of magnitude merely led to increasing losses of 1-butanol (Table III).

These observations confirm that copper and (to a lesser extent) iron catalyze the oxidation of primary alcohols to aldehydes by molecular oxygen under mild conditions. Thus, the relative reactivities of Cu(II) and Fe(III) in the model system match the relative effects of these two ions on beer flavor stability and on the uptake of oxygen from beer headspace (48). In each case, Cu(II) is the more reactive species.

The extent of oxidation in the model system was found to be critically dependent on the nature of the coupled reducing agent. At least two types of beer constituents—cysteine and trihydroxypolyphenols—are capable of functioning as coupled reducing agents (i.e., as prooxidants) during such oxidations. Cysteine is typically present in beer at concentrations of 7–20

TABLE I
Extent of Oxidation of 1-Butanol by $CuCl_2$, O_2, and Various Coupled Reducing Agents

Reducing Agent	Loss of 1-Butanol[a] (%)
None	5.8
Cysteine	19.0
Ascorbic acid	48.1
Pyrogallol	46.3
Gallic acid	81.1
Glucose	5.6
Sodium metabisulfite	6.1
Catechol	5.9
Catechin	5.9

[a] Reaction of 0.6 m*M* 1-butanol, 0.6 m*M* $CuCl_2$, and 0.6 m*M* reducing agent (except glucose: 10 m*M*) in an open flask at 23°C for two days.

TABLE II
Extent of Oxidation of 1-Butanol by $FeCl_3$, O_2, and Various Coupled Reducing Agents

Reducing Agent	Loss of 1-Butanol[a] (%)
None	4.9
Cysteine	5.0
Ascorbic acid	24.8
Pyrogallol	15.7
Glucose	4.8
Sodium metabisulfite	5.5

[a] Reaction of 0.6 m*M* 1-butanol, 0.6 m*M* $FeCl_3$, and 0.6 m*M* reducing agent (except glucose: 10 m*M*) in an open flask at 23°C for two days.

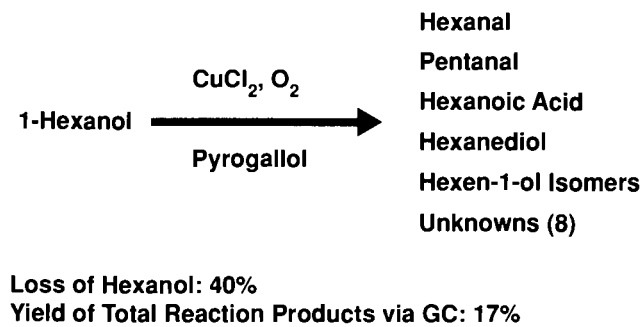

Fig. 4. Products of 1-hexanol oxidation by $CuCl_2$, O_2, and pyrogallol.

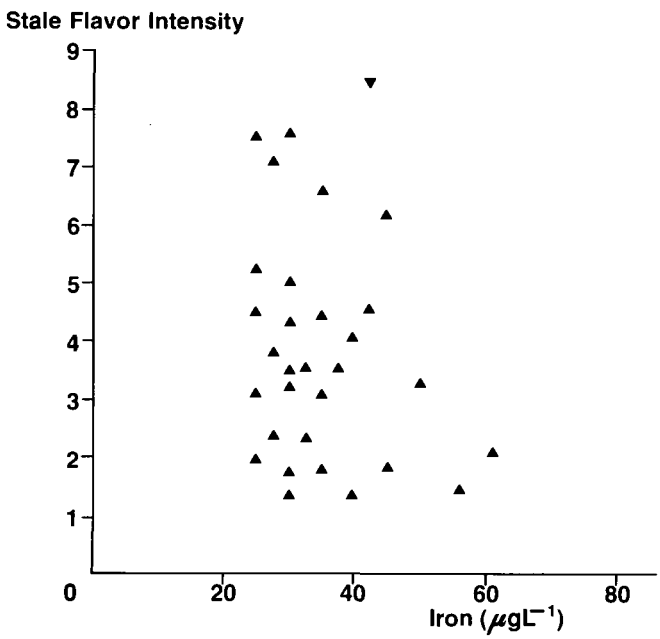

Fig. 3. Effect of endogenous iron concentration on development of stale flavor in beer after storage at 23°C for five months.

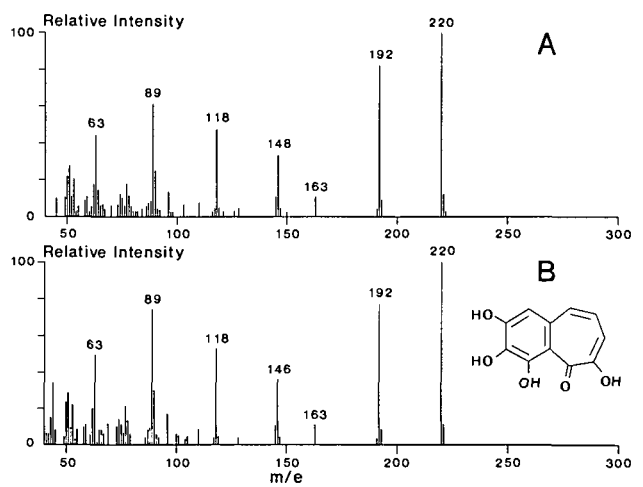

Fig. 5. Mass spectra of the orange solid isolated from the oxidation of 1-hexanol by $CuCl_2$-O_2-pyrogallol (**A**) and authentic purpurogallin (**B**).

mg/L (9), and protein thiols probably behave in a manner similar to that of cysteine. Both Cu(II) and Fe(III) are known to catalyze the oxidation of thiols to disulfides by oxygen. The Cu(II)-catalyzed reaction with cysteine is known to produce H_2O_2, whereas the Fe(III)-catalyzed reaction does not (15). It is tempting then, but not necessarily correct, to conclude that the alcohol-oxidizing activity of the Cu(II)-cysteine system is due to the intermediacy of H_2O_2 and, further, that the inactivity of the Fe(III)-cysteine system is due to the absence of H_2O_2.

Polyphenols containing a 1,2,3-trihydroxybenzene ring, such as pyrogallol and gallic acid, were found to be even more potent than cysteine in driving the 1-butanol oxidation. It has also been observed (40) that the addition of pyrogallol to wine greatly increases the production of aldehydes and acetals during aging. In view of their similar functionality, it seems probable that 3′,4′,5′-trihydroxyflavans also function as coupled reducing agents. Although gallic acid usually is present in beer at only μg/L concentrations (42), 3′,4′,5′-trihydroxyflavan species—such as delphinidin, myricetin, and their polymers—are present at mg/L concentrations (9,18,27,35). Such concentrations should be sufficient to drive the oxidation process.

Somewhat parodoxically for an antioxidant, ascorbic acid caused a large oxidative loss of 1-butanol, which was consistent with results published for similar systems (8).

Cu(II) is known to catalyze the oxidation of sulfite to sulfate by oxygen, yet metabisulfite had no effect on the 1-butanol. Clearly, any reactive oxygen species generated in the presence of sulfite is consumed at a much faster rate by unreacted sulfite than by 1-butanol. This property presumably contributes to sulfite's efficacy as a beer flavor stabilizer and suggested that sulfite might scavenge reactive oxygen species in the presence of prooxidants such as pyrogallol. A further model reaction study subsequently confirmed that an equimolar concentration of metabisulfite completely protected 1-butanol against oxidation in the presence of pyrogallol (Table IV).

1,2-Dihydroxybenzene-containing species such as catechol and catechin were not coupled to the oxidation of 1-butanol (Table I). Furthermore, equimolar concentrations of catechol and catechin were as efficient as metabisulfite in protecting 1-butanol against oxidation in the presence of pyrogallol (Table IV). Thus, polyphenols such as catechin, which contain 1,2-dihydroxybenzene rings, not only are incapable of acting as prooxidants in coupled alcohol oxidations, but they actively protect alcohols

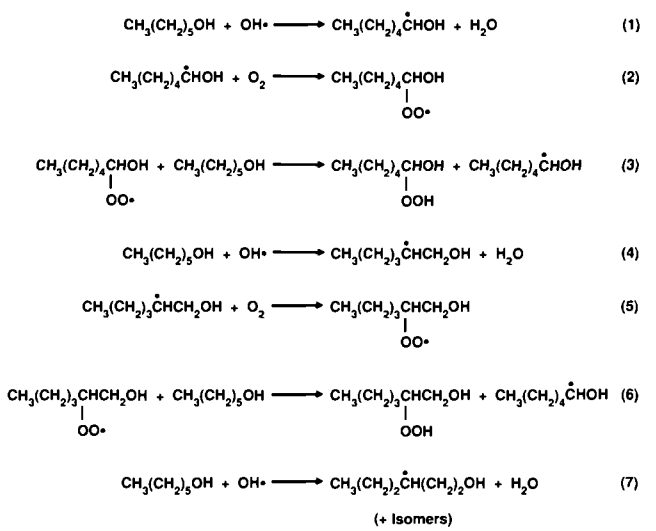

Fig. 6. Chain propagation reactions (numbers in parentheses) between hydroxyl radical, 1-hexanol, and oxygen.

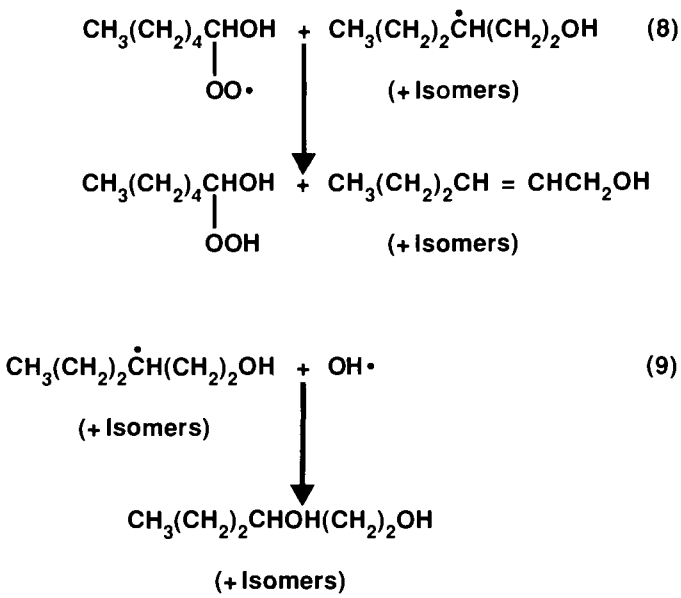

Fig. 7. Chain termination reactions (numbers in parentheses) of 1-hexanol oxidation.

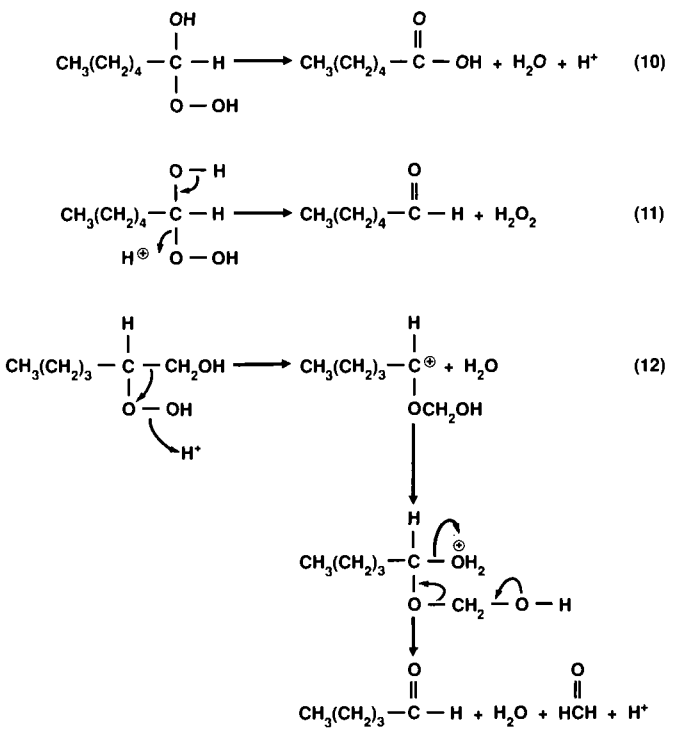

Fig. 8. Hydronium-catalyzed or metal ion-catalyzed reactions (numbers in parentheses) of proposed hexanol peroxide intermediates.

TABLE III
Effect of Increasing Pyrogallol Concentration on 1-Butanol Oxidation by $CuCl_2$, O_2, and Pyrogallol

Concentration of Pyrogallol	Loss of 1-Butanol[a] (%)
0	6
0.6 m*M*	38
2.4 m*M*	38
60 m*M*	69

[a] Reaction of 0.6 m*M* 1-butanol, 0.6 m*M* $CuCl_2$, and pyrogallol in an open flask at 23°C for two days.

from oxidation and therefore function as antioxidants. Monomeric and dimeric catechins can occur at levels of up to 5.5 and 4.0 mg/L, respectively, in beer (12,34) and should therefore help to stabilize beer flavor. Other dihydroxypolyphenols in beer, such as quercetin and leucocyanidin, are present in even higher concentrations (9) and should behave similarly.

Melanoidins are occasionally invoked in beer-staling mechanisms (21–23). These poorly characterized, high molecular weight compounds are formed by reactions between sugars and amino acids and contain reducing groups called reductones. Most authors have found that melanoidins have antioxidant properties (16,30-33,47,50,53). Hashimoto (21), on the other hand, reported that a melanoidin system oxidized 1-butanol to butanal in about 0.1% yield. Consequently, a sample of Hashimoto's melanoidin solution was prepared and stirred with 1-butanol for two days. Although the 1-butanol was recovered unchanged compared with the control (Table V), this was not unexpected, as a 0.1% loss of 1-butanol would be of a smaller magnitude than the experimental error of our analytical method. However, any direct oxidizing activity of the melanoidin solution is clearly much weaker than that of the coupled oxidation systems catalyzed by Cu(II) and Fe(III). When the melanoidin solution was spiked with Fe(III), a small (6%) loss of 1-butanol beyond that of the control was observed. Apparently, reductones in the Hashimoto melanoidin solution have a weak activity as coupled reducing agents in the Fe(III)-catalyzed oxidation of 1-butanol. This activity and the presence of trace levels of iron may be responsible for the trace amounts of butanal detected by Hashimoto in the melanoidin-1-butanol reaction (21).

The combination of Cu(II) and melanoidin solution had no effect on 1-butanol, and it was further found that the melanoidin solution protected 1-butanol against oxidation in the presence of Cu(II)-pyrogallol. The model melanoidin solution is known to contain predominantly unreacted sugars and amino acid (22), and subsequent control experiments showed that its ability to protect 1-butanol against oxidation in the presence of Cu(II) and pyrogallol was due to those unreacted starting materials (Table V). The latter are present at rather high concentrations and effectively scavenge the reactive oxygen species. Consequently, it is not possible to conclude from these results whether reductones have any prooxidant or antioxidant effect on the Cu(II)-catalyzed oxidation of alcohols. There seems to be a weak prooxidant effect with Fe(III). However, this reaction is probably not important in beer, since Cu(II) appears to have greater impact than does Fe(III) on beer flavor instability.

TABLE IV
Effect of Antioxidants on 1-Butanol Oxidation by $CuCl_2$, O_2, and Pyrogallol

Added Antioxidant	Loss of 1-Butanol[a] (%)
None	46.3
Sodium metabisulfite	5.0
Catechol	7.0
Catechin	6.2

[a] Reaction of 0.6 m*M* 1-butanol, 0.6 m*M* $CuCl_2$, and 0.6 m*M* pyrogallol, and 0.6 m*M* added antioxidant in an open flask at 23°C for two days.

TABLE V
Effect of Melanoidin Solution on 1-Butanol Oxidation

Added Reagent	Added Metal Salt	Loss of 1-Butanol (%)
None	None	5.8[a]
Melanoidin	None	5.7[b]
Melanoidin	$FeCl_3$	11.6[b]
Melanoidin	$CuCl_2$	4.3[b]
Pyrogallol (0.6 m*M*)	$CuCl_2$	46.3[a]
Pyrogallol (0.6 m*M*) + Melanoidin	$CuCl_2$	4.3[a]
Pyrogallol (0.6 m*M*) + Glycine (20 m*M*)	$CuCl_2$	5.5[a]
Pyrogallol (0.6 m*M*) + Sugars[c]	$CuCl_2$	5.2

[a] Reaction of 0.6 m*M* 1-butanol and 0.6 m*M* metal salt in distilled water with various added reagents in an open flask at 23°C for two days.
[b] Reaction of 0.6 m*M* 1-butanol and 0.6 m*M* metal salt in melanoidin stock solution in an open flask at 23°C for two days.
[c] Maltose (200 m*M*) + glucose (100 m*M*) + xylose (1 m*M*).

Effect of Complexing Agents on the Metal Ion-Catalyzed Oxidation of Alcohols

Metal-chelating agents and ligands are known to alter the reduction potential and the catalytic efficiencies of copper and iron in reactions with molecular oxygen and hydrogen peroxide. Chelating agents such as EDTA are frequently used as quenchers of metal ion redox activity during biochemical research and as antioxidants or preservatives in various food systems (44). Indeed, Blockmans et al (6) reported that beer treated with EDTA retained its freshness longer.

Transition metal ions are also known to bind amino acids (45), and it seemed probable that they, too, might affect the oxidation of alcohols and the resulting production of aldehydes in beer. The oxidation of 1-butanol in the Cu(II)-O_2-pyrogallol model

TABLE VI
Effect of Lysine on 1-Butanol Oxidation by $CuCl_2$, O_2, and Pyrogallol

Concentration of Added Lysine	Loss of 1-Butanol[a] (%)
None	38.1
0.30 m*M*	36.8
3 m*M*	36.4
15 m*M*	30.2
30 m*M*	16.4
150 m*M*	6.1

[a] Reaction of 0.3 m*M* 1-butanol, 0.3 m*M* $CuCl_2$, and 0.3 m*M* pyrogallol in an open flask at 23°C for two days.

TABLE VII
Effect of EDTA on 1-Butanol Oxidation by $CuCl_2$, O_2, and Pyrogallol

Concentration of Added EDTA[a]	Loss of 1-Butanol[b] (%)
None	38.1
0.30 m*M*	36.1
3 m*M*	36.8
15 m*M*	21.7
30 m*M*	5.8
150 m*M*	5.7

[a] Ethylenediaminetetraacetic acid disodium calcium salt.
[b] Reaction of 0.3 m*M* 1-butanol, 0.3 m*M* $CuCl_2$, and 0.3 m*M* pyrogallol in an open flask at 23°C for two days.

TABLE VIII
Effect of EDTA and Lysine on 1-Butanol Oxidation by $FeCl_3$, O_2, and Pyrogallol

Added Ligand	Loss of 1-Butanol[a] (%)
None	17
Lysine (150 m*M*)	5
EDTA (30 m*M*)	14

[a] Reaction of 0.6 m*M* 1-butanol, 0.6 m*M* $FeCl_3$, and 0.6 m*M* pyrogallol in an open flask at 23°C for two days.

system was accordingly found to be completely blocked by EDTA or lysine at concentrations of 100- and 500-fold excess, respectively (Tables VI and VII). Whereas a 250-fold excess of lysine also completely blocked the Fe(III)-catalyzed oxidation of 1-butanol, a 50-fold excess of EDTA inhibited this system only slightly (Table VIII).

The ability of EDTA and lysine to inhibit aldehyde formation in beer was then evaluated. The relative concentrations of a selection of aldehydes were determined in an untreated control beer, a force-aged control beer, and in force-aged beers containing EDTA and lysine. After their extraction, the aldehydes were converted to oximes under mild conditions, identified by GC-mass spectrometry and quantitated by GC-flame ionization detection. As in the model oxidation system, both EDTA and lysine were able to block the formation of most of these aldehydes (Table IX). The lack of effect of both EDTA and lysine on the increased furfural concentration in force-aged beer suggests that this compound is formed by carbohydrate degradation rather than by oxidation of furfuryl alcohol.

Bamforth and Parsons (1) reported an inhibitory effect of diethylenetriaminepentaacetic acid on the total aldehyde production in beer spiked with Cu(II). The production of aldehydes in beer by added hydrogen peroxide is inhibited by EDTA (28), and the addition of lysine reportedly improves the flavor stability of beer (41). The general parallel effects of lysine and EDTA on aldehyde production in the model oxidation system and in beer further confirm the similarity between these two systems and further emphasize the importance of metals to the oxidation process in beer. Although some of the copper ion in beer is presumably bound to amino acids and protein, the nature and extent of the binding do not completely prevent participation of the copper in oxidation reactions.

Melanoidins bind Fe(II) and Cu(II) more strongly than does EDTA, and it has been suggested (17) that this ability contributes to their antioxidant properties. Although not well documented, dark malts have been alluded to as producing more flavor-stable beers than pale malts (5,14). Perhaps this effect can be ascribed to the higher melanoidin content and hence higher metal-binding capacity of dark malts.

Effect of Model Oxidation System on Precursors of *trans*-2-Nonenal

An array of reactions has been proposed to account for the increased concentration of a large number of individual aldehydes in aged beer (23). The relevance of some of these reactions and of most of the aldehydes to the development of stale flavor is questionable. The aldehydes produced from most of these reactions, including those produced by oxidation of simple primary alcohols, neither have the correct stale flavor characteristics nor rise in concentration above their individual taste thresholds.

One exception is *trans*-2-nonenal, which has the correct papery flavor characteristics and whose concentration rises above taste threshold in stale beer (26,44,47,49). This compound has been proposed to arise from the oxidative degradation of unsaturated, trihydroxyfatty acids that are known to be present in beer (13,46). The recent identification of *cis*-3-nonenal in beer suggests that it may be the penultimate oxidation product of the trihydroxyfatty acids that subsequently isomerizes to *trans*-2-nonenal (4). *trans*-2-Nonenal is present in wort (3), and it has been proposed that some survives fermentation in masked forms such as 3-hydroxynonanal and as bisulfite complexes of the latter and *trans*-2-nonenal (3,19). It was further proposed that the *trans*-2-nonenal would be liberated as the bisulfite concentration fell during aging. Given the oxidation process occurring in beer, 2-nonen-1-ol appeared to be a further possible source of *trans*-2-nonenal. An authentic sample of 2-nonen-1-ol was subsequently found to be 32% oxidized by the Cu(II)-O_2-pyrogallol model system (Table X). The major products were *trans*-2-nonenal and heptanal. As with the 1-butanol oxidation, the 2-nonen-1-ol oxidation was significantly inhibited by both EDTA and lysine. However, any alcohol oxidation in beer has to compete with ethanol for the reactive oxygen species, and the oxidation of 2-nonen-1-ol in the presence of 5% ethanol was found to significantly decrease the percent oxidation of 2-nonen-1-ol and the percent yield of *trans*-2-nonenal. A small amount (0.2%) of the latter was still formed, whereas none was detected in the control. Although the 0.6m*M* 2-nonen-1-ol apparently competes to a small extent with 5% ethanol for the oxidizing species, the significance of such a small yield of *trans*-2-nonenal with respect to stale flavor development depends on the concentration of 2-nonen-1-ol in beer. Assuming a 0.2% yield of *trans*-2-nonenal from the oxidation of 2-nonen-1-ol in beer, 50 μg/L of the latter would be required to produce 0.1 μg/L of *trans*-2-nonenal, that is, an amount equivalent to its approximate taste threshold (51).

2-Nonen-1-ol had not been detected in beer (38). However, wort contains *trans*-2-nonenal, and the alcohol dehydrogenase of *Saccharomyces* spp. has a wide substrate specificity, including the ability to reduce 2-alkenals to the corresponding 2-alken-1-ols (20). Accordingly, it seemed possible that beer might contain detectable levels of 2-nonen-1-ol. An analytical method for this compound was subsequently developed, based on a solid-phase extraction procedure with diatomaceous earth granules (25). The dichloromethane extract was concentrated in a Kuderna-Danish apparatus, and the 2-nonen-1-ol was determined by capillary GC-mass spectrometry with single-ion monitoring. The method was calibrated via standard addition of known amounts of authentic 2-nonen-1-ol to beer. A typical calibration line is shown in Figure 9. The results in Table XI show that four different bottlings of a production adjunct lager (lagers 1–4), and a production all-malt lager (lager 5) contained less than 0.5 μg/L 2-nonen-1-ol. Although a number of different bottlings of one production adjunct ale (ales 1–3) and two other production ales (ales 4 and 5) did contain detectable amounts of 2-nonen-1-ol, the concentrations were very low.

These very low 2-nonen-1-ol concentrations and the very low yield of *trans*-2-nonenal in the presence of 5% ethanol strongly suggest that 2-nonen-1-ol is not a significant precursor of *trans*-2-nonenal in beer. Nor is it likely that alkenols serve as precursors of the other alkenals that may be involved in the development of stale flavor.

The other potential precursors of *trans*-2-nonenal are the partially oxidized, unsaturated fatty acids and the bisulfite complexes of *trans*-2-nonenal and 3-hydroxynonanal. Both appeared to have

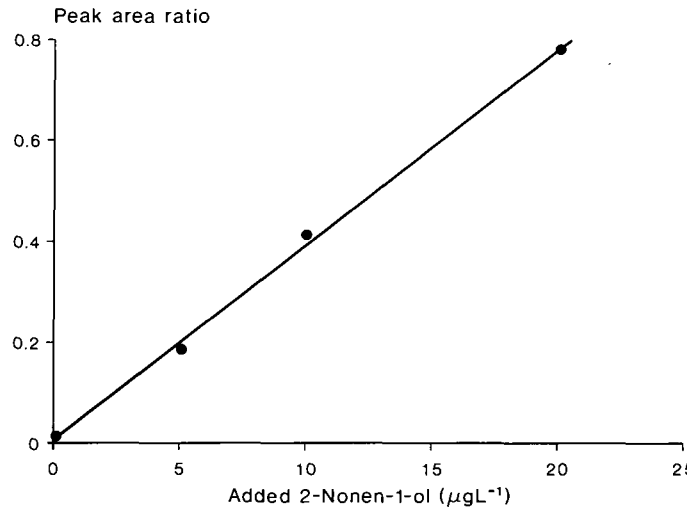

Fig. 9. Calibration line of 2-nonen-1-ol in beer obtained by method of standard additions and gas chromatographic-mass spectroscopic analysis with single-ion monitoring (correlation coefficient, 0.9991). Peak area ratio = area of mass 57 (2-nonen-1-ol) and mass 71 (internal standard) ion peaks.

structures susceptible to oxidation by the Cu(II)-O_2-pyrogallol model system. Although the possible *trans*-2-nonenal-precursor fatty acids, such as 9,12,13-trihydroxy-10-*trans*-octadecanoic acid (13) are not readily obtainable, the structurally related 12-hydroxy-9-octadecenoic acid (ricinoleic acid) was commercially available. When ricinoleic acid was oxidized in the model system, it yielded a mixture of products, including 1-hexanol, which comprised about 80% of the volatile reaction products as detected by GC (Fig. 10). Other reaction products included heptanal, heptanoic acid, and the oxidation products of 1-hexanol. Under the same conditions that oxidatively cleaved ricinoleic acid, 9,12-octadecadienoic acid (linoleic acid) and 12-hydroxyoctadecanoic acid gave no products that were detectable by GC.

It is not certain whether the partially oxidized fatty acids known to occur in beer would actually yield stale-flavored, unsaturated aldehydes in this reaction. However, the results obtained with ricinoleic acid clearly show that unsaturated, hydroxyfatty acids are susceptible to Cu(II)-catalyzed oxidation and that the reaction products include aldehydes. Given this result and the dependence of stale flavor development on copper-catalyzed oxidation processes, it seems most probable that unsaturated hydroxyfatty acids do serve as precursors for unsaturated aldehydes. Simple, saturated hydroxy fatty acids or unsaturated fatty acids are much less reactive and do not yield oxidative degradation products under the same conditions. The inactivity of the simple, unsaturated fatty acids in this oxidation system presumably accounts for their lack of effect on flavor stability when added into the fermenter or to finished beer (1).

TABLE IX
Effect of EDTA and Lysine on Individual Aldehydes Produced in Force-Aged Beer

Aldehyde	Increase in Concentration After Force-Aging, %		
	Control	Control + EDTA[a]	Control + Lysine[b]
Acetaldehyde	3,825	275	25
Propanal	970	400	12
Furfural	800	800	800
2-Methylbutanal	5	10	0
2-Methylpropanal	440	120	20
Benzaldehyde	100	80	0
Phenylacetaldehyde	128	120	0
Nonanal	100	0	0

[a] Ethylenediaminetetraacetic acid disodium salt, 0.27 m*M*.
[b] 3.3 m*M*.

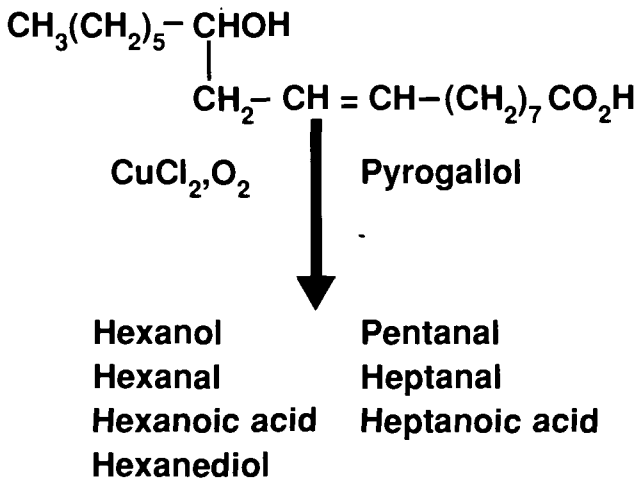

Fig. 10. Products of ricinoleic acid oxidation by $CuCl_2$-O_2-pyrogallol.

When hexanal bisulfite was exposed to the Cu(II)-O_2-pyrogallol model system, 71% of the bound hexanal was released after 24 hr (Table XII). Control reactions without Cu(II) or pyrogallol released less than 2% of the hexanal in the same time. *trans*-2-Nonenal is known to bind bisulfite less strongly than do saturated alkanals such as hexanal, and it should release bisulfite even more rapidly (3). Thus, both unsaturated hydroxy fatty acids and bisulfite complexes are susceptible to Cu(II)-catalyzed oxidation, and these compounds appear to be the most probable precursors of stale-flavored, unsaturated aldehydes in aging beer.

The foregoing results suggest that the overall oxidative process leading to the formation of stale-flavored, unsaturated aldehydes in beer is as shown in Figure 11.

The same process presumably results in the simultaneous production of many other aldehydes via the oxidation of primary alcohols. The direct contribution of these other, predominantly saturated aldehydes to stale flavor is arguable. However, as discussed previously (3), any increase in the concentration of saturated aldehydes will result in the liberation of unsaturated aldehydes from their bisulfite complexes by intermolecular bisulfite transfer.

Alternative reducing agents to those shown in Figure 9 have been proposed to recycle the transition metal ions through their reduced states during the oxidation process in beer. These include

TABLE X
Oxidation of 2-Nonen-1-ol by $CuCl_2$, O_2, and Pyrogallol[a]

Additive	Concentration of Pyrogallol	Loss of 2-Nonenol (%)	Yield of 2-Nonenal (%)	Yield of Heptanal (%)
None	0	2.9	ND[b]	ND
None	0.6 m*M*	31.9	3.0	5.7
Lysine (30 m*M*)	0.6 m*M*	9.3	ND	0.25
EDTA (30 m*M*)	0.6 m*M*	18.4	0.44	0.58
Ethanol (5%, v/v)	0.6 m*M*	7.2	0.21	0.10

[a] Reaction of 0.6 m*M* 2-nonen-1-ol, 0.6 m*M* $CuCl_2$, and pyrogallol in an open flask at 23°C for two days.
[b] Not detected.

TABLE XI
Determination of 2-Nonen-1-ol in Beer

Beer	Concentration of 2-Nonen-1-ol (μg/L)
Adjunct lager	
1	<0.5
2	<0.5
3	<0.5
4	<0.5
All-malt lager	
5	<0.5
Adjunct ale	
1	5.5
2	<0.5
3	0.7
4	0.5
All-malt ale	
5	0.8

TABLE XII
Effect of Model Oxidation System on Hexanal-Bisulfite Complex

Additive	Hexanal Released[a] (%)
None	1.7
$CuCl_2$	1.5
$CuCl_2$ + pyrogallol	71.0

[a] Reaction of 0.37 m*M* hexanal bisulfite, 0.6 m*M* $CuCl_2$, and 0.6 m*M* pyrogallol in an open flask at 23°C for 24 hr.

the use of H_2O_2 to reduce Fe(III) to Fe(II), leading to a cyclic Fenton reaction (1,2,28):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad (13)$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OH_2^{\bullet} + 2H^{+} \quad (14)$$

$$\text{Net:} \quad 2H_2O_2 \rightarrow OH^{\bullet} + O_2^{\bullet} + H_2O + H^{+} \quad (15)$$

In fact, thermodynamic considerations suggest that reaction 15 is unlikely to occur. The Gibbs free-energy change ($\Delta G^{o'}$ J/mol) for a given redox couple is equal to $-nF\Delta E^{o'}$, where n = the number of moles of electrons transferred in the reaction equation, F = the number of coulombs (96,500) of electrons per mole, and $\Delta E^{o'}$ = the difference in volts between the standard reduction potentials of the two reactants. $\Delta G^{0'}$ refers to a standard state of pH 7, but one in which all other activities are unity. The unit of $\Delta G^{0'}$ may be converted from joules to kilocalories by a conversion factor of 0.2390×10^{-3}. Thus, the $\Delta G^{0'}$ for reaction 15 at pH 7 can be calculated from the respective reduction potentials of reactions 16 (29) and 17 (52) as $-1 \times 96{,}500 \times 0.2390 \times 10^{-3} \times -0.48$ (i.e., +11.06 kcal/mol).

	$E^{o'}$ (pH 7)
$H_2O_2 + H^{+} + e \rightarrow OH^{\bullet} + H_2O$	(16) + 0.46V
$H_2O_2 - e \rightarrow O_2^{\bullet} + 2H^{+}$	(17) − 0.94V
Net: $2H_2O_2 \rightarrow O_2^{\bullet} + OH^{\bullet} + H_2O + H^{+}$	(15) − 0.48V

Since the free energy change of reaction 15 would be even more positive at pH 4, this reaction should not occur in beer. Reaction 14 is also thermodynamically unfavorable and unlikely to occur at pH 4 (29).

The Haber-Weiss cycle has also been suggested to occur in beer (1,2,28). The Haber-Weiss reaction is shown:

$$M^{n+} + H_2O_2 + H^{+} \rightarrow M^{(n+1)+} + H_2O + OH^{\bullet} \quad (18)$$

$$M^{(n+1)+} + O_2^{\bullet} \rightarrow M^{n+} + O_2 \quad (19)$$

$$\text{Net:} \quad O_2^{\bullet} + H_2O_2 + H^{+} \rightarrow H_2O + O_2 + OH^{\bullet} \quad (20)$$

$M^{n+} = Fe^{2+}$ or Cu^{+}

The Haber-Weiss reaction consists of a Fenton reaction (reaction 18) coupled with a reaction between oxidized metal ion and superoxide (reaction 19), the superoxide thus serving to recycle the metal through its Fenton-active, reduced state. Although the Haber-Weiss reaction (reaction 20) is thermodynamically favorable, it has been demonstrated only in defined biochemical model reactions that lacked auxiliary reducing agents (43). In the presence of significant concentrations of alternative reducing agents such as trihydroxyaromatic compounds and ascorbic acid, the latter are probably of more importance to metal recycling than is superoxide. Superoxide, for example, appears not to be involved in the Cu(II)-catalyzed oxidation of ascorbic acid (43).

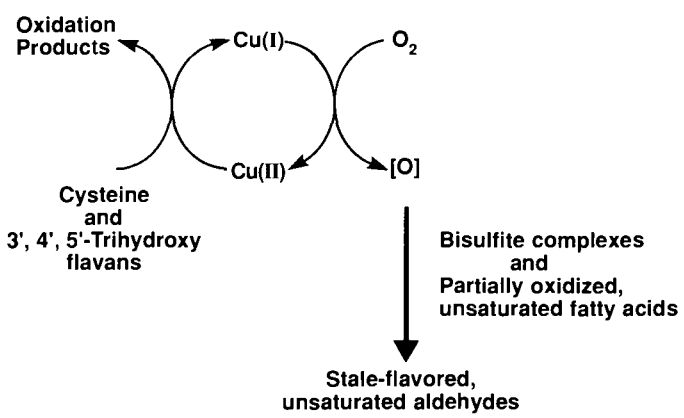

Fig. 11. Proposed mechanism for formation of stale flavor aldehydes in beer.

It has been claimed that polyphenolic species in general, and quercetin in particular, act as antioxidants and improve the flavor stability of beer (10,11). The present results confirm that 3',4'-dihydroxyflavans do behave as antioxidants by protecting alcohols and unsaturated, hydroxyfatty acids against oxidation and that they should indeed serve to improve beer flavor stability.

The present results also suggest that another class of polyphenols, 3',4',5',-trihydroxyflavans, should decrease beer flavor stability. Whether the total polyphenol fraction of a given beer has a net stabilizing or destabilizing effect on its flavor presumably depends on the absolute and relative concentrations of the trihydroxy and dihydroxy species. Both potential precursors of the stale-flavored, unsaturated aldehydes are related, in that the sulfite-bound, unsaturated aldehydes are almost certainly formed by oxidation of partially oxidized fatty acids which, in turn, are formed by enzyme-catalyzed oxidation reactions during malting.

Consequently, the oxidation mechanism in Figure 11 emphasizes not only the need to control oxygen and metal accumulation subsequent to fermentation, but also the importance of the raw materials and the brewhouse processes that determine the concentrations of partially oxidized fatty acids and unsaturated aldehydes entering the fermenter. Considering the higher temperatures of the mash mixer, lauter tun, and kettle compared with the model reaction, metal-catalyzed oxidation of partially oxidized fatty acids and perhaps even unsaturated fatty acids will also occur at these stages of the brewing process. The brewhouse operations result in a decrease in the reducing power of the wort (37), which is due at least in part to the oxidation of amino acid thiol groups and dihydroxyflavan and trihydroxyflavan species. Since overexposure of wort to oxygen leads to poorer beer flavor stability (36,37), the loss of antioxidant dihydroxyflavans in the brewhouse may be more significant than the loss of prooxidant thiols and trihydroxyflavans. However, this last suggestion is tentative at best, as the additional oxygen in those experiments may have resulted in the formation of additional quantities of stale flavor precursors, such as the partially oxidized fatty acids and *trans*-2-nonenal itself.

SUMMARY

The rate of flavor staling in beer is significantly increased by traces of Cu(II), even at levels below 100 μg/L. The Cu(II) ions catalyze the formation of reactive oxygen species from molecular oxygen in a reaction that requires a coupled reducing agent (e.g., cysteine or a 1,2,3-trihydroxypolyphenol) to recycle the Cu(II) through its lower oxidation state. Fe(III) is less reactive than Cu(II) in both a model system and beer.

The reactive oxygen species oxidizes C_n primary alcohols to yield the corresponding C_n and C_{n-1} aldehydes as the major volatile products. 2-Nonen-1-ol is not present in beer at concentrations that would allow it to serve as a significant precursor of *trans*-2-nonenal.

Model studies suggest that stale flavored, unsaturated aldehydes such as *trans*-2-nonenal may be produced in beer by the metal-catalyzed oxidation of unsaturated, hydroxyfatty acids or bisulfite complexes of the aldehydes.

The Cu(II)-catalyzed oxidation of alcohols is inhibited by EDTA, lysine, metabisulfite, and 1,2-dihydroxypolyphenol species. Lysine and EDTA inhibit the formation of aldehydes in aged beer.

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