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Nonoxidative Mechanism for Development of *trans*-2-Nonenal in Beer

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ABSTRACT

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Oxidation is usually recognized as the major cause of development of a stale flavor in beer. However, no significant difference in *trans*-2-nonenal concentration has been observed between oxygen-receiving and oxygen-free beers after aging. Moreover, $^{18}O_2$ was not incorporated into the carbonyl fraction, indicating that the cardboard flavor in beer is not due to lipid oxidation in the bottle. As shown by adding deuterated nonenol to the pitching wort, nonenal oxidation and sulfitic adduct degradation were also inefficient pathways of *trans*-2-nonenal synthesis. On the other hand, wort nonenal linked to amino acids and proteins revealed to be able to release nonenal at the beer pH. The measured nonenal potential proved a good indicator of beer staling; therefore, UV spectroscopy was used to find which experimental conditions destabilize *trans*-2-nonenal precursors in wort. Laboratory-scale experiments confirmed that SO_2 can reduce both lipid autooxidation and the nonenal potential rise while the wort is boiling.

Keywords: Aging, Oxidation, Schiff base, Stability

RESUMEN

La oxidación es usualmente reconocida como la principal causa de desarrollo de sabor añejo en cerveza. Sin embargo, no se han observado diferencias significantes en la concentración de *trans*-2-nonenal entre cervezas con oxigeno y cervezas libres de oxigeno, después de envejecimiento. Aunque $^{18}O_2$ causó dramática deterioración de polifenoles, este no fue incorporado dentro de la fracción carbonilica, indicando que el sabor acartonado en cerveza no es debido a la oxidación de lípidos. Como se demostró por adición de nonenal deuterado a mosto inoculado, la oxidación de nonanol y la degradación del aducto sulfítico fueron también rutas ineficientes para la síntesis de *trans*-2-nonenal. Todos estos datos nos conducen a proponer un mecanismo no-oxidativo para la producción de alquenales en cerveza envasada. El potencial nonenal del mosto resulto ser un buen indicador del sabor añejado de la cerveza. Espectroscopia ultravioleta nos permite visualizar el enlace químico que es roto durante los experimentos de oxigeno-libre y determinar condiciones experimentales que desestabilicen los precursores de *trans*-2-nonenal en mosto. Adición de SO_2 después de la filtración del mosto fue de mucha utilidad: esto redujo tanto la autooxidación de ácido linoléico, como el incremento del potencial nonenal, mientras el mosto esta en ebullición.

Oxidative changes occurring in packaged beer constitute one of the most serious problems in brewing. Although the mechanisms of beer staling have not been fully elucidated, oxidation is recognized as the major cause of the development of a stale flavor in beer. Oxygen in the headspace is consumed during storage of packaged beer and, the more air in the headspace, the more the bottled beer deteriorates (12). Apart from air exclusion, no practical solution has been found to date. An oxygen-free headspace, moreover, does not always effectively prevent the appearance of a cardboard flavor in aged beers. Grigsby et al (6) have shown that samples stored with increased levels of O_2 did develop a more pronounced oxidized character, but the chief flavor change was to the sweet, caramelized note which is quite different from the cardboard character usually associated with beer staling. It is well known that the major contributor to this stale flavor is *trans*-2-nonenal (8,21), which can be formed by oxidation of linoleic acid (19). Many authors (4,7,17,22) have demonstrated that linoleic acid could be converted by lipoxygenase through mashing. However, none of the oxidized forms issued from the breakdown of linoleic hydroperoxide (such as the trihydroxy acids) has been shown to be able to release *trans*-2-nonenal in the bottle.

In the present work, we sought to clarify the issue of the impact of oxygen in the headspace of bottled beer. Beers were bottled with and without oxygen and stored; *trans*-2-nonenal was quantified in both beers after aging. Using $^{18}O_2$, we also determined the amount of carbonyls resulting from oxidation of lipids in the final product. In some experiments, deuterated *trans*-2-nonenal was added to the pitching wort to see if nonenal oxidation or sulfitic adduct degradation could explain synthesis of the alkenal. Finally, the impact of the oxygen level during wort mashing was assessed and discussed.

EXPERIMENTAL PROCEDURES

Aging of Bottled Beer with Oxygen in the Headspace

A volume of 15 ml of oxygen (isotope 18) was injected with a gas-tight syringe into the headspace of commercial beer (initial oxygen level <0.1 ppm) through a silicone top (Vel No. 4). The bottles were then crown-sealed and the beer aged at 40°C for five days in a dark room (accelerated aging) or at room temperature for three months (natural aging).

Chromatographic Analysis of Carbonyl Compounds

Vacuum steam distillation. Carbonyl compounds were extracted by vacuum distillation, based on the method of Currie et al (3). The samples (1.5 L) and sodium chloride (300 g) were poured into flask A and heated in a 30°C water bath. The mixture was stirred at 250 rpm. Valves 2, 3, 4, and 5 were opened and valve 1 was closed. Vacuum was applied to the system (2–5 mm Hg). Traps B, C, and D were cooled with liquid nitrogen. Vacuum was applied to flask A by gradually opening valve 1. The samples were distilled at 30°C for 1 hr and at 35°C for 30 min. The volatile fraction was collected in cold trap B (Fig. 1).

Solvent transfer. After thawing, the distillate (≈300–400 ml) was passed through a C18 Bond Elut column (Varian, Zaventem, Belgium), 500 mg, conditioned beforehand with 40 ml of technical methyl alcohol (Vel, Leuven, Belgium) and 30 ml of dichloromethane (99.9% purity; Prosan, Merelbeke, Belgium). Nonpolar volatiles were eluted with 25 ml of dichloromethane.

Concentration. The eluate and 5 ml of external standard, nonane (99% purity; Janssen, Geel, Belgium), 0.5 mg/L in dichloromethane, were then reduced by Kuderna-Danish evaporation to ≈0.5 ml. Dichloromethane extracts were analyzed for the presence of *trans*-2-nonenal by gas chromatography-mass spectrometry (GC-MS).

Gas chromatography-mass spectrometry. For GC, we used a Hewlett Packard model 5890 gas chromatograph fitted with a 50- × 0.32-mm, wall-coated, open tubular (WCOT) nonpolar CP-SIL5 CB capillary column (film thickness 1.2 µm). The carrier gas was

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helium at a flow rate of 1.3 ml/min. The oven temperature was programmed to rise from 30 to 80°C at 20°C/min, and then to 200°C at 2°C/min. Splitless injections (2 µl) were made at an injector temperature of 250°C. The column was directly connected to a Hewlett Packard 5988 quadrupole mass spectrometer. Electron impact mass spectra were recorded at 70 eV. Spectral recording throughout elution was automatically performed with the HP59970C software. Using the selected ion monitoring (SIM) mode (selected ions 57, 70, 83, and 85), *trans*-2-nonenal was detected and quantified. The detector response was calibrated with authentic standards. The method allows a recovery factor >80% for *trans*-2-nonenal with a variation coefficient of 3–5% (2).

Quantification of ^{18}O in the Carbonyl Fraction of Aged Beers

The carbonyl compounds from five vacuum distillations were transferred after extraction and concentration to 50 µl of isooctane (99.8% purity; E. Merck AG, Darmstadt, Germany), of which 30 µl was subjected to cyclotron analysis of ^{18}O. Cyclotron (U.C.L., Louvain-la-Neuve, Belgium) analysis of ^{18}O involves bombarding the samples with energetic protons, causing production of ^{18}F, a radioactive fluorine isotope (16). The 30-µl carbonyl fraction was placed in a sample case and sealed with tantalum foil. Fractions were then irradiated for 30 min with a 7-MeV proton beam at 15 nA on target. The ^{18}F isotope produced decays with a half-life of 110 min and emitted radiation which was easily measured with a gamma detector. Gamma emissions were measured every 20 min for 8 hr after irradiation. The amount of ^{18}O present in the sample before bombardment was calculated from the ^{18}F decay profile.

Fermentation with Addition of Deuterated Nonenal to the Pitching Wort

Deuterated *trans*-2-nonenal, 10 ppb, (C4D9-CH2-CH2-CH=CH-CHO; *synthesis procedure to be published*) was added to a 12°P wort (90% malt, 10% corn) just before fermentation. Fermentation was conducted on 20 L of wort with a lager yeast (12.5×10^6 cell/ml at pitching) at 12°C for four days, 14°C for two days, and 16°C for four days. Maturation was at 10°C for one day, 7°C for two days, and 0°C for three days.

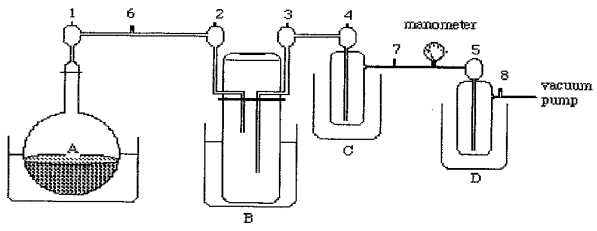

Fig. 1. Distillation apparatus.

TABLE I
***trans*-2-Nonenal Contents Measured in Industrial Beer After Aging[a]**

	trans-2-Nonenal Content (ppb)			
	Injection		No Injection	
Sample	Values	Mean	Values	Mean
Fresh beer	0.09, 0.09	0.09	0.09, 0.09	0.09
Beer after accelerated aging	0.27, 0.31	0.29	0.31, 0.39	0.35
Beer after natural aging	0.21, 0.24	0.23	0.20, 0.22	0.21

[a] Beer measured after accelerated (five days at 40°C) or natural (three months at room temperature) aging, with and without injection of $^{18}O_2$ (96 ppm) into the headspace before storage.

Retention and Release of *trans*-2-Nonenal in a Protein Solution

Malt albumins were extracted according to Byers et al (1). A total of 21.4 ppb of *trans*-2-nonenal was added to the aqueous protein solution (886 ppm of bovine serum albumin equivalent) at pH 5.4. The amount of free alkenal was quantified before and after heat treatment at 50°C for 25 min. *trans*-2-nonenal released after the nonenal potential experiment was also determined.

trans-2-Nonenal-Lysine Schiff Base Synthesis

A total of 183 mg of lysine-HCl (99% purity; E. Merck) was added to 10 ml of a 10-m*M* *trans*-2-nonenal (97% purity; Aldrich, Bornem, Belgium) solution prepared in 50% ethanol (amine-to-aldehyde molar ratio 10:1). The pH was further adjusted to 5.4 with 0.05*M* HCl. After 30 min of reaction at 100°C, the absorbance was measured at 20°C with a UV-VIS 240 Schimadzu spectrophotometer. The influence of temperature was determined by comparison with reactions conducted at 0, 20, 40, 60, 80, and 90°C. A lysine-to-*trans*-2-nonenal molar ratio of 2:1 was used to assess the influence of pH (3–11 range). In one experiment, 15 mg of potassium metabisulfite (UCB, Drogenbos, Belgium) was added to the mixture before the reaction (14 m*M*).

Boiling with Sulfites

A total of 1.275 g of potassium metabisulfite was added to 15 L of wort (12°P, 90% malt, 10% corn) as it began to boil (boiling time 1 hr 15 min). Fermentation was conducted in 3-L EBC tubes with a top-fermentation yeast (*Saccharomyces cerevisiae*, 10×10^6 cell/ml at pitching) at 20°C for seven days and 7°C for seven days.

TABLE II
Proton Bombardment Analysis of Carbonyl Extracts Issued from Beers Aged in Presence of Oxygen Headspace

Sample[a]	Cyclotron Signal	^{18}O Content	^{18}O (µg)[b]	Carbonyl (ppb)[c]
Beer, accelerated aging	· · ·	· · ·	0.00004	0.001
With 84 ppm $^{16}O_2$	33.71 ± 5.45	0.200 %	· · ·	· · ·
With 96 ppm $^{18}O_2$	37.98 ± 4.76	0.225 %	· · ·	· · ·
Beer, natural aging	· · ·	· · ·	0.00003	0.001
With 84 ppm $^{16}O_2$	57.77 ± 1.35	0.200 %	· · ·	· · ·
With 96 ppm $^{18}O_2$	62.69 ± 6.41	0.217 %	· · ·	· · ·

[a] Beer measured after accelerated (five days at 40°C) or natural (three months at room temperature) aging.
[b] ^{18}O incorporated in 250 ml of beer.
[c] Carbonyl compounds binding an ^{18}O atom.

TABLE III
Relationships Between Nonenal Potential of Worts and the Flavor Stability of Corresponding Beers[a]

Wort	Boiling[b]	Fermentation[c]	Accelerated Aging[d]	Natural Aging[e]
With CO_2[f]	0.3	1.4	0.22	0.27
Good hot break[g]	3.9	3.3	0.40	0.98
Bad hot break[h]	4.5	5.1	0.65	2.69

[a] Measured in ppb.
[b] Nonenal potential before boiling.
[c] Nonenal potential before fermentation.
[d] *trans*-2-Nonenal after accelerated aging (five days at 40°C).
[e] *trans*-2-Nonenal after natural aging (three months at room temperature).
[f] Wort prepared with 4 L of CO_2 bubbled for the first 15 min of mashing (57 L of deoxygenated water and 18.2 kg of deoxygenated flour).
[g] Wort prepared with high level of oxygen: 4 L of O_2 bubbled for the first 15 min of mashing (57 L of deoxygenated water and 18.2 kg of deoxygenated flour).
[h] Amount and aspect.

Nonenal Potential Determination

The pH of 1.5 L of wort was adjusted to 4 by phosphoric acid 85% (E. Merck). After a 15-min purging with argon to reduce the oxygen level, the wort was heated at 100°C in a 2-L closed vessel for 2 hr, then cooled during one night before *trans*-2-nonenal analysis (4).

RESULTS AND DISCUSSION

Why a Nonoxidative Pathway?

A total of 96 ppm of $^{18}O_2$ was injected into the bottle headspace of a low-sulfite (2 ppm) commercial lager beer (initial oxygen level below 0.1 ppm). After five days at 40°C (accelerated aging) or three months at 20°C (natural aging), *trans*-2-nonenal was extracted by vacuum distillation and C_{18}-water-dichloromethane partitioning. Despite the large amount of oxygen injected into the headspace, GC-MS revealed no significant difference in *trans*-2-nonenal concentration between oxygen-receiving and oxygen-free samples (Table I).

In all cases, the level increased from 0.1 ppb in fresh beer to 0.2–0.3 ppb in aged beer, whatever the oxygen level. Although *trans*-2-nonenal is known to be also degraded in beer after a long period (5,21), our data suggest that the cardboard flavor would not be produced in the bottle by an oxidative pathway alone.

The alkenal dichloromethane extract, also containing nonenoic acid and 3-hydroxynonanal, the two major degradation products of *trans*-2-nonenal (13), was then analyzed by proton bombardment after transfer from dichloromethane to isooctane. This experiment differs from the work of Owades and Jakovac (15), who derivatized their carbonyls by 2,4 dinitrophénylhydrazine. Very low amounts of ^{18}O were measured in our extracts (exceeding the natural frequency of ^{18}O by only 0.025 and 0.017 atoms/100 oxygen atoms) (Table II). Assuming that the extracted carbonyls and related flavoring compounds (average molecular weight 140) represent a maximum concentration of 5 ppb in the initial beer sample, it appears from our calculations that carbonyls having incorporated ^{18}O represent no more than 1 ppt. This incorporation level is very close to the sensitivity threshold of our method, and well below the 0.2 ppb of *trans*-2-nonenal that appear through aging. All our experiments thus confirm that the cardboard flavor is not due to the oxidation of lipids in the final product.

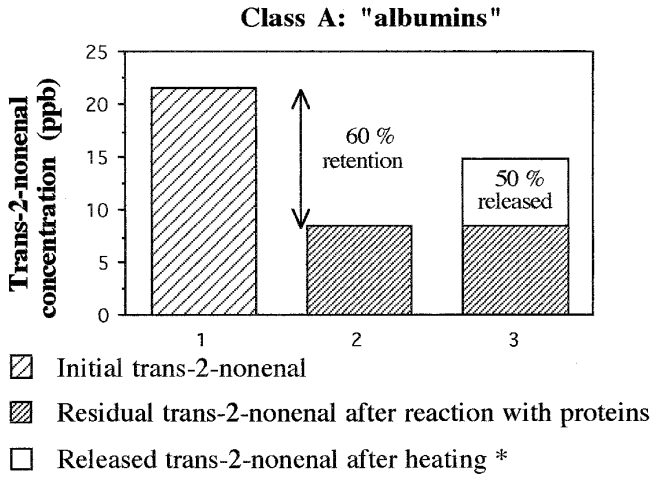

Fig. 2. *trans*-2-Nonenal concentration before (1) and after (2 and 3) heating a solution of *trans*-2-nonenal (21.4 ppb) and malt albumins (886 ppm of bovine serum albumin equivalent) for 25 min at 50°C. * = Drost experiment, 2 hr at 100°C under argon, pH 4

Moreover, 10 ppb of deuterated nonenal added at the beginning of the fermentation failed to yield deuterated nonenal in the aged beer (concentration of labeled nonenal below 0.03 ppb). Since yeast reduces nonenal to nonenal during fermentation (4), this experiment suggests that nonenol oxidation cannot occur in the bottled beer. Our data also confirm the results of Kaneda et al (10), showing that no complex between nonenal and sulfites is created during fermentation. Only sulfitic adducts formed in the final product could be a source of *trans*-2-nonenal during aging (14).

We propose that *trans*-2-nonenal is synthesized by oxidation before fermentation but protected from yeast reduction by binding to amino acids and proteins. Previous data (13) show that this kind of complex is the major degradation product of *trans*-2-nonenal during mashing and boiling. Applying the nonenal potential experiment (4) on an albumin-alkenal model mixture, free nonenal was released (50%), suggesting that this mechanism is realistic at the pH of the beer (Fig. 2). Moreover, the nonenal potential experiment applied to fresh beers obtained after the addition of deuterated nonenal in the kettle (leading to 15 ppb of deuterated nonenal potential at the beginning of fermentation) allowed the release of 1.2 ppb of labeled nonenal. This result confirms that Drost's experiment is not only the acidic breakdown of trihydroxy acids coming from mashing.

Influence of the Brewing Process

We logically detect higher nonenal potentials when oxidation occurs during mashing (higher lipoxygenasic activity) or when the hot break is insufficiently eliminated (slight nonenal potential decrease) (Table III). Moreover, the nonenal potential of the wort is clearly related to staling of the flavor of the corresponding beers, confirming that flavor stability is not related to beer packaging but to wort preparation.

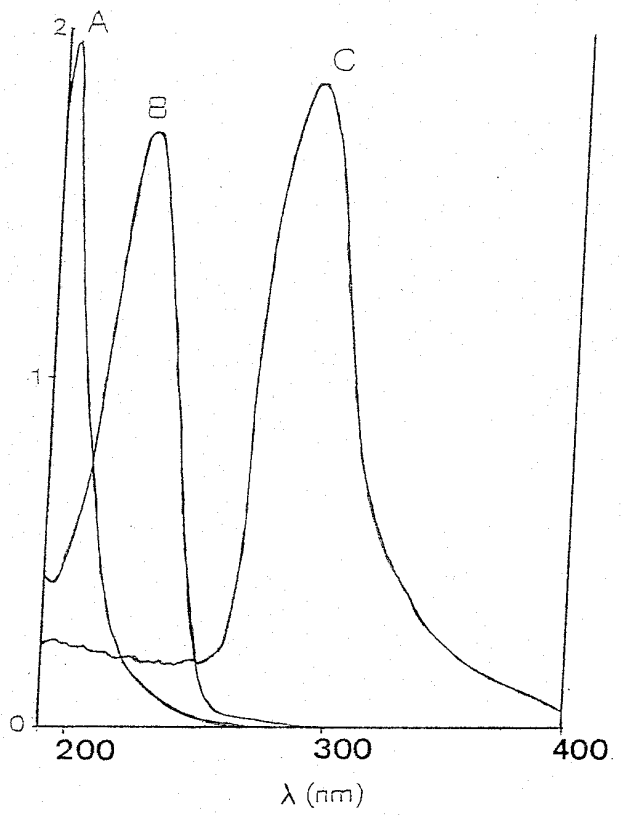

Fig. 3. (A) UV spectrum of lysine, **(B)** *trans*-2-nonenal, and **(C)** *trans*-2-nonenal-lysine Schiff base issued from reaction at 100°C and pH 5.4 for 30 min.

TABLE IV
Nonenal Potential Concentration in the Pitching Wort and *trans*-2-Nonenal Content in Aged Beer in Relation to Sulfite Concentration[a]

	Nonenal Potential in Pitching Wort	*trans*-2-Nonenal in Aged Beer		SO_2 in Pitching Wort		SO_2 in Fresh Beer		SO_2 in Aged Beer	
Sample	**pH 5.4**	**Free pH 4.3**	**Total pH 9**	**Free pH 5.4**	**Total pH 9**	**Free pH 4.3**	**Total pH 9**	**Free pH 4.3**	**Total pH 9**
Blank (+10 ppm of SO_2 in fresh beer)	5.1	0.31	0.59	0.0	0.0	0.8 (+10.0)	1.0 (+10.0)	0.3	3.6
Adding 50 ppm of SO_2 after wort filtration	3.5	0.18	0.21	6.0	10.0	1.8	6.3	1.5	4.4

[a] Beer aged for five days at 40°C; measurements in ppb.

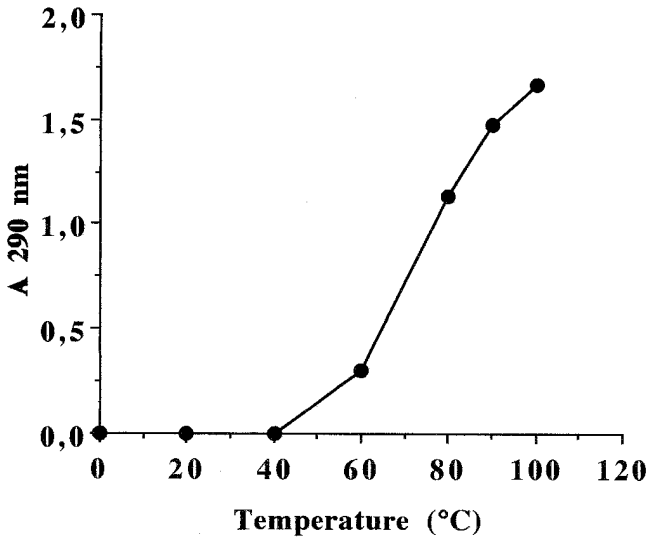

Fig. 4. Influence of temperature (heating for 30 min, pH 5.4) on the Schiff base lysine-*trans*-2-nonenal formation.

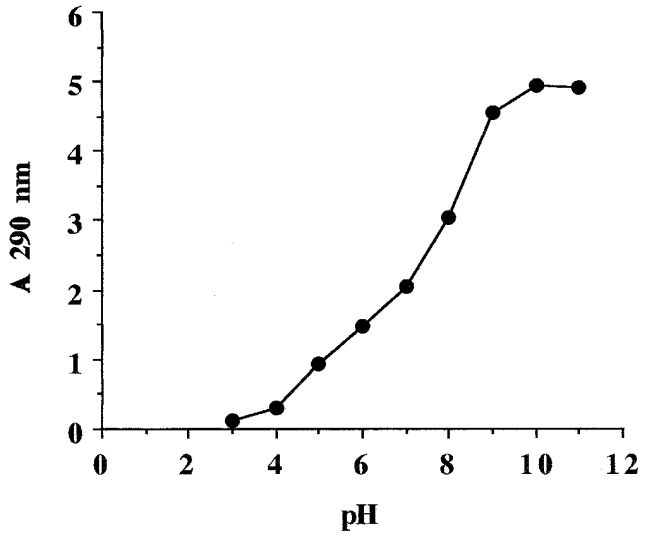

Fig. 5. Influence of pH (heating at 100°C for 30 min) on the Schiff base lysine-*trans*-2-nonenal formation.

UV Spectroscopy to Determine the Stability of Schiff Bases Under Various Conditions

Measuring the nonenal potential is proposed as a means of quantifying the amount of bound nonenal in the wort and hence to assess the future cardboard flavor in beer; therefore, we have tried to determine how various parameters affect the stability of the alkenal-nitrogen compound bond. Ultraviolet absorbance at 290 nm enabled us to visualize such Schiff bases under various conditions (Fig. 3).

Our results clearly indicate that temperature is a parameter increasing imine synthesis (Fig. 4). Another factor influencing the absorbance at 290 nm is pH: the higher the pH, the higher the Schiff base concentration (Fig. 5).

Most interesting was the effect of sulfites, because they suppressed formation of 50% of the C=N bonds at 100°C (pH 5.4). This led us to try to decrease the amount of nonenal precursors by adding 50 ppm SO_2 after wort filtration. The addition of SO_2 also reduces lipid oxidation as the wort boils; therefore, very low nonenal potentials were measured in the final wort. Very good stability also characterized the beer obtained in this way (Table IV). Correlations previously described by Kaneda et al (9) and Uchida et al (20) between the reduction power of the final beer and staling may be due to the fact that the higher the level of antioxidants in beer, the lower the rate of linoleic acid oxidation during boiling.

CONCLUSION

In conclusion, a nonoxidative degradation product of *trans*-2-nonenal synthesized before fermentation is proposed to be the major precursor of the cardboard flavor in aged beers. Due to their Schiff base structure, nonenal precursors can be destabilized by low pH or sulfites. Addition of amino acids to beers, as recommended by Grigsby et al (6), should logically delay alkenal release. The mechnism described here for nonenal synthesis through aging most probably also concerns other aldehydes and ketones produced in the final beer, such as 5-hydroxymethylfurfural, furfural (11), or β-damascenone (18).

ACKNOWLEDGEMENTS

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