Effect of the Removal of Sensitive Proteins and Proanthocyanidins on the Colloidal Stability of Lager Beer

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ABSTRACT

Nine different trial beers were produced on a pilot scale from a common feedstock by using, either singly or in combination, three dosage rates (0, 15, and 30 g/hl) of polyvinylpolypyrrolidone (PVPP) and three dosage rates (0, 100, and 200 g/hl) of silica hydrogel (SHG). The colloidal stabilities of the beers were assessed by three forcing tests and by storage under simulated trade conditions. Colloidal stability varied widely but was greatest for beers dosed at the higher dosage rates with both SHG and PVPP. The data obtained were used to construct a model for predicting economical dosage rates compatible with a given stability specification. Suitable stabilization regimes included dosage with PVPP at 25 g/hl, dosage with SHG at 125 g/hl, or combined dosage with PVPP and SHG at 15 and 50 g/hl, respectively. Treatments with SHG diminished the contents of sensitive proteins in beers, whereas PVPP treatments decreased the polyphenol content. Dimeric proanthocyanidins were measured by direct-injection high-performance liquid chromatography, using an electrochemical detector. Other assays for polyphenols included less specific colorimetric and turbidometric methods. A close relationship was found between the rates of chill haze development in the nine beers and the products of their assay values for sensitive proteins and dimeric proanthocyanidins.

Keywords: Haze stabilization, Polyphenols, Polyvinylpolypyrrolidone, Proanthocyanidins, Sensitive proteins, Silica hydrogel

Using modern brewing technology, brewers can now produce beers with a colloidal stability exceeding 12 months (16,31). Much has been learned about the different hazes that can form in beer, and of these, the most intensively studied has been chill haze (31). The greater part of this nonbiological haze is proteinaceous, but existing analytical methods do not yet permit the specific measurement of this component (13,15,19). Much more information has been obtained on the polyphenolic constituents of chill haze, which exist in beers not only as simple monomers and dimers (Fig. 1) but also as more complex associations with other substances, especially proteins (2,12,13,15,21,22,26, 30,32,37,40). The flavanoid polyphenols have been referred to loosely as *anthocyanogens* (5,12), but the term *proanthocyanidins* (2,7,12,17,32,34,35,37,40) has gained favor for identified structures such as prodelphinidin B3 and procyanidin B3 (Fig. 1). The names, *proanthocyanidins* and *anthocyanogens*, signify that these compounds yield anthocyanidins on hydrolysis. In brewing, the most frequently encountered anthocyanidins are delphinidin, cyanidin, and pelargonidin. Many of the technological factors that influence haze stability do so through their effects on one or another of the major haze precursors (13,31,32), although frequently the chemical mechanism remains obscure (12,13).

Few uncertainties exist, however, concerning the modes of action of two of the several sorbents (20,31) used widely for the amelioration of colloidal instability. Silica hydrogel (SHG) and polyvinylpolypyrrolidone (PVPP) act by sorbing proteins and polyphenols, respectively. Increasing the dosage of beer with PVPP up to 50 g/hl removes increasing proportions of the total polyphenols or anthocyanogens, accompanied by increases in colloidal stability (6,20,30,32,36,40,). Dosage with SHG up to 200 g/hl improves colloidal stability (11,38), but studies of the

proteins removed by this treatment are relatively few (15). Experience indicates that initial concerns over flavor, bitterness, color, and foam quality losses resulting from these treatments were exaggerated (6,14,15,20,38). Indeed, new products are being made with claims for both enhanced effectiveness and specificity as compared with their predecessors (11,16,19). Although total replacement of conventional diatomaceous filter aids by silica gel powders may be possible (11,38), kieselguhr is presently the most popular filter medium. Notably, filtration through kieselguhr is a prerequisite for stabilization with PVPP (14), and the potentially costly treatment with PVPP can be diminished through a pretreatment with a low dosage of SHG (31,36). Such combined treatments (4) are recommended especially for some beers that are difficult to stabilize. There is, therefore, scope for optimizing stabilization treatments by critically adjusting the dosage rates for PVPP and SHG.

The definitive test for the effectiveness of the stabilization treatment is the rate of haze formation in the product, judged usually by a haze-forcing test (9,31,33). The effectiveness of each sorbent can be determined by measuring the amounts of the haze precursors removed from the beers (13). Several of the more empirical assays have been criticized (31) because they lack specificity and correlate poorly with colloidal stability. Indeed, it has been claimed (13) that the only assays of value in the prediction of haze stability are the measurements of "sensitive proteins" and "oxidizable polyphenols."

The limitations of the conventional assays prompted Vancraenenbroeck et al (40) to devise a gas chromatographic method with which dimeric proanthocyanidins and monomeric flavanols in beers were separately quantifiable. Their study showed that PVPP had a stronger affinity for the proanthocyanidins than for catechin and epicatechin and that decreases in the contents of the proanthocyanidins correlated well with increases in haze stability. In another study (32), high-performance liquid chromatography (HPLC) was used to follow the fates of the simple flavanoids through the brewing process, and the effects on flavanoid contents of different stabilization procedures were compared. Again, the strong adsorbing capacity of PVPP for proanthocyanidins, and to a lesser extent for catechins, was demonstrated. The effect of PVPP dosage on the contents of monomeric, dimeric, trimeric, and polymeric flavanoids in an experimental ale were studied using HPLC and colorimetric and and enzymatic methods of analysis (30). Whereas most of the flavanoids were removed from the beer by low dosages of PVPP (0–20 g/hl), dosages greater than 100 g/hl were required to substantially decrease the monomers. This selective adsorptive action of PVPP was not revealed by empirical colorimetric measurements.

The general objectives of this study were to construct a pilot-scale filtration system suitable for use as a test bed for production-scale stabilization procedures and to develop analytical procedures with potential application as process control measurements. The initial target was to define suitable dosages of PVPP and SHG, used singly or in combination, to stabilize a certain production lager sufficiently to comply with an expected haze shelf life of 12 months. Thereafter, the aim was to define acceptable colloidal stability in terms of measurable beer constituents.

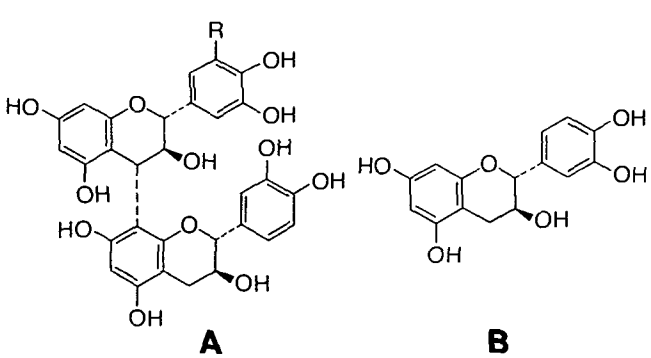

Fig. 1. General structure of dimeric and monomeric flavanols found in beer. **A**, Proanthocyanidins (dimeric): Procyanidin B3, R = H; prodelphinidin B3, R = OH. **B**, (+)-Catechin (monomeric).

EXPERIMENTAL

Lager Beer

Beer was a standard product from a conventional lager brewery, brewed from a barley malt and maize mixed-grist by decoction mashing. Sweet wort (original gravity = 14.3°P) was boiled with hop pellets and hop extract, and the hopped wort was fermented in a cylindroconical vessel at 10°C for 10 days. Fermented beer was chilled, dosed with isinglass finings, and then stored at −1°C for 21 days. Dosage with isinglass was found to greatly assist the settlement of particulate matter, including chill haze, during storage. Whereas the filtration performance was thereby considerably enhanced, no evidence was obtained that isinglass treatments prolonged haze shelf life. At the end of storage, a 10-hl sample was collected from a vat and put into 20 50-L kegs, taking appropriate precautions to exclude oxygen. The kegs were then transferred to a cold room at −2°C, where they were stored before filtration.

Pilot-Scale Filtration

The mobile filtration unit (Fig. 2) was sized to filter 50- to 100-L batches of beer and was designed to incorporate optional treatments with SHG and PVPP. All vessels and pipework were fabricated in stainless steel (SS 304 or 316) and were configured to permit ease of purging with deaerated water and carbon dioxide. Apart from cleaning, all filter operations were conducted in a cold room (−2°C). Before each trial, the system was thoroughly purged free from oxygen and then the kieselguhr plate-and-frame filter (type SF 200/21E, 20 × 20 cm; Beco Filtration Systems, Sudbury, Suffolk, England) containing three filter sheets (0.091 m^2 total area, type KGO/400 DT; Beco Filtration Systems) was precoated at 0.4 kg/m^2 with Hiflo Supercel (Manville Sales Corporation, Lompac, CA) by circulation of a liquid slurry (10% solids) from the precoat mixing vessel through a positive displacement pump (model M210, variable 0–2 hl/hr; Ibex Engineering Co., Ltd., Hastings, Sussex, England). A second precoat, of Dicalite 438 (Steetley Ltd., Worksop, Nottinghamshire, England), was then applied at the same rate. Storage beer (100 L) was transferred to the bulk dosage vessel and dosed by gentle stirring with Dicalite 438 (65 g/hl) and with a quick-dispersal free-flow grade of SHG (Lucilite QD7, mean particle size = 15 μm, mean pore size = 95 Å; A.B.M. Chemicals, Stockport, Cheshire, England) at rates of 0–200 g/hl, both being added as suspensions in a subsample of the same beer (10% w/v). The dosed beer was then pumped (1.5 hl/hr) through the kieselguhr filter and routed either to the PVPP dosage vessel or directly to the polishing filter via a bypass. Optional treatments (0–30 g/hl) with PVPP (Polyclar R, GAF [Great Britain] Co. Ltd., Manchester, England) involved the addition of slurried powder (10% w/v in beer) followed by recirculation of the dosed beer for 30 min through the circulation pump (type GM-1A, Alpha-Laval Flow Equipment, Kolding, Denmark; 12 hl/hr). Treated beer was then pumped (model M120, Ibex Engineering Co., Ltd.) through the polishing filter (type SF 200/21E, Beco Filtration Systems) containing three filter sheets (type KDS-15, Beco Filtration Systems) at 50 L/hr for collection in a bright beer tank (100 L). Bright beer was diluted with deaerated water to an original gravity of 10.9°P and carbonated (6 g of CO_2/L) by circulation under a carbon dioxide head pressure of 193 kN/m^2 for approximately 2 hr. The carbonated beer was then packaged in dark brown 29-cl glass bottles (mean total air in

bottle = 0.8 ml, mean dissolved oxygen content = 0.15 mg/L) and pasteurized in a small tunnel box pasteurizer (Briggs Engineers, Barton and Trent, England).

Haze Measurement and Haze Stability

All haze measurements were made with a beer haze meter (model HZ-103, Lg-automatic aps, Hillerod, Denmark), calibrated in EBC units using turbidity standards (Advanced Polymer Systems, Inc., Redwood City, CA). Permanent haze was measured in samples of degassed beers after their attemperation to 18°C. Total haze was then measured on the same samples after they had been held overnight at 0°C. Chill haze was calculated as the difference between these two values. The haze stabilities of all trial beers were measured in duplicate under three different storage conditions. Rapid haze-forcing was obtained by immersing bottles in water at 60°C for 48 hr, before measurement of total and permanent haze (10). Other bottles were stored at 37°C in air-incubators, and haze measurements were made at weekly intervals on samples withdrawn over a period of 12 weeks. Natural haze stability was assessed from haze measurements made at monthly intervals on bottles removed from storage at 18°C, over six months. Haze stability was also assessed by the alcohol cooling test (9), in which samples of beers containing additions of ethanol (3% v/v) were chilled for 45 min in a cooling bath (−5°C).

Sensitive Proteins

The hazes (in EBC units) that developed when 10 ml of a solution (100 mg/L) of tannic acid was added dropwise over 2 hr to 190-ml samples of beer (39) were measured as indicators of the content of "sensitive" proteins.

High-Molecular-Weight Proteins

The Coomassie Brilliant Blue binding microassay procedure (3) was used with γ-globulin as the standard.

Oxidizable Polyphenols

Beer samples were oxidized by treatment with hydrogen peroxide and peroxidase (39). The hazes (in EBC units) that developed on subsequent addition of a solution of cinchonine sulfate were measured as indicators of the content of "oxidizable polyphenols."

Total Polyphenols

Total polyphenols in beers were measured by reaction with ferric reagent according to standard procedures (1,10), but using (+)-catechin as an alternative standard.

Total Flavanols

Beer samples (2.5 ml) were pretreated by dilution to 10 ml with methanol, and after precipitation was allowed to develop fully over 1 hr, the precipitate was removed by centrifugation. Samples (1 ml) of the clear supernatant were mixed with 5 ml of DAC reagent (0.3% 4-dimethylaminocinnamaldehyde in acidic methanol) and the increases in absorbance at 640 nm were measured (28). The flavonol contents were calculated by reference to a calibration prepared with (+)-catechin (0–25 μg/ml). This test was used also on other methanolic and acetonic solutions of polyphenols without the pretreatment step. Catechin and the simple flavanol oligomers are particularly reactive with DAC reagent, and the color reaction is not only specific but also very reproducible.

Anthocyanogen Value

The beer samples were treated with Polyclar AT (BDH Chemicals Ltd., Poole, England) to adsorb anthocyanogens, and the polyamide powder was then refluxed in acidic butanol to hydrolyze the adsorbed substances to colored flavylium ions (5). Anthocyanogen values were then calculated from absorbance measurements made at 545 and 445 nm.

Analysis for Monomeric and Dimeric Flavanols in Beers by Direct-Injection HPLC

Samples (10 μl) of beer were injected through a WISP auto-injector (Waters Associates, Milford, MA) onto a column (30 cm × 4 mm) of 10-μm Nucleosil C_{18} (Machery-Nagel, Duren, West Germany), protected by a Waters C_{18} Guard-Pak precolumn. Elution from the column was at 1 ml/min, with a gradient from 100% mobile phase A (0.1 *M* KH_2PO_4, pH = 2.5) changing linearly to a mixture of 20% A and 80% mobile phase B (95% methanol, 5% water) in 90 min. This gradient was generated from two Waters model 510 pumps operated through a Waters 680 gradient controller. The elution of analytes was monitored with a Waters 460 electrochemical detector (applied detection voltage = 850 mV versus Ag/AgCl electrode, range = 20 nA) linked to a Waters 820 data management system.

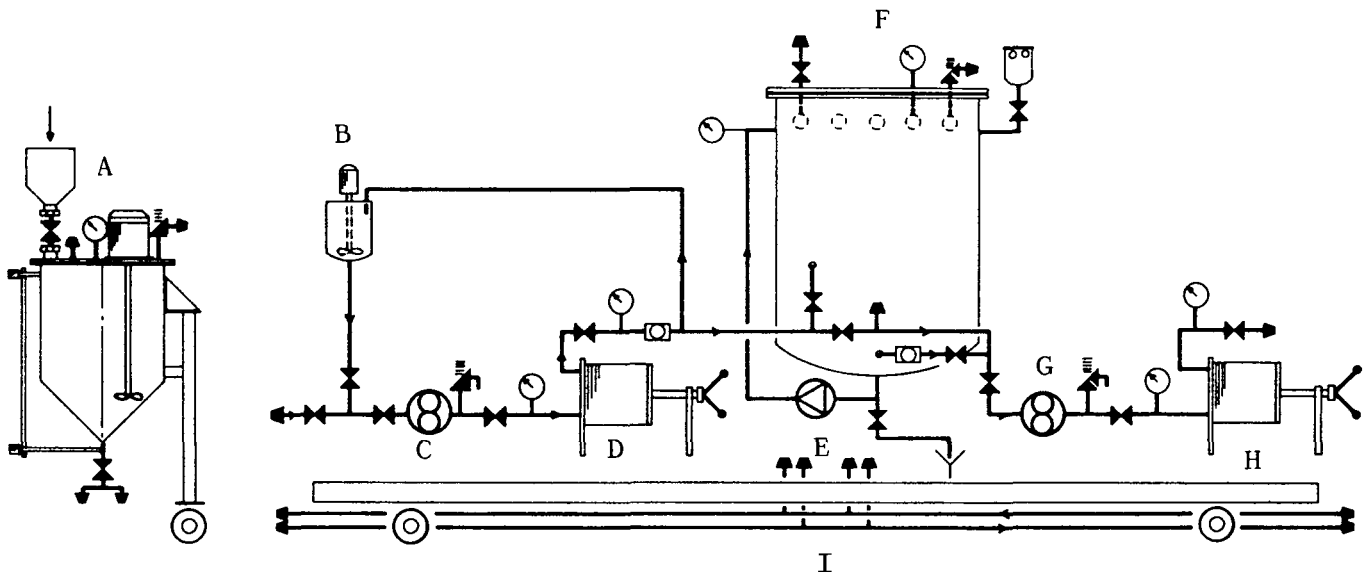

Fig 2. Pilot-scale filtration system. A = dosage vessel for kieselguhr and silica hydrogel, B = precoat slurrying tank, C = positive displacement pump, D = primary (kieselguhr) filter, E = circulation pump, F = polyvinylpolypyrrolidone (PVPP) dosage vessel, G = positive displacement pump, H = polishing and PVPP-removal filter, I = CO_2 inlet and outlet lines.

Isolation of Proanthocyanidins from Beer (Sephadex LH20 Separation)

Polyphenols were adsorbed from 10 L of unstabilized lager by pumping the beer through a column (2.5 × 30 cm) of water-washed Sephadex LH20 (Pharmacia LKB Biotechnology, Uppsala, Sweden) at 4 ml/min. The column was washed with 1.2 L of distilled water to remove weakly adsorbed beer constituents, and then the more strongly bound polyphenols were eluted with 400 ml of acetone and water (3:1). This acetonic eluate was concentrated to 100 ml by evaporation at 25°C under reduced pressure, and the red aqueous solution obtained was extracted three times with equal volumes of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness, taking precautions to minimize contact of the residues with air, and the recovered solids were dissolved in 5 ml of methanol. The pale yellow methanolic solution obtained was termed the crude polyphenol extract A. The aqueous phase remaining after extraction was lyophilized, and the recovered solids (110 mg) were dissolved in methanol (10 ml) to form a red-brown solution, which was designated the crude polyphenol extract B.

Thin-Layer Chromatography

Aqueous or methanolic solutions (10–20 μl) were spotted as appropriate for two-dimensional chromatography onto glass plates (20 × 20 cm) coated with cellulose (PEI-cellulose F, 0.1 mm; E. Merck, Darmstadt, Germany). Chromatograms were developed in the first dimension with acetic acid and water (6:94 v/v) and in the second dimension with sec-butanol, acetic acid, and water (14:1:5 v/v). Following development, the air-dried plates were sprayed with DAC reagent, as used in the colorimetric test for flavanols. Catechin and epicatechin were stained emerald green, whereas spots of proanthocyanidins were stained brown-green. When the plates were lightly sprayed later with distilled water, the spots of separated flavanoids were transformed from green to a more intense dark blue.

Analysis by HPLC of Phenolics in Extracts from Beers

Samples (10–20 μl) of concentrated extracts of phenolic substances recovered from beers were separated by gradient elution on a reversed-phase column, using a Perkin Elmer Integral 4000 chromatograph (Perkin Elmer, Beaconsfield, Buckinghamshire, England). The chromatograph consisted of an autoinjector, ternary gradient pump, photodiode detector, and data handling capability. Samples were eluted from a column (30 × 4 mm) of 10-μm Nucleosil C_{18} with a mobile phase changing linearly in 120 min from 100% A (acetic acid and water, 2:98) to 100% B (acetic acid, methanol, and water, 2:95:3). Elution of analytes was monitored by light-absorption measurements at 280, 310, and 360 nm.

TABLE I
Stabilization Treatments and Colloidal Stabilities of Trial Beers 1–9

Trial No.	Dosage Rates (g/hl) SHG[a]	PVPP[a]	Chill Haze[b] (EBC units) Alcohol Cooling Test	Forcing Test 60°C, 2 days	37°C, 12 wks	Storage Test 18°C, 6 mos	Chill Haze Development[c] (EBU units/wk)
1	0	0	9.2	1.0	4.3	1.2	0.42
2	0	15	7.3	0.7	2.1	0.4	0.22
3	0	30	3.5	0.2	0.9	0.4	0.09
4	100	0	4.5	0.5	1.9	0.6	0.20
5	100	15	2.0	0.4	0.7	0.3	0.06
6	100	30	1.0	0.2	0.4	0.1	0.03
7	200	0	1.1	0.4	0.5	0.1	0.05
8	200	15	1.0	0.3	0.3	0.1	0.02
9	200	30	0.9	0.2	0.1	0.1	0.01

[a]SHG = silica hydrogel, PVPP = polyvinylpolypyrrolidone.
[b]Permanent haze = 0.4–0.5 EBC units.
[c]Rate at 37°C. Linear regression of >8 data points.

Analysis by HPLC of Anthocyanidins in Hydrolysates of Anthocyanogens

Samples (50 μl) of hydrolysates were separated by the same system as used for the separation of phenolics in beer extracts except that light absorption was monitored at 550 nm. The anthocyanidins (delphinidin, cyanidin, and pelargonidin) were well separated and identified by cochromatography with authentic standards.

Nylon 66 Solid Phase Extraction of Phenolics from Beer

Samples (100 ml) of beers were pumped (2 ml/min) through glass columns (12 × 1 cm) containing 1.5 g (bed depth = 5.5 cm) of slurry-packed Nylon 66 powder (DS 3276, Imperial Chemical Industries, Billingham, England). Each column was then washed with distilled water and these washings were discarded. Beer polyphenols were recovered by eluting each washed column with 50 ml of acetone and water (3:1 v/v) pumped through at 2 ml/min. Further purification of the individual acetone extracts was obtained after their evaporation to 10 ml followed by extraction of each of the resulting aqueous phases three times with ethyl acetate (30 ml total). Combined ethyl acetate extracts were evaporated to dryness, and each residue was dissolved in 5 ml of methanol to yield the simple flavanol extracts. The remaining aqueous phases were retained and termed polymeric flavanol extracts.

Isolation of Barley Proanthocyanidins

Procyanidin B3 and prodelphinidin B3 were extracted from ground barley grains and then isolated by preparative liquid chromatography as described previously (27). The purified substances were used as chromatographic standards.

Chemicals

Monomeric flavanols, flavonol glycosides, phenolic acids, 4-dimethylaminocinnamaldehyde, and HPLC-grade solvents were obtained from sources mentioned previously (23–29).

RESULTS

Haze Stabilities of Treated Beers

The permanent haze contents of beers subjected to a wide range of treatments (Trials 1–9) with either PVPP or SHG or both sorbents (Table I) were similar (0.4–0.5 EBC units) when measured at bottling. However, several tests indicated sizeable differences in the colloidal stabilities of these beers. During these tests no

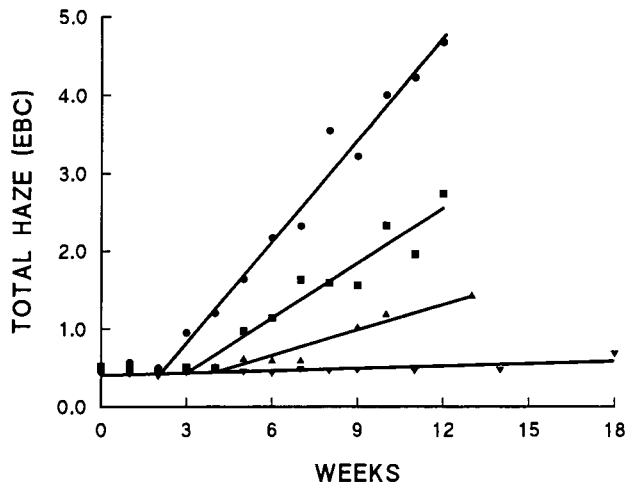

Fig 3. Effect of storage time on the development of total haze (chill + permanent) in four bottled beers stored at 37°C after different stabilization treatments: Trial 1 beer (●), Trial 2 beer (■), Trial 3 beer (▲), Trial 9 beer (▼).

increases in permanent haze were obtained even though increases in chill haze were obvious.

Forcing test 1: Chill haze development rate at 37° C. Typically, chill haze developed after the second to fourth storage week in beers held at 37°C (Fig. 3) and increased linearly with time until the termination of the test. By measuring the rates of increase in chill haze between the fourth and twelfth weeks of storage, a quantitative expression of colloidal instability in different beers was obtained. The rates (Table I) were computed as linear regressions ($r = 0.8920$–0.9967, mean = 0.9426) of the data points obtained at weekly intervals for each of the nine beers. These calculations showed that chill haze in the unstabilized beer (Trial 1) increased at almost 40 times the rate of increase in the most heavily treated beer (Trial 9). The effects of variable dosage with PVPP and SHG on the development rate of chill haze are shown in Figure 4.

The results for discrete measurements of chill haze made after 12 weeks storage are given in Table I, and these indicated the same differences between the colloidal stabilities of the beers as the did the development rate measurements.

Natural storage test: Chill haze development rate at 18° C. The rates of development of chill haze in beers stored under simulated trade conditions were much slower than those of the corresponding samples forced at 37°C. The customary interpretation of the 37°C-forcing test is that the amount of haze developed in 12 weeks at this temperature is roughly equivalent to that developed over 12 months in beers stored at 18°C. We confirmed that the chill haze development rates at 18°C were approximately one quarter of the corresponding rates measured at 37°C for the least stable beers (Trials 1–4). A similar comparison for the most stable beers (Trials 5–9) was not possible because the rates at 18°C were too low for accurate measurement.

Forcing test 2: Alcohol-cooling test. Table I shows that following the addition of extra ethanol (3% v/v) the beers produced different amounts of chill haze when subjected to rapid cooling. As expected, the beer that received the least stabilization treatment (Trial 1) produced the most haze, and the most heavily dosed beer (Trial 9) produced the least haze. The results correlated well ($r = 0.9547$) with haze development after 12 weeks of storage at 37°C.

Forcing test 3: 60° C storage. Chill haze developed in all beers that had been held at 60°C for two days (Table I). Differences between the beers were much less than those of the alcohol cooling test, but the results were well correlated ($r = 0.9406$) with haze development after 12 weeks of storage at 37°C.

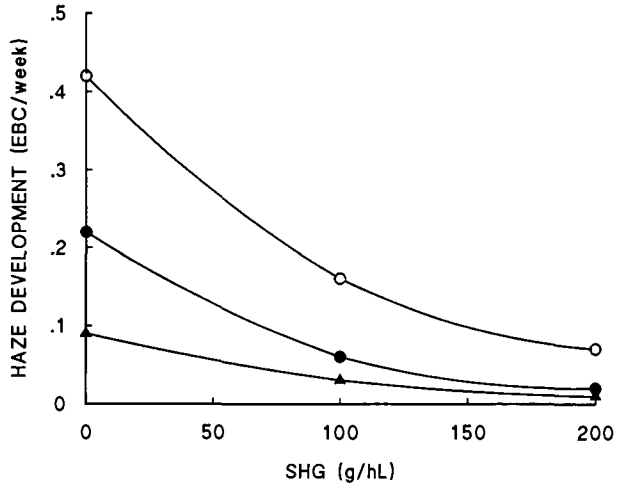

Fig 4. Effect of variable dosage with silica hydrogel (SHG) and polyvinylpolypyrrolidone (PVPP) on the rate of chill haze development in bottled beers stored at 37°C. PVPP treatment rates: 0 g/hl (○), 15 g/hl (●), 30 g/hl (▲).

Gross Contents of Beer Haze Precursors

Three empirical tests were used to assess the effects of the sorbents on the contents of the proteinaceous and polyphenolic constituents of haze in the trial beers.

Sensitive and high-molecular-weight proteins. The results (Table II) indicated unequivocally that the content of sensitive proteins in the beers was diminished reproducibly by treatments with silica hydrogel but was unaffected by dosage with PVPP. In contrast, no differences of significance were detected between the content of high-molecular-weight proteins in the beers.

Oxidizable polyphenols. Oxidizable polyphenols (Table II) were diminished by dosage with both PVPP and silica hydrogel. The decreases in the assay values were approximately in proportion to the dosage rates of the sorbents, with slightly greater decreases caused by SHG used at 200 g/hl than by PVPP at 30 g/hl and by SHG at 100 g/hl than by PVPP at 15 g/hl.

Total Polyphenols, Total Flavanols, and Anthocyanogen Values

Three colorimetric assays were used to evaluate the removal from the beers of different types of polyphenols by the sorbents.

Total polyphenols. Dosage with increasing amounts of PVPP progressively diminished the contents of total polyphenols in the beers by up to 40% (Table III), displaying an average adsorption capacity of 90 mg of total polyphenols per gram of PVPP. In contrast, SHG showed no capacity to remove total polyphenols from the beers.

Total flavanols. About 40% of the total flavanols were adsorbed by PVPP used at 30 g/hl (Table III), and the corresponding adsorption capacity was approximately 20 mg of total flavanols per gram of PVPP. Dosage with SHG did not decrease the content of total flavanols in the beers.

Anthocyanogen values. Table III shows the anthocyanogen values of the beers to be decreased with increasing dosage of

TABLE II
Effect of Dosage with Silica Hydrogel (SHG) and Polyvinylpolypyrrolidone (PVPP) on Sensitive Proteins, High-Molecular-Weight Proteins, and Oxidizable Polyphenols in Trial Beers

Trial No.	Dosage Rates (g/hl) SHG	PVPP	Sensitive Proteins (EBC units)	High-Molecular-Weight Proteins (mg/L)	Oxidizable Polyphenols (EBC units)
1	0	0	6.9	270	1.9
2	0	15	6.9	290	1.9
3	0	30	6.9	300	1.5
4	100	0	1.6	310	1.6
5	100	15	1.7	300	1.1
6	100	30	1.7	310	1.1
7	200	0	0.3	270	1.2
8	200	15	0.3	320	1.1
9	200	30	0.3	280	0.6

TABLE III
Effect of Dosage with Silica Hydrogel (SHG) and Polyvinylpolypyrrolidone (PVPP) on Total Polyphenols, Total Flavanols, and Anthocyanogen Values in Trial Beers

Trial No.	Dosage Rates (g/hl) SHG	PVPP	Total Polyphenols (mg/L)	Total Flavanols (mg/L)	Anthocyanogen Values (units)
1	0	0	76	15.9	67
2	0	15	66	13.1	52
3	0	30	49	9.5	27
4	100	0	78	15.6	65
5	100	15	67	12.3	45
6	100	30	48	9.2	32
7	200	0	79	15.7	64
8	200	15	64	12.6	45
9	200	30	44	8.8	32

PVPP but to be not materially affected by dosage with SHG.

Although the three different assay methods vary in their specificities, the results correlated with one another (r = 0.8644–0.9608) and with PVPP dosage levels (r = −0.9030 to −0.962). Correlations with the dosage levels of SHG were not significant (r = −0.0420 to −0.196).

Proanthocyanidins in Unstabilized Beer (Sephadex LH20 Separation)

The crude polyphenol extract A recovered from 10 L of unstabilized lager contained 93 mg of total polyphenols and was shown by HPLC (Fig. 5) to contain flavanols, flavonol glycosides, and phenolic acids. The different constituents were identified by cochromatography with authentic standards and by their absorbance spectra using photodiode array detection. The only flavanols that were identified were prodelphinidin B3, procyanidin B3, catechin, and epicatechin. Many other smaller peaks appeared not to be flavanols because they absorbed more strongly at 310–330 nm than at 280 nm. Moreover, none of those peaks coincided in retention times with flavanol trimers isolated from barley (23,26,27). When a sample of the crude polyphenol extract A was examined by thin-layer chromatography (Fig. 6), the only spots revealed by the flavanol-specific DAC spray reagent (4-dimethylaminocinnamaldehyde) were the same compounds as identified by HPLC.

Crude polyphenol extract B contained 100 mg of total polyphenols but did not contain any simple flavanols that were readily identifiable. The constituents of this fraction did not migrate as resolved peaks on HPLC but were eluted as a broad band of absorbance, more intense at 315 nm than at 280 nm, that spread throughout the chromatogram. Similarly, on thin-layer chromatography, the constituents located by their weak positive reaction with DAC were either immobile or migrated from the origin as poorly defined smears. Hydrolysis of a sample of the crude extract in refluxing methanol and HCl (5:1) yielded both cyanidin and delphinidin.

Nylon 66 Solid-Phase Extraction of Phenolics from Beer

This solid-phase extraction system was tested with solutions of standard compounds before its application to beer samples. Samples (100 ml) of a cocktail containing catechin, rutin, and ferulic acid (2 mg/L each), as examples of a flavanol, flavonol glycoside and phenolic acid, respectively, were pumped through columns of Nylon 66, which were then washed with water, eluted with acetone-water, and subsequently extracted with ethyl acetate. Analysis by HPLC of the column effluents revealed that, whereas ferulic acid and rutin were moderately retained on the Nylon 66 columns during the initial application and washing, catechin was fully retained. Accordingly, on subsequent desorption with aqueous acetone, the average (n = 3) recoveries of ferulic acid, rutin, and catechin were 70, 87, and 97%, respectively. Moreover, the variability of the recoveries for the same solutes, expressed as the relative standard deviation (RSD), were 35, 9, and 5%, respectively. In separate experiments, the recoveries of catechin (5 mg/L), procyanidin B3 (11 mg/L), and prodelphinidin B3 (7.5 mg/L) were 84, 78, and 44%, respectively. The latter compound was found to elute from the column as a small peak followed by a pronounced tail, which was incompletely recovered by the standard washing procedure with aqueous acetone.

Subsequent liquid-liquid extraction varied in the efficiency of recovery for the analytes in the simple flavonol extracts (SFE) fraction. Four extractions into ethyl acetate were sufficient to remove all of the catechin and epicatechin from a solution containing the four flavanoids, all at 5 mg/L, in ethanol and water (5:95). In contrast, this procedure removed only 92% of the procyanidin B3 (K_D [partition coefficient] [ethyl acetate-water] = ~0.9), whereas only 66% of the prodelphinidin B3 was removed (K_D [ethyl acetate-water] = ~0.3). Consequently, the overall recoveries for the combined solid-phase and liquid-liquid extraction clean-up procedure were estimated at 84% for catechin and epicatechin, 72% for procyanidin B3, and only 29% for prodelphinidin B3. When samples of trial beers 1–3 were analyzed

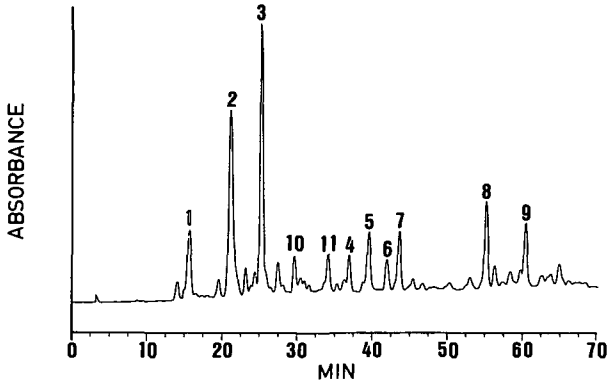

Fig 5. High-performance liquid chromatographic profile of phenolic substances in the ethyl-acetate-soluble fraction of beer desorbate from a Sephadex LH20 column. Analytes were separated by elution (1.0 ml/min) from a column (30 × 4 mm) of 10 μm Nucleosil C_{18} with a mobile phase changing linearly in 120 min from acetic acid and water (2:98) to acetic acid, methanol, and water (2:95:3). Elution of analytes was monitored by light absorption at 280 nm. Identified peaks: 1 = prodelphinidin B3, 2 = procyanidin B3, 3 = (+)-catechin, 4 = (−)-epicatechin, 5 = *p*-coumaric acid, 6 = ferulic acid, 7 = sinapic acid, 8 = quercetin rutinoside, 9 = kaempferol rutinoside, 10 = caffeic acid, 11 = syringic acid.

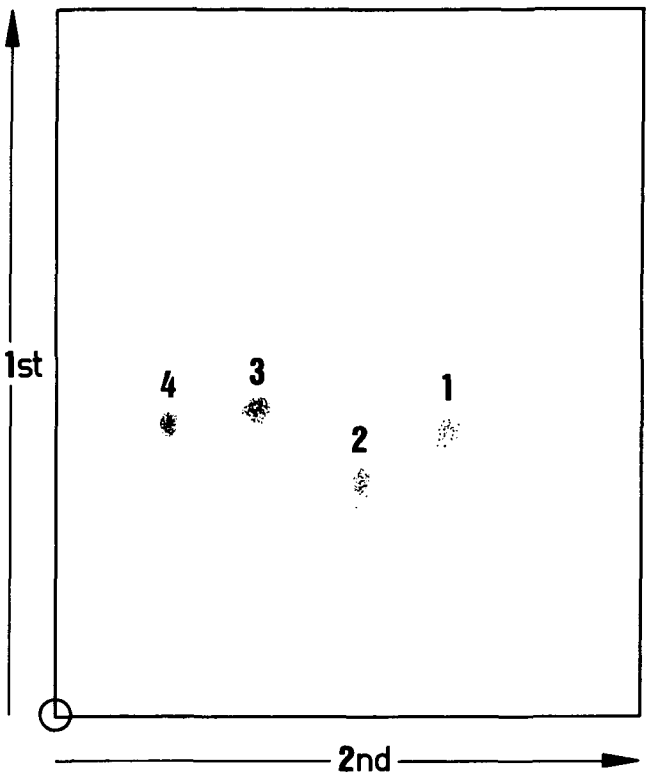

Fig 6. Two-dimensional cellulose thin-layer separation of simple flavanols recovered from beer by adsorption onto Sephadex LH20. The chromatogram was developed in the first dimension with acetic acid and water (6:94) and in the second dimension with sec-butanol, acetic acid, and water (14:1:5). Flavanols were located by spraying with a solution containing 0.3% dimethylaminocinnamaldehyde in acidified methanol. Located spots: 1 = (+)-catechin, 2 = (−)-epicatechin, 3 = procyanidin B3, 4 = prodelphinidin B3.

in duplicate for the four flavanols, the results were very variable, but the general trend was for the content of each flavonol in the SFE to be highest in beer 1 and lowest in beer 3. For instance, the measured catechin content of beer 1 varied from 1.9 to 3.2 mg/L and catechin in beer 3 varied from 0.8 to 1.4 mg/L. Procyanidin B3 gave a constant assay value (1.7 mg/L) in beer 1 and was present only in trace amounts in beer 3. Similarly, prodelphinidin B3 gave a slightly variable value (0.3–0.4 mg/L) for beer 1 and was present in trace amounts in beers 2 and 3.

Following the cleanup of each sample, the spent Nylon 66 was recovered from the column and then hydrolyzed in refluxing butanol-HCl (5:1) for 30 min. Samples of the SFE and polymeric flavanol extracts (PFE) were concentrated and also hydrolyzed. The relative amounts of anthocyanidins released were 2:5:3 on average, for the SFE, PFE, and nylon-bound fractions, respectively.

Analysis for Beer Flavanols by Direct-Injection HPLC

A system was devised to assay flavanols in beers without a cleanup. This was done by using an electrochemical detector operated in the chronoamperometric mode. Although the detector was not totally specific for phenolic substances, there was much less interference from nonphenolics than was found with light-absorbance monitoring. Moreover, the electrochemical detector was so sensitive to phenolics that a preconcentration step was not necessary. Flavanols in 10-μl samples were readily detectable and were identified by cochromatography with standards (Fig. 7a). Those peaks identified as the analytes of interest were almost totally removed by sorption on Nylon 66 (Fig. 7b).

For quantitative analysis, the system was calibrated using nylon-treated beer (Fig. 7b) spiked with known amounts (0.5–4.0 mg/L) of catechin, epicatechin, prodelphinidin B3, and procyanidin B3. Peak area integration for all four analytes was acceptably precise (RSD = 0.4–2.5%, mean RSD = 1.2%), and linear regression of the data sets (Fig. 8) gave very satisfactory correlation coefficients (0.99823–0.99998). Although protocatechuic acid (1 mg/L) was added to the beer samples as an internal standard, quantitation by external calibration was found satisfactory and became the method of choice. The precision obtained for measurements of the four flavanols in the set of trial beers was, however, less than that obtained during calibration, with an average RSD of 5% for assays in triplicate.

In Table IV results are given for the nine trial beers immediately after stabilization treatment and bottling. The contents of identifiable flavanoid polyphenols were much less than the indications of the assays for total polyphenols and total flavanols. Treatment of the beers with increasing dosages of PVPP caused progressive decreases in the content of each of the simple flavanoids. At the higher dosage rate (beers 3, 6, and 9), PVPP removed, on average, 75% of the prodelphinidin B3, 92% of the procyanidin B3, 58% of the catechin, and 66% of the epicatechin contents. Increasing dosage with SHG (beers 1, 4, and 7) also caused decreases, but the effect was inconsistent and less than that obtained with PVPP. On average, beers treated at the highest rate of SHG (beers 7–9) contained 23% less of the simple flavanols than their untreated counterparts (beers 1–3).

A less extensive examination of beers (Table V) that had been stored for 12 months at 18°C revealed that the sums of simple flavanol concentrations decreased on average by 25%, and whereas the proanthocyanidins decreased by about 30%, catechin decreased only by 17%. Because epicatechin was originally present in low concentrations, measured losses during storage of about 27% must be regarded with caution.

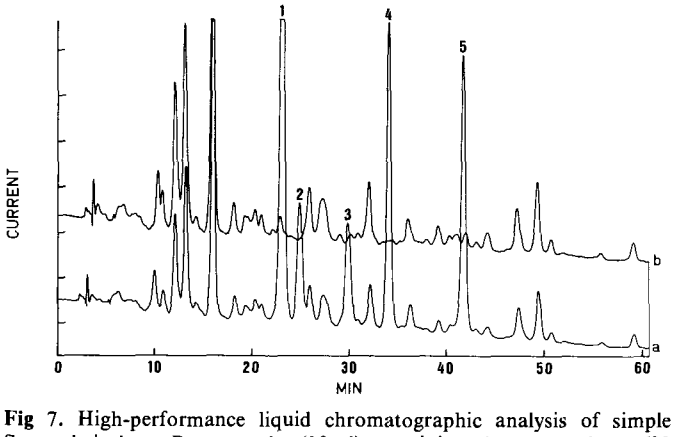

Fig 7. High-performance liquid chromatographic analysis of simple flavanols in beer. Beer samples (10 μl) were injected onto a column (30 cm × 4 mm) of 10-μm Nucleosil C_{18} eluted at 1 ml/min with a gradient from 0.1 *M* KH_2PO_4 (pH 2.5) changing linearly to 0.1 *M* KH_2PO_4-methanol (20:80) in 90 min. Analytes were detected amperometrically. a, Beer sample spiked with internal standard and (−)-epicatechin. b, Beer sample treated with excess Nylon 66. Identified peaks: 1 = protocatechuic acid (internal standard), 2 = prodelphinidin B3, 3 = procyanidin B3, 4 = (+)-catechin, 5 = (−)-epicatechin.

DISCUSSION AND CONCLUSIONS

With respect to specifications for dissolved oxygen contents and air contents in the bottle, the pilot plant was either equal

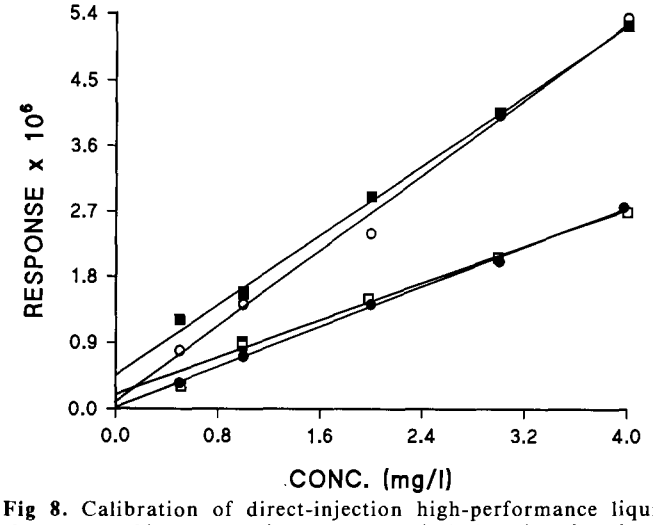

Fig 8. Calibration of direct-injection high-performance liquid chromatographic assay, using electrochemical detection for simple flavanols. Each data point is the mean of duplicate determinations. Regression lines show (+)-catechin (○), (−)-epicatechin (■), procyanidin B3 (□), prodelphinidin B3 (●).

TABLE IV
Effect of Dosage with Silica Hydrogel (SHG) and Polyvinylpolypyrrolidone (PVPP) on Contents of Prodelphinidin B3, Procyanidin B3, Catechin, and Epicatechin in Fresh Beers

Trial No.	Dosage Rates (g/hl)		Flavanols,[a] mg/L				
	SHG	PVPP	Prod.	Proc.	Cat.	Epi.	Sum
1	0	0	1.7	1.6	3.4	0.3	7.0
2	0	15	1.0	0.7	2.6	0.4	4.7
3	0	30	0.4	0	1.4	0.1	1.9
4	100	0	1.5	1.5	3.3	0.5	6.8
5	100	15	1.0	0.5	2.3	0.2	4.0
6	100	30	0.6	0.2	1.7	0.1	2.6
7	200	0	1.5	0.9	3.1	0.3	5.8
8	200	15	0.7	0.3	2.2	0.3	3.5
9	200	30	0.1	0	1.1	0.2	1.4

[a] Data are means of triplicates, Prod. = prodelphinidin B3, Proc. = procyanidin B3, Cat. = (+)-catechin, Epi. = (−)-epicatechin.

or slightly superior to the production plant, so the beer shelf-life results obtained with the former were not compromised (22). In judging the colloidal stabilities of the trial beers, the primary test was the rate of haze development at 37°C, and the indications of the two rapid methods (60°C storage and alcohol-cooling test) were regarded merely as supporting evidence.

All three forcing tests identified the three least-stable beers as trials 1, 2, and 4, in order of increasing stability, with trial 9 beer being the most stable (Table I), although there was less agreement over the remaining beers. Chill haze development at 37°C clearly was influenced by the dosage rate of both sorbents (Fig. 4). Replotting of the data to show the effects on haze development of systematic variations in PVPP dosage (Fig. 9) produced three curves for different dosage rates of SHG (0, 100, and 200 g/hl), similar to curves in Fig. 4. Following from this, the rates of haze development resulting from different combinations of treatments could be predicted. The maximum chill haze development rate that was tolerable in the subject production beer was 0.125 EBC units per week, and four treatment regimes were predicted from the contours that corresponded with this level of stability. These calculated conditions were then used to construct a model of "optimal" stabilization treatments (Fig. 10). This model predicted that beer within specification would result from dosage with PVPP at 25 g/hl, or by dosage with SHG at 125 g/hl, or by many other combination treatments with both sorbents (e.g., 15 g/hl PVPP with 50 g/hl SHG or 5g/hl PVPP with 100 g/hl SHG). The model indicated that 1 g of PVPP was roughly equivalent to 5 g of SHG in stabilizing potency, and there was no evidence of any synergistic influences.

The cost implications of different treatment regimes will vary according to circumstances at particular sites (14). For instance, in the pilot brewery described, recovery and regeneration of spent PVPP is not normal practice, so treatment with this material alone is more than 20 times more expensive than treatment with SHG. In production operations where PVPP is recovered, the ongoing cost of beer stabilization treatment can be equated to the expense of regeneration plus the replacement cost of PVPP lost during processing. Accordingly, treatment with PVPP is then approximately twice as expensive as SHG treatment.

For several years it has been known that PVPP adsorbs from beer a complex mixture of phenolic substances and nitrogenous constituents (24). Our analyses of unstabilized beer showed that the only simple flavanoid constituents were prodelphinidin B3, procyanidin B3, catechin, and epicatechin. The flavanoid polyphenols that were not recovered in the simple flavanol fraction are believed to be polymeric or associated with high-molecular-weight material. Whereas the presence of small amounts of flavanoid trimers has been detected in worts and also in a miniature-brewed ale (26), their absence from several other beers (21,31) attests to the belief that trimers are usually eliminated as insoluble complexes during commercial brewing and fermentation (2,35). Although solid-phase extraction of beer with Nylon 66 has been used to recover and concentrate the simple flavanoid fraction sufficiently for HPLC or gas chromatographic analysis (22,32,40), we did not find this method quantitatively reliable. As an alternative to sample pretreatment, the use of more specific detection with the electrochemical detector was preferred. The amounts of monomeric and dimeric flavanols measured were similar to those reported by others (7,18,26, 30,31,32) and the significance of such low concentrations (1–5 mg/L) in beer is in their effects on colloidal stability (2,7,8,17,22,24,35,37,40). Whereas it is certain that such small amounts of dimeric flavanols in beer can considerably increase the rate of chill haze formation, it seems that much larger concentrations of catechins may be required to exert the same destabilizing effect (2,7,8,22,37). While the concentration of

TABLE V
Effect of Dosage with Silica Hydrogel (SHG) and Polyvinylpolypyrrolidone (PVPP) on Contents of Prodelphinidin B3, Procyanidin B3, Catechin, and Epicatechin in Bottled Beers Stored for 12 Months at 18°C after Treatment

Trial No.	Dosage Rates (g/hl)		Flavanols,[a] mg/L				
	SHG	PVPP	Prod.	Proc.	Cat.	Epi.	Sum
4	100	0	1.2	0.9	2.8	0.3	5.2
5	100	15	0.8	0.6	2.1	0.2	3.7
6	100	30	0.3	0.1	1.4	0.1	1.9
7	200	0	1.0	0.5	2.4	0.1	4.0
8	200	15	0.5	0.2	1.7	0.0	2.4
9	200	30	0.1	0.0	0.9	0.0	1.0

[a]Prod. = prodelphinidin B3, Proc. = procyanidin B3, Cat. = (+)-catechin, Epi. = (−)-epicatechin.

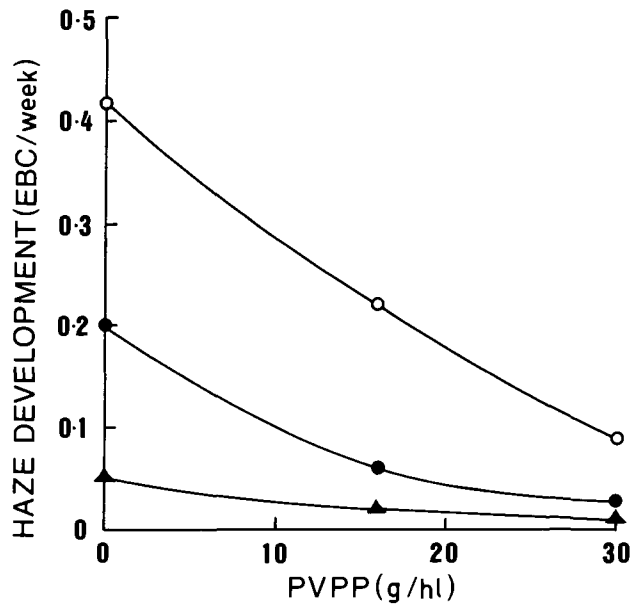

Fig. 9. Effect of variable dosage with silica hydrogel (SHG) and polyvinylpolypyrrolidone (PVPP) on the rate of chill haze development in bottled beers stored at 37°C. SHG treatment rates: 0 g/hl (○), 100 g/hl (●), and 200 g/hl (▲).

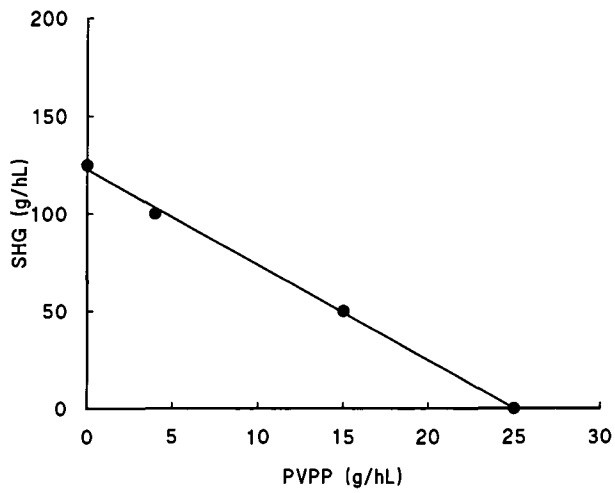

Fig 10. Haze stabilization model. Points represent the calculated dosages of silica hydrogel (SHG) and polyvinylpolypyrrolidone (PVPP) required to stabilize subject lager to a specified shelf life of 12 weeks when stored at 37°C . Shelf life was defined as the time for deterioration to a total haze (chill + permanent) value of 2 EBC units.

dimeric flavanols in beer is important, it is evident from Tables I and IV that differences in haze stability cannot be accounted for entirely by differences in any of the measured flavanols.

As a result of extensive studies (reviewed in 31), L. Chapon stressed the importance of the equilibrium between quantities of protein (P) and tannin (T) in the formation of chill haze. This idea was developed further by Moll (33), who devised an automated instrument for measuring "sensitive proteins" and tannoid reactants in beer. In our study, we measured sensitive proteins by the same basic method as used by Chapon and by Moll and used several different methods to quantify the "tannin" reactant. Having obtained several measures for "tanninogen," we pursued the notion that the rate of haze development should be proportional to the product of the reactants, in accordance with second-order reaction kinetics, such that rate = k [P] [T].

Values for haze development rates (Fig. 4) were, therefore, plotted against the products of sensitive protein contents (Table II) and dimeric proanthocyanidin contents (Table IV) for each beer. These results were well correlated (Fig. 11), indicating that a simple relationship could be drawn between measurable components of the system and chill haze development. It was not assumed, however, that the only polyphenolic reactants involved in the formation of chill haze were the dimeric proanthocyanidins. For this reason, correlations were sought with all the other

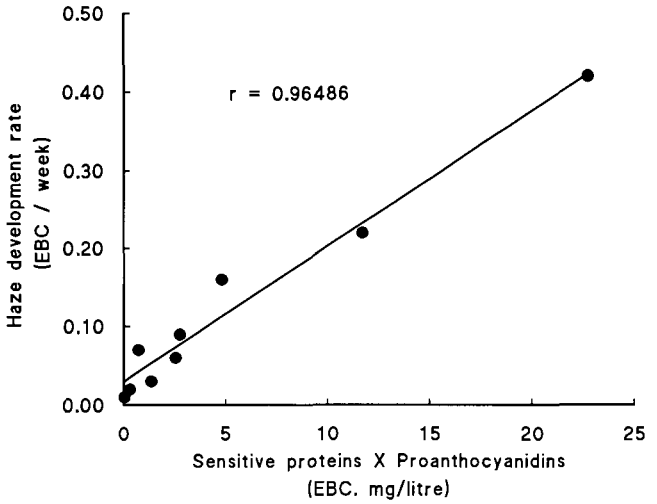

Fig 11. Colloidal stability model. Data points relate the rates of chill haze development in nine trial beers to the products of the measurements for sensitive proteins and proanthocyanidins (prodelphinidin B3 + procyanidin B3).

TABLE VI
Linear Regression Equations Relating the Rate of Chill Haze Development[a] in Beer Samples Stored at 37°C to Their Contents of Sensitive Proteins and Tanninogens

	Solutions		
Tanninogen Measurement	***a***	***b***	r^2
High-performance liquid chromatography			
Dimeric proanthocyanidins (DP)	0.0315	0.0169	0.9340
Catechin + epicatechin (CE)	0.0137	0.0133	0.8568
Sum (DP + CE)	0.0178	0.0077	0.9304
Colorimetric			
Total polyphenols	0.0127	0.0057	0.7349
Total flavanols	0.0129	0.0029	0.7679
Anthocyanogens	0.0131	0.0008	0.8647
Turbidometric			
Oxidizable polyphenols	0.0265	0.0202	0.6513

[a]Haze development rate = $a + b$ [P] [T], where P = content of sensitive proteins, measured by reaction with tannic acid, and T = content of tanninogens, measured by different assays.

measurements made of polyphenolic constituents. Accordingly, values for total polyphenols, total flavanols, anthocyanogens, oxidizable polyphenols, catechins, etc. were substituted separately for [T] in the rate model. The resulting regression lines for these data are given in Table VI. The best fit to the rate model was obtained with data points for dimeric proanthocyanidins (Fig. 11), with the next best fit resulting from substituted values for the sums of the contents of both monomeric and dimeric flavanols. The correlation obtained when the data points for anthocyanogens were used was slightly better than that obtained with data for monomeric flavanols and better still than that for total flavanols. The correlation with total polyphenol values was barely significant, and least well correlated of all were the results for oxidizable polyphenols.

These correlations imply that the rate of chill haze development is closely dependent on the contents of sensitive proteins and dimeric proanthocyanidins present in the beers at bottling and that analytical methods less specific than HPLC reveal this connection less clearly. Moreover, our results (Tables IV and V) indicate also that dimeric flavanols decrease, presumably due to oxidation, during the storage of beer. It is certain that dimeric flavanols require oxidation to a more active form (tannin) before they can participate in chill haze formation (2,7,8,13,18, 21,22,24,31,35,37). So it may seem contrary that the assay for oxidizable polyphenols fitted least well in the haze development model. We have, however, demonstrated already that the values obtained in the assay for oxidizable polyphenols depend not only on the content of simple flavanols, but also on the content of SHG-absorbable higher-molecular-weight substances (25). Our results, therefore, are in agreement with the proposal (40) that the key polyphenolic constituents in the determination of colloidal stability are the dimeric proanthocyanidins.

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