# Effect of Free Radicals on Haze Formation in Beer

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An analytical method for determining proanthocyanidins by HPLC–electrochemical detection was developed, and the effect of free radicals on chill haze formation in beer was studied. The determination limits of procyanidin B3, catechin, and epicatechin by this analytical method were 30, 10, and 10 μg/L, respectively. Addition of $H_2O_2$ and irradiation with $^{60}Co$ γ-rays accelerated the degradation of the proanthocyanidins and formation of chill haze in beer, while the addition of a metal scavenger, a $H_2O_2$ decomposer, and free-radical scavengers inhibited these phenomena. Therefore, it was speculated that free radicals generated by Fenton and Haber–Weiss reactions in beer could play an important role in chill haze formation.

## INTRODUCTION

To the consumer, beer should be an agreeable drink of pleasing flavor and attractive color and clarity and should contain sufficient gas to carry aroma and form a characteristic foam. However, these characteristics are gradually degraded during the storage of beer after packaging. The formation of haze is an especially serious problem, and many researchers have studied the mechanism of haze formation. It is generally thought that proteins and polyphenols in beer are responsible for the nonbiological haze and that the oxidation of proanthocyanidins, especially procyanidin B3 and catechin, plays an important role in the formation of chill haze in beer (Asano et al., 1986; Gramshaw, 1969, 1970). There are, however, very few studies concerning the changes in proanthocyanidins during the storage of beer. The mechanism of haze formation has not been fully clarified. One of the reasons is that the analysis of proanthocyanidins in beer requires some complicated procedures which have not yet been established.

Since high-performance liquid chromatography with electrochemical detection (HPLC–ECD) analysis was developed by Kissinger et al. (1973), much attention has been directed to the application of HPLC–ECD for trace organic analysis because of its high sensitivity and selectivity. The determinations of ascorbic acid and phenolic compounds in beer have been also studied (Kenyhercz and Kissinger, 1977; Knudson and Siebert, 1987; Roston and Kissinger, 1981). Proanthocyanidins have many OH groups and can be expected to be determined by the HPLC–ECD method.

In previous papers (Kaneda et al., 1988, 1989), it was shown that free radicals were generated during the process of beer oxidation and they degraded isohumulones to produce some carbonyl compounds in beer. It was also speculated that the free radicals, such as the hydroxyl radical, might be produced by Fenton and Haber–Weiss reactions from hydrogen peroxide generated in beer and play an important role in flavor staling. It is also thought that these free radicals may be responsible for the chill haze formation. In this paper, we report a new analytical method for determination of proanthocyanidins in beer by HPLC–ECD and the effect of free radicals generated during beer storage on the contents of proanthocyanidins and the formation of chill haze.

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## MATERIALS AND METHODS

**Beer.** Bottled lager beer, obtained before pasteurization and without addition of L-ascorbic acid, was used.

**Reagents.** *N-tert*-Butyl-α-phenylnitrone (PBN) and epicatechin were purchased from Aldrich Chemical Co. Catechin was purchased from the Sigma Chemical Co. Procyanidins B3, B4, B8, and C2 were obtained from Prof. P. Dondeyne of Katholieke Universiteit Leuven. Rutin, morin dihydrate, ethylenediaminetetraacetic acid disodium salt (EDTA), potassium thiocyanate (KSCN), *tert*-butyl alcohol (*t*-BuOH), and sodium formate were obtained from Wako Pure Chemicals. Hydrogen peroxide (31% w/v) was obtained from Mitsubishi Gas Co. (Tokyo) and stored at 4 °C. All other reagents used in the HPLC analysis were of HPLC grade.

**Apparatus.** The HPLC instrument was a Waters 600 E system controller equipped with a Waters 712 WISP automatic sample injector, a column thermostating oven, an ECD-100 electrochemical detector (Eicom Ltd.), and a Waters 741 data module. As the analytical column, μBondasphere 5 μm, C18, 100 Å (3.9 × 150 mm, Waters) was used.

**Incubation of Beer.** Twelve milliliters of beer with or without 2.6 mM $H_2O_2$ and 5 mM EDTA or 0.05 M PBN was transferred into a brown bottle with a headspace of 5 mL. The bottle was sealed and incubated at 60 °C.

**Irradiation.** Twelve milliliters of beer was placed in a stoppered test tube, and the headspace was replaced with $CO_2$. Irradiation was carried out with γ-rays from a $^{60}Co$ source at a dose of 2.41 × 10–3.75 × $10^2$ Gy/h. Beer was irradiated with 0.193, 0.431, 0.677, or 2.79 kGy of $^{60}Co$ γ-rays.

**Haze Measurement.** After treatment, the beer was held at 0 °C for 1 day and haze was measured with a NDH-1001 DP haze meter (Nippon Denshoku Kogyo Co. Ltd).

**Chromatography.** Chromatographic conditions used for the analytical method were as follows: detection potential, +850 mV vs Ag/AgCl reference electrode; flow rate, 1.1 mL/min; column temperature, 35 °C; eluting solution A, 0.08 M $KH_2PO_4$ (pH 4.5); eluting solution B, 0.12 M $KH_2PO_4$ in 50% acetonitrile (pH 4.5). The eluting program sequence was as follows: (1) 0–5 min isocratic 100% A; (2) 5.01–30 min, linear gradient, 0–10% B; (3) 30.01–40 min, isocratic, 90% A, 10% B; (4) 40.01–85 min, linear gradient, 10–50% B; (5) 85.01–90 min, isocratic, 50% A, 50% B; (6) 90.01 min, isocratic, 100% A. Ten microliters of degassed and filtered beer was directly injected into the HPLC.

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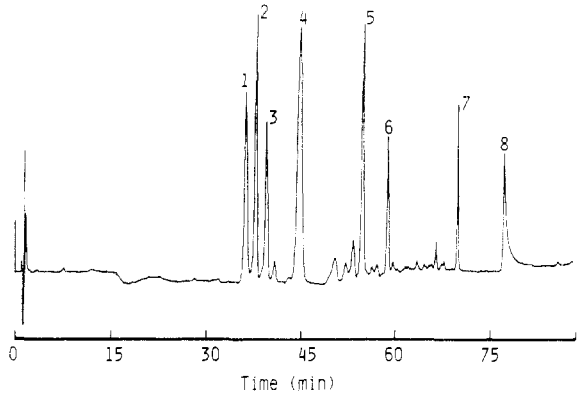


**Figure 1.** HPLC chromatogram of pure polyphenols. 1, Procyanidin B3; 2, catechin; 3, procyanidin C2; 4, procyanidin B4; 5, epicatechin; 6, procyanidin B8; 7, rutin; 8, morin.

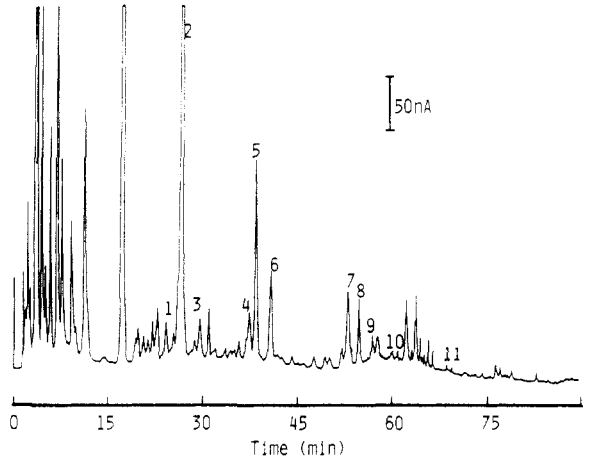


**Figure 2.** HPLC chromatogram of phenolic compounds in beer. 1, Vanillic acid; 2, tyrosol; 3, caffeic acid; 4, procyanidin B3; 5, catechin; 6, *p*-coumaric acid; 7, ferulic acid; 8, epicatechin; 9, sinapic acid; 10, procyanidin B8; 11, rutin.

## RESULTS AND DISCUSSION

**Determination of Proanthocyanidins in Beer.** It was generally difficult to use gradient procedures in HPLC–ECD analysis because the base line is significantly affected by the concentration changes in salts and organic solvents. In this study, concentrations of both salts and acetonitrile were linearly changed with a good balance, pH was kept at 4.5, and a stable base line could be obtained. A glassy carbon was used as a work electrode and the measurement potential was +850 mV vs Ag/AgCl. The peak pattern of pure polyphenols is shown in Figure 1. Each polyphenol peak showed a good separation with high sensitivity. Phenolic acids (vanillic, syringic, *p*-coumaric, ferulic, and sinapic acids), polyphenol monomers (gallic acid, gentisic acid, protocatechuic acid, resorcinol, catechol, chlorogenic acid, and caffeic acid), and other phenolic compounds can also be detected on this chromatogram, though the data are not shown.

The peak pattern of the phenolic components in beer is shown in Figure 2. Procyanidin B3, catechin, and epicatechin could be detected, but procyanidins B4, B8, and C2, rutin, and morin were in trace levels or could not be detected. Vanillic, caffeic, *p*-coumaric, ferulic, and sinapic acids and tyrosol were also detected, except for proanthocyanidins. Procyanidin B3, catechin, and epicatechin, which are concerned with haze formation, were thereafter studied by the HPLC–ECD method. The calibration graphs of proanthocyanidins were linear over the range 0–10 mg/L (Figure 3). The determination limits

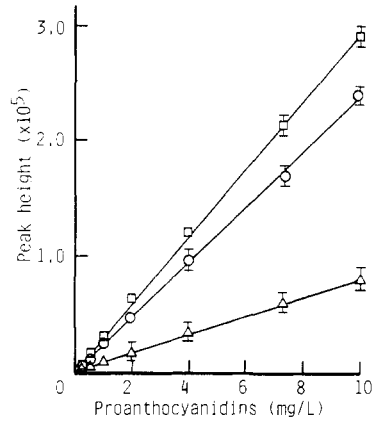


**Figure 3.** Determination of proanthocyanidins. △, Procyanidin B3; ○, catechin; □, epicatechin. Each data point is the average of three replicates, and the vertical lines represent the standard deviations.

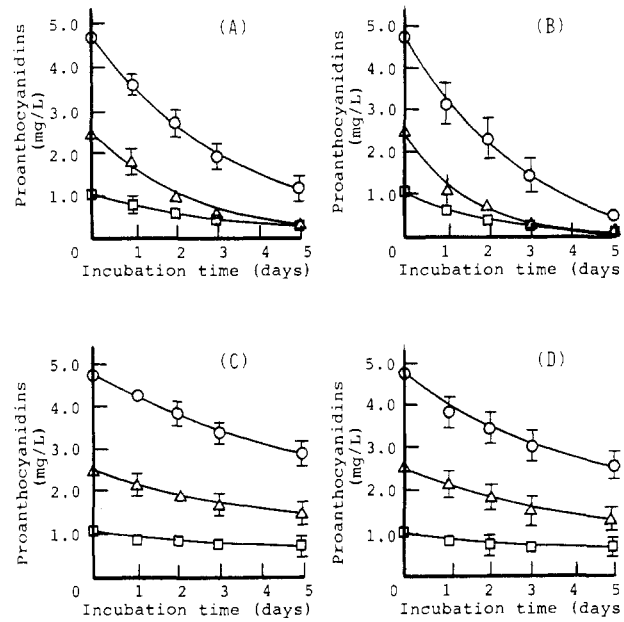


**Figure 4.** Effect of $H_2O_2$, EDTA, and PBN on contents of proanthocyanidins in beer. Beer was incubated at 60 °C. (A) Control (no additions); (B) addition of 2.6 mM $H_2O_2$; (C) addition of 5 mM EDTA; (D) addition of 50 mM PBN. △, Procyanidin B3; ○, catechin; □, epicatechin. Each data point is the average of three replicates, and the vertical lines represent the standard deviations.

of procyanidin B3, catechin, and epicatechin in beer were 30, 10, and 10 μg/L, respectively. This method needs no complicated pretreatment procedures and has higher sensitivity and selectivity when compared to the UV detection method, whose determination limits were about 0.5 mg/L. It can also be applied to the determination of phenolic compounds not only in beer but also in malts, hops, and wort. The determinations were thereafter carried out by absolute calibration methods.

**Effect of Free Radicals on the Formation of Chill Haze in Beer.** When beer was incubated at 60 °C, the contents of procyanidin B3, catechin, and epicatechin decreased and chill haze increased with the incubation times (Figures 4A and 5). When 2.6 mM $H_2O_2$ was added, the decrease in proanthocyanidins and the haze formation were accelerated (Figures 4B and 5), but when 5 mM EDTA or 50 mM PBN was added, these phenomena were inhibited (Figures 4C,D and 5). Procyanidin B3 decreased the most significantly and catechin did the least during the storage of beer. When 20, 40, or 100 mg/L of sulfite

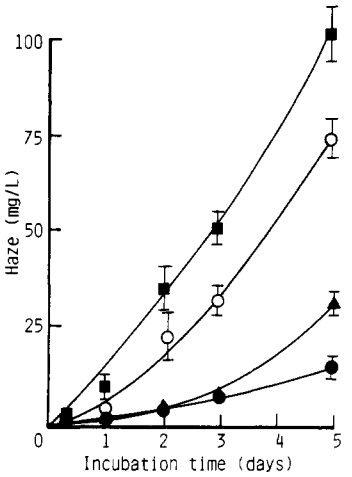


**Figure 5.** Effect of $H_2O_2$, EDTA, and PBN on formation of chill haze in beer. ○, Control (no additions); ■, addition of 2.6 mM $H_2O_2$; ▲, addition of 5 mM EDTA; ●, addition of 50 mM PBN. Haze shows kaolin concentration (milligrams per liter). Each data point is the average of three replicates, and the vertical lines represent the standard deviations.

**Table I. Effect of Sulfite[a] on Proanthocyanidin Contents and Formation of Chill Haze in Beer**

| addition of $SO_3^{2-}$, mg/L | incubation time, days | proanthocyanidins,[b] mg/L: procyanidin B3 | catechin | epicatechin | chill haze,[b] mg/L |
|---|---|---|---|---|---|
| 0 | 0 | 2.80 (100)[c] | 4.50 (100)[c] | 1.03 (100)[c] | 1.0 |
| | 3 | 0.60 (21.5) | 2.20 (48.9) | 0.40 (38.8) | 38.1 |
| 20 | 3 | 1.11 (39.6) | 3.06 (68.0) | 0.63 (61.2) | 28.3 |
| 40 | 3 | 1.30 (46.4) | 3.35 (74.4) | 0.70 (68.0) | 22.2 |
| 100 | 3 | 2.10 (75.0) | 4.16 (92.4) | 0.90 (87.4) | 6.7 |

[a] Beer with or without sulfite was incubated at 60 °C. [b] The data represent the means of three determinations. [c] Each value shows a percentage of each proanthocyanidin.

was added to beer, the decrease in proanthocyanidins and the formation of chill haze were inhibited in proportion to the $SO_3^{2-}$ concentrations (Table I).

Fenton and Haber–Weiss reactions produce free radicals from hydrogen peroxide with metal catalytic actions (Halliwell and Gutteridge, 1984) and are accelerated by the addition of $H_2O_2$ and inhibited by EDTA. Sulfite is generally lower than 10 mg/L in beer and reduces $H_2O_2$ (Blockmans et al., 1987; Chapon et al., 1982). PBN has been used for ESR analysis as a spin trapping reagent and is a good free-radical scavenger (Evans, 1979; Kaneda et al., 1988, 1989). It was also confirmed that proanthocyanidins decreased by Fenton reaction in a model system ($H_2O_2$ - $Fe^{2+}$, pH 4.3), though data are not shown. Therefore, it was thought that the Fenton and Haber–Weiss reactions produced free radicals in beer and played an important role in the degradation of proanthocyanidins and the haze formation.

When beer was irradiated with $^{60}Co$ γ-rays under a $CO_2$ atmosphere, the contents of three kinds of proanthocyanidins decreased and the chill haze increased in proportion to the irradiation doses (Figures 6 and 7). When aqueous solutions are irradiated with γ-rays under anaerobic conditions, hydroxyl radicals are peculiarly produced (Simic, 1983; Swallow, 1972). When beer with added 100 mM KSCN, 100 mM *t*-BuOH, and sodium formate, which are hydroxyl radical scavengers, was incubated at 60 °C, the decrease in proanthocyanidin contents and the

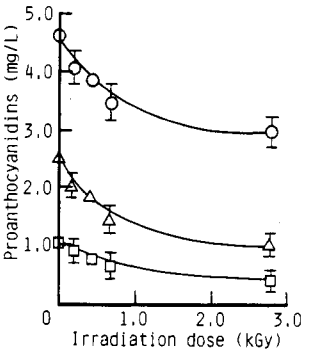


**Figure 6.** Effect of irradiation with $^{60}Co$ γ-rays on contents of proanthocyanidins in beer. △, Procyanidin B3; ○, catechin; □, epicatechin. Each data point is the average of three replicates, and the vertical lines represent the standard deviations.

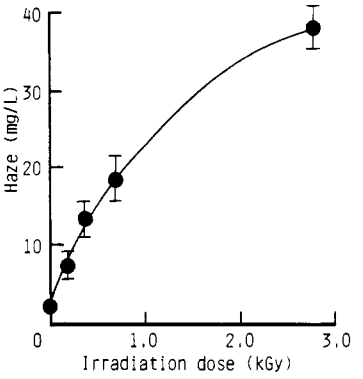


**Figure 7.** Effect of irradiation with $^{60}Co$ γ-rays on formation of chill haze in beer. Haze shows kaolin concentration (milligrams per liter). Each data point is the average of three replicates, and the vertical lines represent the standard deviations.

**Table II. Effect of Radical Scavengers[a] on Proanthocyanidin Contents and Formation of Chill Haze in Beer**

| treatment (additions) | incubation time, days | proanthocyanidins,[b] mg/L: procyanidin B3 | catechin | epicatechin | chill haze,[b] mg/L |
|---|---|---|---|---|---|
| control (no additions) | 0 | 2.65 (100)[c] | 4.40 (100)[c] | 0.94 (100)[c] | 0.6 |
| | 3 | 0.67 (25.3) | 2.16 (49.1) | 0.43 (45.7) | 41.0 |
| 100 mM KSCN | 3 | 0.89 (33.6) | 2.80 (63.6) | 0.55 (58.5) | 30.0 |
| 100 mM *t*-BuOH | 3 | 1.37 (51.7) | 3.63 (82.5) | 0.71 (75.5) | 20.6 |
| 100 mM sodium formate | 3 | 1.50 (56.6) | 3.80 (86.4) | 0.75 (79.8) | 10.1 |

[a] Beer with or without radical scavengers was incubated at 60 °C. [b] The data represent the means of three determinations. [c] Each value shows a percentage of each proanthocyanidin.

formation of chill haze was inhibited (Table II). Therefore, it was thought that hydroxyl radicals degraded proanthocyanidins to form the chill haze in beer. Chapon et al. (Chapon, 1965; Chapon and Chapon, 1977, 1979) already showed that $Fe^{2+}$ and $Cu^{2+}$ accelerated the formation of chill haze and speculated that the catalytic actions of metals had an important role in the oxidative reactions in beer. The results of this study agreed with the speculations of Chapon et al. and, moreover, made clear that free radicals are involved in the formation of chill haze.

Gramshaw (1969, 1970) suggested the important roles of the catechin and proanthocyanidin dimer in the formation of beer haze and thought that they were the main precursors of beer haze. Asano et al. (1986) studied the affinity of proanthocyanidins and their oxidation products

for haze-forming proteins in beer and concluded that procyanidin B3 and catechin surviving in packaged beer underwent oxidative polymerization and increased their affinity for haze-forming proteins to form a chill haze. Delcour and Dondeyne (1982) reported that acetaldehyde took part in the polymerization of polyphenols. In this study, we could not detect oxidation products and clarified the oxidative polymerization of proanthocyanidins in beer. Further studies of the oxidation products of proanthocyanidins have to be undertaken.

On the basis of the results presented so far, a pathway for the chill haze formation in beer can be speculated. First, free radicals, especially the hydroxyl radical, that were produced by Fenton and Haber–Weiss reactions during the storage of beer attacked proanthocyanidins directly or indirectly to produce oxidation products in the beer. Second, the oxidants had a large affinity for proteins and took part in the haze-forming reactions.

## CONCLUSIONS

This HPLC–ECD is a useful analytical tool for determining phenolic compounds and can be used for their determination in not only beer but also malts, hops, and wort. It will provide valuable information for the control of beer qualities.

Brewers have tried to reduce the level of proteins and polyphenols in fresh beer and to increase colloidal stability. However, polyphenols and proteins also contribute to the flavor, taste, and foam of the beer. In this study, it was shown that free radicals could be responsible for the formation of chill haze in beer and that the inhibition of free-radical reactions in beer led to colloidal stability. Further studies of haze-forming reactions are essential to brew a more stable and agreeable beer.

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**Registry No.** Procyanidin B3, 23567-23-9; catechin, 154-23-4; epicatechin, 490-46-0.