Beer Hazes. I. Isolation and Preliminary Analysis of Phenolic and Carbohydrate Components[1]

M. Dadic and G. Belleau, *Molson Breweries of Canada Limited, Montreal, Quebec, Canada H2L 2R5*

ABSTRACT

Haze was collected from fresh beer by centrifugation (Haze 1), and the water-soluble portion (over 90% of the total weight) was isolated (Haze 2). Water-soluble portions of hazes from an aged beer (Haze 3) and from a beer forced at 60° C for 72 hr (Haze 4) were also isolated. Phenolic concentrates of Hazes 1–4 (Preparations 5–8) were prepared by acid-alkali treatment followed by ether extraction. Phenolic compounds were then separated by high performance liquid chromatography on a Microbondapack C_{18} column. Eleven phenolic acids, two flavonols (quercetin and kaempferol), D-catechin, and L-epicatechin, were identified and quantified in the hazes. Carbohydrates were separated on an Aminex HPX-87 column, and glucose, maltose, fructose, and a dextrin determined after Sep-pack treatment.

Key words: *Aminex HPX-87, Catechins, Flavonols, HPLC, Microbondapak C_{18}, Phenolic acids*

The general composition and formation of nonbiological hazes in beer have been reviewed by several authors (4,7,14). The main components of chill and permanent hazes are polypeptides, polyphenols, and carbohydrates, with minor amounts of metals in the inorganic ash. In spite of the existing analytical methods, neither the overall contents of the major groups of haze constituents nor the concentrations of the individual compounds are known with any certainty. Metals in beer hazes are usually determined by atomic absorption spectrophotometry or by color reactions (7). Their role has been studied by Chapon (3), among others. The available procedures for estimating other principal haze constituents are not specific and yield unreliable results. This is largely because of interactions between polyphenols (particularly those polymerized) and polypeptides or polysaccharides. Such interactions may affect the immunoelectrophoretic analysis of the polypeptide moiety, which is usually determined by the assay of amino acids following hydrolysis. Comparison of the overall amino acid composition of hazes with that of barley shows that no specific barley protein is responsible for haze formation (6).

Determination of the carbohydrate spectrum of beer hazes, following hydrolysis, has at least two serious drawbacks: a mixture composed solely of monosaccharides usually results, and losses are incurred by interaction with both polypeptides and polyphenols during hydrolysis. On the other hand, measurement of "reducing sugars" before hydrolysis is subject to extensive interferences by polyphenols (7). Thus, with beer hazes containing polysaccharides, the principal (if not the only) material measured as "reducing sugars" will be the polyphenols. This may be one reason why, in a few reports on beer haze composition, inordinately low phenolic contents have been reported, another reason may be the complexity of analysis and the reactivity (sensitivity) of phenolics (4,5).

Upon hydrolysis, beer hazes yield a number of monosaccharides: glucose, xylose, and arabinose as major products; ribose, fructose, fucose, rhamnose (which may be derived from flavonol glycosides such as rutin), galactose, and mannose (which may be derived from mannans excreted by yeast during fermentation) as minor products. Carbohydrates present in beer sediments were studied, eg, by Letters (10).

Anthocyanogens (procyanidin, prodelphinidin, and propelargonidin) were recognized as major phenolic components of hazes relatively early (2,11). Catechin, methoxylated phenolics (probably related to lignin), and phenolic acids (ferulic, sinapic, vanillic, syringic, gallic, protocatechuic, and caffeic) were also identified in hazes (8,9); the presence of "flavonol residues" was postulated (7). Chill haze contains comparatively more simple polyphenols, and permanent haze contains more condensed tannins; this is consistent with the fact that chill hazes are grayish when freshly isolated but rapidly turn dark brown on oxidation.

In addition to the lack of available data on the identity of individual chemical compounds present in beer hazes, quantitative data are even more lacking and unreliable. A selective method of choice for separation, identification, and quantification of individual haze components appears to be high performance liquid chromatography (HPLC). Preliminary results of our work on the application of HPLC to determine phenolic and carbohydrate components of beer hazes are presented in this report.

We hope that this study will help to distinguish between hazes of various origins and contribute an additional tool for use in combatting beer instability.

EXPERIMENTAL

Equipment

This work involved centrifugation and freeze-drying to prepare the samples and HPLC for their analyses. Our laboratory equipment consisted of a centrifuge (International, model B-20A), a freeze-drier (New Brunswick Scientific), and an HPLC system made of the following units (all Waters): injector U6K; WISP (Waters Intelligent Sample Processor) 710-A; pumps 6000 A and M-45; detectors, absorbance model 440 and differential refractomonitor R 401; solvent programmer, model 660; recorder and integration system data module, model 730. Other materials included Microbondapak C_{18} (Waters) and Aminex HPX-87 (Bio-rad) column packings, Sep-pak C_{18} cartridge (Waters), and reagent grade reference compounds. Eluents were of chromatographic grade.

Preparation of Samples

Haze 1. Ten liters of degassed beer, previously filtered at 0° C to remove sediment, was centrifuged at 13,500 rpm and 0° C for 1 hr. The supernatant beer was decanted and the precipitated haze was washed with 20 ml of double-distilled deionized (ddd) water (boiled and subsequently chilled). Washings were discarded. Haze was transferred quantitatively with 100 ml of ddd water to a 250-ml round-bottomed flask. Water was removed by freeze-drying, and the residual haze was dried to constant weight in a vacuum desiccator at room temperature (average yield: 9.0 mg/L). Haze was stored under nitrogen or in a vacuum desiccator with protection from light. The haze was a fluffy grayish solid, insoluble in organic solvents but partially soluble in water/methanol (1:1).

Haze 2. One hundred milliliters of ddd water was added to a 250-ml round-bottomed flask containing 100 mg of Haze 1 and stirred magnetically at room temperature for 30 min. The suspension was centrifuged at 13,500 rpm and 0° C for 15 min. The water extraction of the insoluble portion was repeated and the supernatants were combined. Water was removed by freeze-drying, and the residual haze was dried to constant weight in a vacuum desiccator at room temperature (average yield: 8.4 mg/L of beer). The water-soluble portion was a fluffy beige solid. It was stored under nitrogen or in a vacuum desiccator with protection from light. (The water-insoluble portion was not further examined due to its insolubility.)

Haze 3. Haze was isolated as described for Haze 1 from degassed beer stored for 2–4 weeks at 4° C (average yield: 11 mg/L). The

[1]Presented at the 46th Annual Meeting, Minneapolis, MN, May 1980.

0361-0470/80/04015405/$03.00/0

water-soluble portion was prepared as described for Haze 2 (average yield: 10.3 mg/L). The fluffy beige solid was stored under nitrogen or in a vacuum desiccator with protection from light.

Haze 4. This haze was prepared as Haze 3 was, starting with degassed beer forced at 60° C for 72 hr (average yield of the water-soluble portion: 170 mg/L). The water-soluble portion was a brown solid. (The water-insoluble portion, about 21 mg/L, was not further examined because of its insolubility.)

Preparations 5–8 (from Hazes 1–4, Respectively). Five milliliters of a 1.25*N* solution of sodium hydroxide in distilled water was added to 100 mg of each haze sample and boiled for 5 min. The solution was acidified to a pH of approximately 2, using hydrochloric acid (1:1). It was then extracted with ether (2× 100 ml) and the combined ethereal layers were backextracted with a saturated solution of sodium bicarbonate (2 × 50 ml). The solution was reacidified and extracted with ether (2 × 100 ml). Ether was removed under reduced pressure. One hundred milliliters of ddd water was added to the oily residue and freeze-dried to obtain a white solid (yield: 25 mg).

Identification and Quantification of Products

Phenolics by HPLC on Microbondapak C_{18}. Samples 1–8 were dissolved in water/methanol (1:1) and clarified by filtration through a Nucleopore filter (1 μ). Concentration of the samples varied from 5 to 25 μg/μl as follows: Hazes 1–3, 5 μg/μl; Haze 4, 25 μg/μl; Preparation 5, 7 μg/μl; Preparation 6, 8 μg/μl; Preparation 7, 18 μg/μl; Preparation 8, 10 μg/μl. Solubility of the samples was: Haze 1, low; Hazes 2 and 3, not completely soluble; Haze 4 and Preparations 5–8, completely soluble.

All samples were then subjected to HPLC with the following reference phenolics: gallic, protocatechuic, gentisic, *p*-hydroxybenzoic, vanillic, chlorogenic, caffeic, syringic, *p*-coumaric, ferulic, *trans-o*-coumaric, and sinapic acids; D-(+)-catechin and L-(−)-epicatechin; quercetin and kaempferol. HPLC conditions were: column, 30 cm × 3.9 mm, Microbondapak C_{18} (10–20 μl injected); linear gradient elution (solvent programmer, curve 6); solvent A, H_2O/0.01*M* propionic acid/methanol; solvent B, 0.01*M* propionic acid/methanol. The gradient, initially, 10% solvent B in solvent A was increased until 25% (flow rate, 1 ml/min; pressure, 1,200 psi) or 40% (flow rate, 2 ml/min; pressure, 2,500 psi) of solvent B was in solvent A, in 40 min. Detection (absorbance model) was conducted at 254,280 and 313 nm.

Carbohydrates by HPLC on Aminex HPX-87. Samples 1–8 were prepared as for HPLC on Microbondapak C_{18} and subjected to HPLC with the following reference compounds: glucose, fructose, maltose, sucrose, and an alcohol-precipitated dextrin (Fisher). Sep-pak treatment was carried out as described for Waters Sep-pak C_{18} cartridges. HPLC conditions were: column, 30 cm × 7.8 mm, Aminex HPX-87, kept at 80° C (10–20 μl injected);

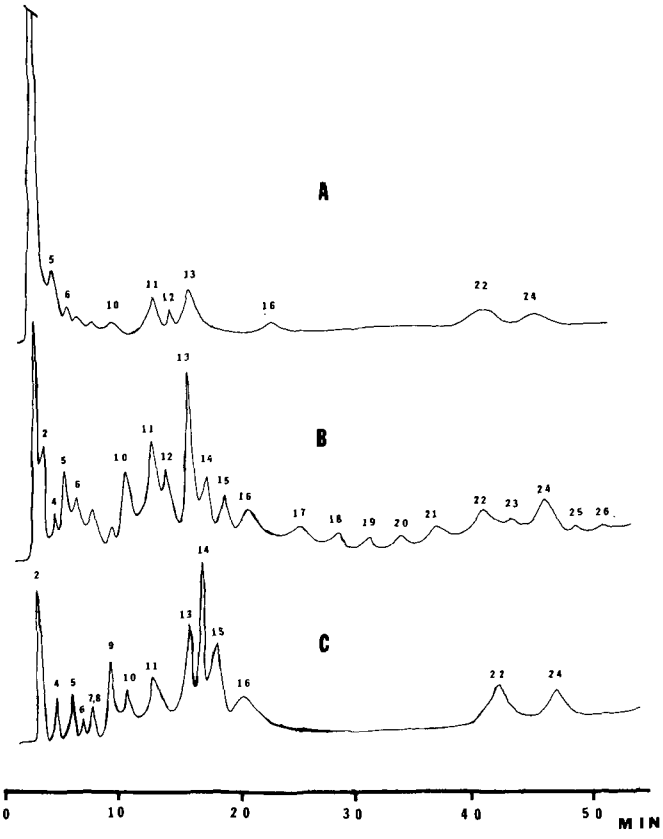


Fig. 1. High performance liquid chromatography (Microbondapak C_{18}) of fresh beer haze (**A**, Haze 2; **B**, Preparation 6) and a cocktail of 16 reference phenolics (**C**). Gradient: 10–40% solvent B and solvent A. Detection at 280 nm. Peak numbers given in Table I.

TABLE I
Phenolic Monomers in Hazes 1–4 by High Performance Liquid Chromatography on Microbondapak C_{18} (280 nm)

Peak Number[a]	Compound	R_t (min) at Elution Gradient, %		Content[b] in Hazes			
		10–40	10–25	1	2	3	4
2	Gallic acid	2.2	4.6	5.3	5.9	3.5	7.5
4	Protocatechuic acid	3.2	6.7	6.0	5.0	5.4	17.8
5	*p*-Hydroxybenzoic acid	4.8	9.9	18.5	13.4	43.0	22.5
6	D-(+)-Catechin	5.8	12.0	19.6	17.1	32.6	72.5
7	Vanillic acid	6.5	14.0	5.7	5.6	5.3	18.2
8	Caffeic acid	6.5	16.0	3.6	3.7	3.6	12.5
9	Chlorogenic acid	8.0	18.0	2.1	2.8	5.1	14.7
10	Syringic acid	9.7	20.9	7.1	6.7	12.9	22.0
11	*p*-Coumaric acid	12.4	24.0	4.6	4.6	7.3	20.0
13	Ferulic acid	15.0	29.5	15.3	15.0	22.9	40.5
14	*trans-o*-Coumaric acid	16.0	31.5	2.5	2.8	2.5	4.7
15	Sinapic acid	17.9	34.6	6.4	7.5	7.3	19.5
16	L-(−)-Epicatechin	19.9	42.5	17.8	17.7	19.0	43.7
22	Quercetin	41.8	···	8.2	8.7	6.4	5.7
24	Kaempferol	46.0	···	10.7	9.0	7.1	9.0
Total				133.4	123.5	183.9	330.0
Percent				0.13	0.12	0.18	0.33

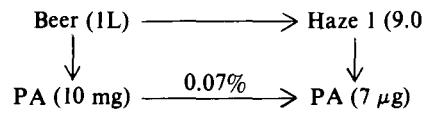
[a] Numbers correspond to peak numbers in Figs. 1–4.
[b] Given as μg/100 μg × 10^3.

flow rate, 0.5 and 0.6 ml/min; pressure, 500 psi; eluent, water (ddd) kept at 80° C. Detection was conducted with differential refractomonitor R 401.

RESULTS AND DISCUSSION

Phenolics

The portion of total phenolic monomers (PM) (Table I) that passes from beer into hazes is minimal. An example for Haze 1 can be diagrammed. Fresh beer (an ale) contains about 10 mg/L of phenolic acids (PA) (1) and gives about 9 mg/L of Haze 1. Haze 1, in turn, contains only about 7 μg of PA per 9 mg (Table I). Thus, less than 0.1% of the PA present in beer passes to fresh hazes.



Beer forced at 60° C for 72 hr yields over 17 times as much haze (Haze 4) as does the fresh beer (Hazes 1 and 2), but beer aged for 2–4 weeks at 4° C gives only slightly more haze (Haze 3) than does the fresh beer. Simple calculation, using the yield of various hazes and their PM content (Table I), shows that the PM portion of the aged beer that passes into Haze 3 is only slightly larger than the one that passes into fresh beer hazes (Hazes 1 and 2); however, the PM portion passing from the forced beer into Haze 4 is much larger than the one passing into fresh beer hazes. This may relate to recent reports (12,13) showing that levels of most PM (except D-catechin, L-epicatechin, and chlorogenic acid) decrease in aging beer.

Gentisic acid, with a retention time (R_t) close to that of protocatechuic acid under the HPLC conditions used, showed extremely low absorbance at 280 nm for the concentrations of Hazes 1–4 subjected to HPLC. Consequently, Table I shows the contents of 15 reference phenolic monomers (instead of the 16 used in the cocktail), arranged in order of increasing R_t for Hazes 1–4 measured at 280 nm and with two different elution gradients (10–40 and 10–25% solvent B in solvent A). Particularly abundant in all hazes are *p*-hydroxybenzoic, ferulic acids, and monomeric catechins. The increase in the PM content of Haze 4 compared to those of Hazes 1–3 is significant for all 15 compounds. The total PM content of Haze 1 (0.13%) is higher than that of its water-soluble portion (Haze 2, 0.12%); the increase of PM contents in aged-beer haze (0.18%) and forced-beer haze (0.33%) is apparent (Table I). Although the total percentages of PM in Hazes 1–4 are minimal, they may still be significant in studying the beer aging pattern. This may also apply to the individual PM, which all increase on forcing beer, and particularly to those that are relatively abundant (compounds 5, 6, 13, and 16).

Phenolic polymers (such as anthocyanogens) have been recognized (2,4,11) as major components of beer hazes. Tanninogen analyses (5) of some hazes derived from various ales yielded about 20–25% of tanninogens (anthocyangens + catechins). With the addition of the total percentages (0.13–0.33%) of the 15 phenolic monomers identified and quantified in an ale (Table I) and of the nonidentified phenolic monomers (such as quercitrin and rutin), the total percentage of phenolic components in such hazes may approach a grand total of about 30%. This is well in conformance with the percentage of the phenolic portion usually given for beer hazes (4,7,14), although it may vary significantly with the type of beer. In addition, the total distribution of tanninogens (20–25%) and nontanninogen phenolics (close to 5%, tentatively) in beer hazes resembles that reported for beer (4). This relationship may be of value in tracing the origin of beer hazes.

Monomeric, oligomeric, and polymeric tanninogens present in Hazes 1–4 either remain on the column under the applied chromatographic conditions or do not pass into phenolic preparations 5–8 because of insolubility in ether. These substances

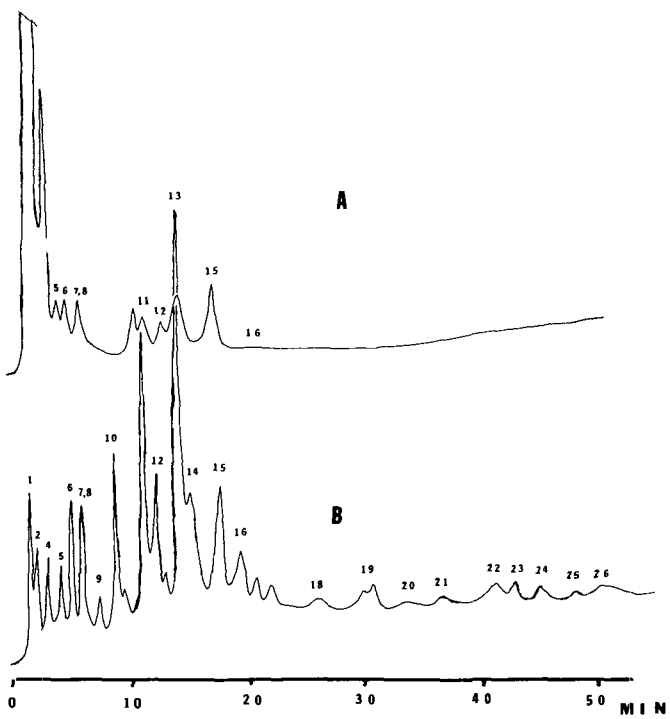


Fig. 2. High performance liquid chromatography (Microbondapak C_{18}) of forced beer haze (**A**, Haze 4; **B**, Preparation 8). Gradient: 10–40% solvent B in solvent A. Detection at 280 nm. Peak numbers given in Table I.

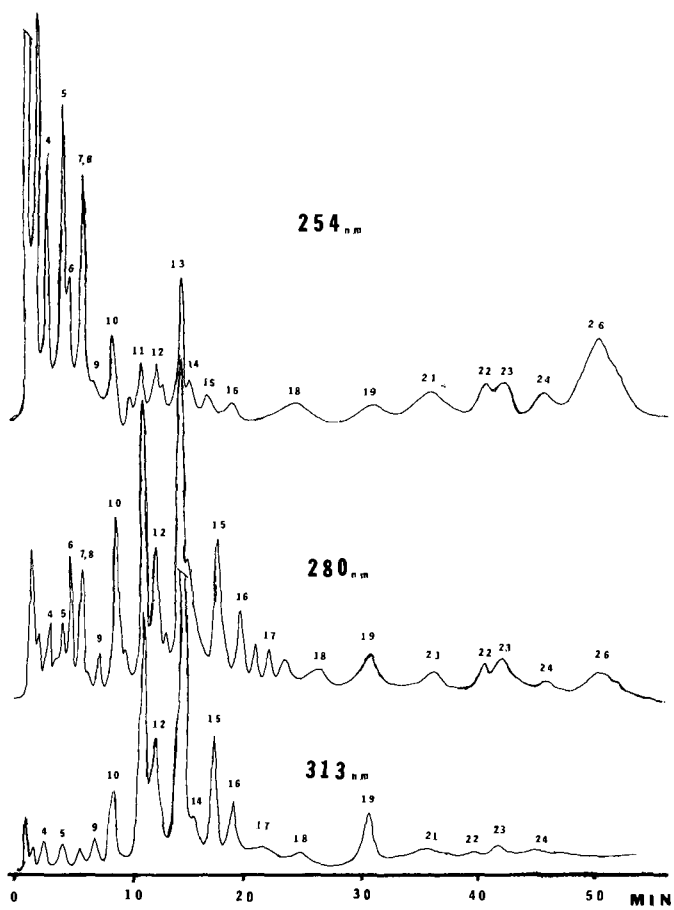


Fig. 3. High performance liquid chromatography (Microbondapak C_{18}) of Preparation 8. Gradient: 10–40% solvent B in solvent A. Detection at 254, 280, and 313 nm. Peak numbers given in Table I.

can be determined simultaneously in hazes as anthocyanogens and catechins (tanninogens), however (5).

The peak numbers in Table I identify the corresponding phenolic compounds in Figs. 1–4 and were used for identification and quantification of these compounds in the haze concentrates (Preparations 5–8). The maximum number of peaks was 26, which means that 10 components still remain unidentified.

The identity of the phenolic monomers was confirmed by chromatography, using various eluents and detection at three wavelengths (254, 280, 313 nm). Additional confirmation was obtained by comparing the absorbancies at 254, 280, and 313 nm with those at the corresponding wavelengths in the ultraviolet spectra of the pure compounds.

Chromatography of fresh beer haze (Haze 2) and its derived phenolic concentrate (Preparation 6) clearly showed an increase in concentration of phenolic compounds in the concentrate (Fig. 1). The difference in the order of R_t for chlorogenic and ferulic acids and for quercetin relative to their R_t in a beer HPLC study (12) is probably due to different eluent systems. In the study of beer phenolics (12), acetic acid was used instead of propionic, and PIC (tetrabutylammonium phosphate) was added.

The concentration of phenolic compounds was also clearly higher in Preparation 8 than in the forced beer haze (Haze 4) from which it was derived (Fig. 2). The amounts of Hazes 2 and 4 and of Preparation 6 and 8 used to obtain chromatograms in Figs. 1 and 2 were the same, clearly showing the much higher concentrations of phenolic materials in Haze 4 compared to those in Haze 2 and in Preparation 8 compared to those in Preparation 4.

Chromatograms of Preparation 8 at three detection wavelengths (Fig. 3) showed varying absorbancies of the same compounds at different wavelengths. Selection of the optimal wavelength for HPLC detection and quantification of a particular phenolic in hazes is therefore possible.

Use of a gradient elution of 10–25% solvent B in solvent A at 280 nm detection (Fig. 4) produced a superior resolution than was obtained with the 10–40% gradient at this wavelength (Figs. 1 and 3).

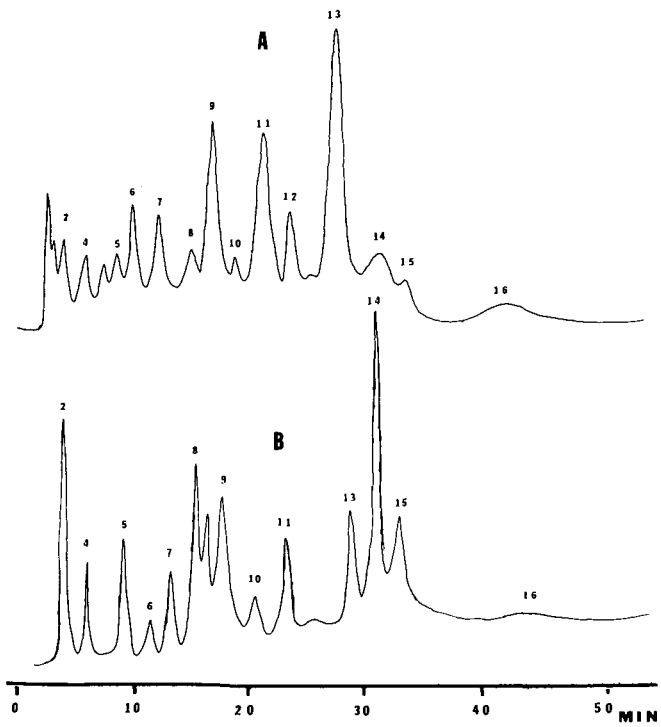


Fig. 4. High performance liquid chromatography (Microbondapak C_{18}) of Preparation 8 (**A**) and a cocktail of 16 reference phenolics (**B**). Gradient: 10–25% solvent B in solvent A. Detection at 280 nm. Peak numbers given in Table I.

Carbohydrates

Purification of Hazes 1–4 for carbohydrate determination was done using Sep-pak C_{18} cartridge technique. The identification and quantification were performed with the aid of reference compounds, whose standard solutions (1.1 g of glucose, 1.0 g of fructose, 1.05 g of maltose, and 1.05 g of dextrin alcohol-precipitated) were prepared in 50 ml of ddd water. The R_t and percentage of each carbohydrate component are shown in Table II.

When fresh beer haze (Haze 2) and forced beer haze (Haze 4) were chromatographed with and without Sep-pak treatment (Figs. 5 and 6), refractive index monitoring revealed that Sep-pak treatment removes from hazes some material responding to refractomonitoring. Because Sep-pak does not retain carbohydrates, the removed material is not of carbohydrate nature. Consequently, the carbohydrate components in Hazes 1–4 were determined after

TABLE II
Carbohydrates in Hazes 1–4 by High Performance Liquid Chromatography on Aminex HPX-87 with Refractomonitor Detection

Component	R_t (min)	Percent in Haze 1	2	3	4
Dextrin	8.4	24.5	10.3	12.0	36.7
Maltose	10.7	3.5	3.6	3.5	3.2
Glucose	12.7	0.5	1.1	0.9	0.1
Fructose	14.9	Traces	0.15	Traces	Traces
Total		28.5	15.1	16.4	40.0

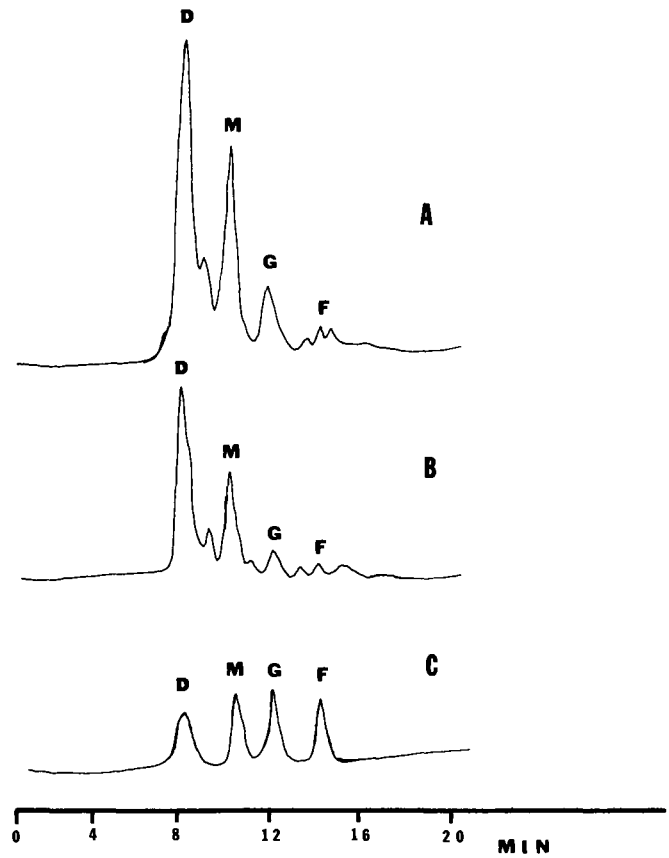


Fig. 5. High performance liquid chromatography (Aminex HPX-87) of fresh beer haze (**A**, Haze 2; **B**, Haze 2 Sep-pak treated) and a cocktail of four reference carbohydrates (**C**). Detection: differential refractomonitor. D = dextrin, alcohol-precipitated; M = maltose; G = glucose; F = fructose.

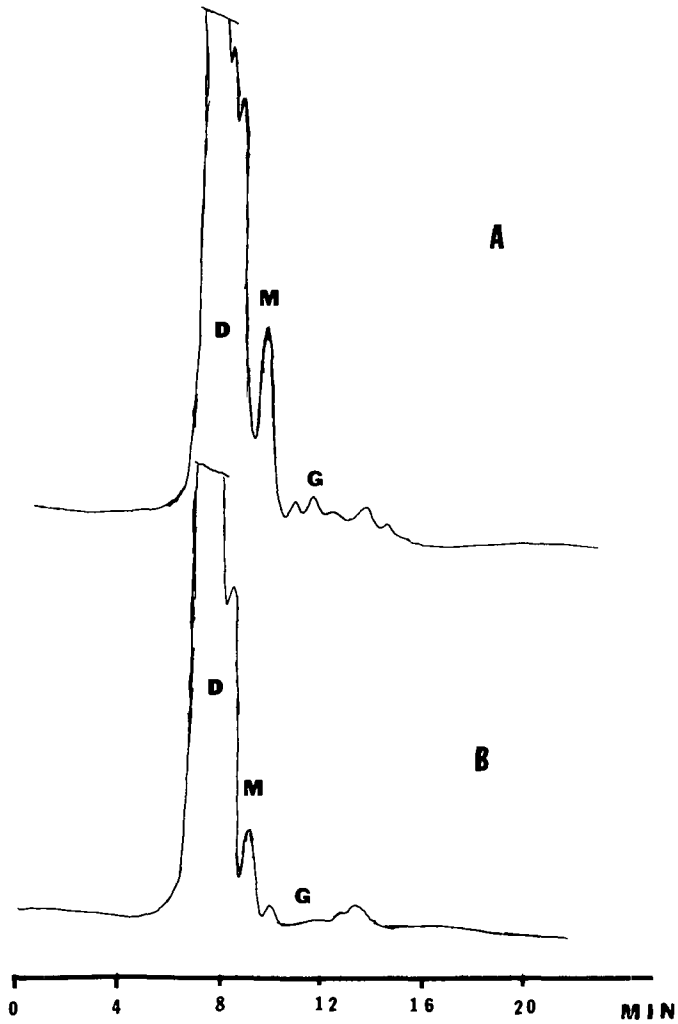


Fig. 6. High performance liquid chromatography (Aminex HPX-87) of forced beer haze (**A**, Haze 4; **B**, Haze 4 Sep-pak treated). Detection: differential refractomonitor. D = dextrin, alcohol-precipitated; M = maltose; G = glucose; F = fructose.

Sep-pak purification (Table II). However, a possibility exists that hazes treated with Sep-pak may contain traces of noncarbohydrate contaminants. Comparison of Figs. 5 and 6 also shows that Sep-pak treatment results in a better resolution of peaks.

Some other interesting observations can be made. First, the total percentage of carbohydrate material in Hazes 1–4 (ranging from 15% in Haze 2 to 40% in Haze 4), when added to the approximate total percentage (25–30%) of phenolic components in beer hazes, gives a range of 40–70% and leaves a range of 30–60% for nitrogenous matter (protein, polypeptides, amino acids) and metals. Second, the decrease in the dextrin component in the water-soluble poriton of Haze 1 (Haze 2) indicates that over 50% of polysaccharides in Haze 1 are water-insoluble (due to their high degree of polymerization). Third, a slight increase (about 20%) in the dextrin component in the aged Haze 3 and a large increase in dextrin in forced-beer Haze 4 in comparison to that in their precursor, the water-soluble Haze 2, confirms the role of polyphenol-polysaccharide interactions in the haze formation in aged (or forced) beer.

ACKNOWLEDGMENTS

Thanks are due to R. L. Weaver and J. E. A. Van Gheluwe for their continuous interest, to J. G. Lavallee and L. Randall for technical assistance, and to Molson Breweries of Canada Ltd. for permission to release these results.

LITERATURE CITED

1. Belleau, G., and Dadic, M. *Am. Soc. Brew. Chem., Proc. 1974,* p. 5.
2. Bengough, W. I., and Harris, G. *J. Inst. Brew.* 61:134, 1955.
3. Chapon, L. *J. Inst. Brew.* 71:299, 1965.
4. Dadic, M. *Tech. Q. Master Brew. Assoc. Am.* 13(3):182, 1976.
5. Dadic, M. *Brew. Dig.* 51(4):38, 1976.
6. Djurtoft, R. *J. Inst. Brew.* 71:305, 1965.
7. Gramshaw, J. W. *Tech. Q. Master Brew. Assoc. Am.* 7(2):122, 1970.
8. Harris, G. *J. Inst. Brew.* 71:292, 1965.
9. Harris, G., and Ricketts, R. W. *J. Inst. Brew.* 65:252, 1959.
10. Letters, R. *J. Inst. Brew.* 75:54, 1969.
11. McFarlane, W. D., Wye, E., and Grant, H. L. *Eur. Brew. Conv., Proc. Cong. 5th, Baden-Baden, 1955,* p. 298.
12. Qureshi, A. A., Burger, W. C., and Prentice, N. *J. Am. Soc. Brew. Chem.* 37:153, 1979.
13. Qureshi, A. A., Burger, W. C., and Prentice, N. *J. Am. Soc. Brew. Chem.* 37:161, 1979.
14. Wainwright, T. *Brew. Dig.* 49(5):38, 1974.

[Received May 13, 1980]