Formation of Protein−Polyphenol Haze in Beverages

Karl J. Siebert,* Aurea Carrasco, and Penelope Y. Lynn

Food Science and Technology Department, Cornell University, Geneva, New York 14456

Protein−polyphenol hazes form in beer, wine, and fruit juices and can limit shelf life. Haze-active protein is higher in beer than the other beverages. Haze-active polyphenol is highest in apple juice and red wines, variable in grape juices, and low in beer and white wine. A systematic study of factors that influence haze formation was carried out in a model system. Pectin, arabinogalactan, and poly(galacturonic acid) led to increased haze while free amino acids and other carbohydrates had no effect. Maximum haze occurred near pH 4 with less haze at higher and lower pH's. As ethanol concentration increased near pH 4, the haze at first declined, but further increases in ethanol led to increased haze. It appears that haze formation is similar in all the beverages examined and may be explained by a single mechanism. This has implications for analysis of haze-active constituents and beverage stabilization.

Keywords: *Haze-active protein; haze-active polyphenol; pH; alcohol; turbidity; beer; wine; fruit juices*

INTRODUCTION

Beers, fruit juices, and wines all contain proteins and polyphenols that can combine to form colloidal suspensions that scatter light and make the product appear cloudy. These beverages are all stabilized during processing to delay haze formation, using similar methods. The sources of the substances involved in haze formation are, however, rather different in the different products. In beer, the source of the haze-active protein has been shown to be the barley hordein (Asano et al., 1982), a prolamin or alcohol-soluble protein that is rich in proline (as much as 20 mol % of the amino acids). It is known from work with model systems that haze-active proteins contain significant amounts of proline and that proteins that lack proline form little or no haze with added polyphenols (Asano et al., 1982; Siebert et al., 1996). Wine haze-active proteins are "poorly understood", as protein instability correlates only weakly with total protein content (Jackson, 1994). The nature of haze-active proteins in fruit juices and wine has been studied and some of their characteristics have been described (Beveridge and Tait, 1993; Hsu et al., 1987, 1989; Johnson et al., 1968), but no clear identifications have been made.

Beer polyphenols come partly from barley and partly from hops. The beer polyphenols most closely associated with haze formation are the proanthocyanidins (dimers of catechin and epicatechin). These have been shown to interact strongly with haze-active proteins (Asano et al., 1982, 1983; Outtrup et al., 1987), and their level in beer has been shown to be directly related to the rate of haze formation (McMurrough et al., 1992). It has been demonstrated that anthocyanogen-free barley produces beer that, without any stabilizing treatment, is extremely resistant to haze formation provided that hopping is done with a product that does not contribute polyphenols (Ahrenst-Larsen and Erdal, 1979). The prominent proanthocyanidin species present in beer are procyanidin B_3 and prodelphinidin B_3. The haze-active polyphenols in fruit juices are also thought to be procyanidins (Heatherbell, 1984; Johnson et al., 1968; Lea, 1984; Spanos and Wrolstad, 1992) and apple juice contains a large amount of procyanidin B_2 (Coseteng and Lee, 1987; Spanos and Wrolstad, 1992).

The beverages mentioned above span a range of alcohol concentrations and pH and contain constituents other than protein and polyphenols that may influence haze formation; the effects of these additional factors on haze formation have not been clearly defined. It is also not clear whether haze-forming behavior is a continuum spanning all the products or is discretely different for some of them. It is interesting to consider the similarities and differences in composition that relate to haze formation in the various beverages and the general features that apply across the spectrum of products.

EXPERIMENTAL PROCEDURES

Chemicals. Gliadin, gelatin (G-0510 calfskin, Type IV, 60 bloom; bovine, Type B, 75 bloom; or porcine, Type A, 175 bloom), free amino acids, carbohydrates, and catechin were purchased from Sigma Chemical (St. Louis, MO). Tannic acid was purchased from Baker (FCC#0380-04) or Mallinckrodt (#1764).

Stock Solutions. Sodium phosphate buffer, 0.02 M, pH 4.2, was prepared fresh daily in HPLC-grade (deionized, distilled, and filtered) water.

In several experiments, gelatin was weighed and added to each test container as the dry powder. In the other cases, gelatin was dissolved in hot HPLC-grade water. Ethanol (20% of the final stock solution volume) was then added, and the solution was brought to final volume with HPLC-grade water.

Tannic acid stock solution was prepared by dissolving tannic acid in ethanol (20% of the final stock solution volume) and making to volume with HPLC-grade water.

Beverages. Commercial, clear bottled apple and grape juices and beer were purchased in the supermarket. Cider (cloudy, nonstabilized, unfermented apple juice) was purchased locally and was clarified by centrifugation (20 min at 13700*g*) and filtration (through glass fiber filter circles and then Whatman No. 5 filter paper, both with the aid of vacuum). The beer samples were degassed by filtration through Whatman No. 5 filter paper assisted by application of vacuum. The wine samples were produced on either the commercial or pilot scale and were not treated with adsorbents or fining. These samples were degassed, centrifuged (30 min at 9000*g*) and

* Author to whom correspondence should be addressed [telephone (315) 787-2299; fax (315) 787-2284; e-mail Karl_siebert@cornell.edu].

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Table 1. Comparison of Beverage Stabilization Procedures[a]

treatment	apple juice	beer	wine
protein removal			
bentonite adsorption	X	X	X
silica gel adsorption	X	X	X
tannic acid fining		X	X
proteolytic enzyme treatment		X	
ultrafiltration	X		X[b]
polyphenol removal			
PVPP adsorption	X	X	X[b]
gelatin fining	X	X	X
egg albumin fining			X
casein fining			X
isinglass fining		X	X
carbohydrate removal			
"pectinase"	X		X
β-glucanase			X[c]

[a] X indicates commonly used stabilization procedure for the indicated product. [b] Not used for red wines due to too much flavor and color loss. [c] β-Glucanase is used for *Botrytis*-contaminated wines.

filtered through Whatman No. 2V and then Whatman No. 5 filter papers with the aid of vacuum.

Induction of Haze in Beverages. Hazes were produced by adding catechin (as the dry powder), gelatin, or tannic acid (as a stock solution) to the test beverage to achieve the desired concentration (typically in a 20 mL volume in a 100 mL beaker). The samples were incubated for 30 min in a water bath at 25 or 80 °C, depending on the experiment. The 80 °C treated samples were then placed in a 25 °C bath for attemperation before haze measurement.

Model Systems. In the model system, a protein (gelatin or gliadin) and a polyphenolic compound (catechin or tannic acid) were combined in 0.02 M, pH 4.2 phosphate buffer in a beaker. The mixture (typically 100 mL in a 250 mL beaker) was held in a water bath at 25 or 80 °C for 30 min. The 80 °C treated samples were then placed in a 25 °C bath for attemperation before haze measurement.

Haze Measurements. Light scattering measurements were carried out with a Hach Model 2100AN ratio turbidimeter (Hach Co., Loveland, CO) using either 24 or 13 mm diameter cuvettes. Results were expressed in nephelos turbidity units (NTU).

Response Surface Methodology. The effects of four independent variables, protein (gliadin) concentration, polyphenol (tannic acid) concentration, pH, and alcohol, on haze formation were investigated in the model system using a response surface method. The initial experiment design used was a 24-point central composite, face-centered design produced with the aid of the MODDE computer program (Umetrics, Winchester, MA). The experiments were carried out in a randomized sequence. MODDE was used to model the results with multiple linear regression and display them graphically. Additional points were added to the design and used to refine the model.

RESULTS AND DISCUSSION

Stabilization Methods. Analysis of collected haze material from beer typically shows a substantial portion of carbohydrate (as much as 80%), together with protein and a small (1%−2%) amount of polyphenol (Siebert et al., 1981). However, all of the commonly used approaches to improve beer colloidal stability (see Table 1) are based on reducing either the level of protein or polyphenol. This indicates that the carbohydrate found in beer hazes is entrained or coprecipitated with protein or polyphenol but is not involved in the haze formation mechanism.

The approaches used to stabilize wines and fruit juices are similar to those employed in brewing (see Table 1). Bentonite adsorption is more commonly applied for wine and juice making, while silica hydrogel use predominates for beer chill proofing, where removal of foam-active protein is undesirable. Polyphenol reduction can be accomplished in beer, fruit juices, or wine either by fining with a protein (most commonly gelatin) or by adsorption with polyvinylpolypyrrolidone (PVPP).

Carbohydrase enzymes are often used in juice production to improve liquefaction of the fruit, which leads to greater efficiency of juice recovery. It is known that many of the commercial carbohydrase enzyme preparations contain minor but significant amounts of other enzyme activities. Since a small amount of protease can have a significant effect on beer stabilization (papain was used by a number of brewers for many years as the sole colloidal stabilization treatment), minor protease activity could account for the stabilization effect sometimes associated with the use of carbohydrase preparations.

Table 2. Haze (NTU) Resulting from Addition of Tannic Acid and Incubation at 25 °C

tannic acid (g/L)	apple juice 1	apple juice 2	clarified cider	beer 1	beer 2
0.0	16	0.5	56	4.3	1.7
0.50	18	1.8	62	2357	1811
1.25	19	2.1	68	4174	3393
2.50	23	2.9	71	5302	4244

Table 3. Haze (NTU) Resulting from Addition of Tannic Acid and Incubation at 80 °C

tannic acid (g/L)	apple juice 1	apple juice 2	clarified cider	beer 1	beer 2
0.0	11	1.0	99	3.6	1.7
0.50	13	0.8	110	2142	1855
1.25	16	2.8	120	3839	2100
2.50	21	1.7	134	3713	1907

Comparison of Levels of Haze-Active Proteins and Polyphenols in Beverages. Many of the commonly used methods for measuring protein or polyphenol do not give a good assessment of their haze-forming activity. The Bradford dye binding assay for protein, for example, hardly responds to beer haze-active protein (McMurrough et al., 1992); this is because Coomassie blue is highly biased toward basic and aromatic amino acids and these amino acids comprise only a small percentage of the haze-active protein (Siebert and Knudson, 1989).

Estimates of the relative amounts of "sensitive" or haze-active proteins in beer can be made by adding tannic acid (TA); this haze-active polyphenol combines with the haze-active protein in the sample to form haze that can be measured by light scattering (Chapon, 1993). When this procedure was applied to both apple juice and beer the results in Tables 2 and 3 were obtained. The data in Table 2 were produced by holding the sample−TA mixture at 25 °C for 30 min before measurement with a ratio turbidimeter, while those in Table 3 were produced by holding at 80 °C for 30 min. The two commercial apple juices showed a small (2−7 NTU) increase in haze as a result of the tannic acid addition at 25 °C. The increase was larger for the mechanically clarified cider that had not been stabilized (which would have removed much of the haze-active protein or polyphenol). The beers, on the other hand, exhibited a very large increase in haze from the same TA additions. The results were generally similar at 80 °C, except for the cider. There are likely two temperature effects operating here. Higher temperature can dissolve loosely or freshly associated haze, called "chill

Table 4. Haze (NTU) Resulting from Addition of Tannic Acid to Commercial Grape Juices and Incubation at 25 °C

tannic acid	white			red	
(g/L)	1	2	blush 1	1	2
0.0	4.7	1.2	0.9	0.4	0.7
0.50	5.3	3.9	1.3	2.6	1.3
1.25	5.7	1.7	1.9	1.3	2.1
2.50	6.1	2.8	2.5	2.1	2.3
5.00	6.9	3.9	3.7	4.8	4.1

Table 5. Haze (NTU) Resulting from Addition of Tannic Acid to Wines and Incubation at 25 °C

tannic acid		red	
(g/L)	white 1	1	2
0.0	4.9	1.6	0.8
0.5	5.0	2.2	0.9
1.0	5.6	3.3	2.2
2.0	6.2	6.0	1.9
3.0	6.4	8.9	2.1
4.0	6.8	16.4	4.8
5.0	6.8	10.0	5.4

Table 6. Haze (NTU) Resulting from Addition of Gelatin and Incubation at 25 °C

gelatin	apple juice		clarified	beer	
(g/L)	1	2	cider	1	2
0.0	17.3	2.4	92.9	4.26	1.95
0.1	254	61.5	168	4.81	2.14
0.2	307	23.7	243	4.92	2.00
0.4	329	11.2	289	5.40	2.71

haze" in brewing, leading to less light scattering. On the other hand, work with model systems showed that higher temperature incubation led to stronger protein–polyphenol binding (Artz et al., 1987) and more haze development (Oh et al., 1980; Siebert et al., 1996), apparently because partial denaturation of the protein exposes additional polyphenol binding sites. In the case of beer, where the haze-active barley protein has typically been boiled for 90 min and is presumably thoroughly denatured, it is likely that heating at 80 °C can contribute little additional access to polyphenol binding sites. Cider, on the other hand, has had little heat exposure (not even the pasteurization treatment commercial apple juices typically receive) and is more likely to show a temperature effect.

Tannic acid was also added to a number of commercial grape juices and wines. These mixtures were held at 25 °C and the hazes were measured (see Tables 4 and 5). The increase in haze in the grape juices caused by the tannic acid additions ranged from about 2 to 4 NTU. Results were similar for the one white wine sample examined and slightly higher for the two red wines (4–15 NTU). These results resemble those seen with commercial apple juices in Table 2. It is clear that the commercial fruit juices have only a small amount of haze-active protein while beer has a much larger amount.

It appeared that it might be possible to estimate the relative amounts of haze-active polyphenols in a sample by adding gelatin (a haze-active protein) and observing the haze produced. This was done with 30 min holding to develop haze at 25 °C. The results are shown in Tables 6–8. In this case, the results are just the opposite from the situation with TA addition. Here, beer formed almost no haze (near 1 NTU or less) while the apple juices and cider gave a pronounced response. Grape juice responses ranged from modest to pronounced and wines from little in white wine to considerable in the red wines. Taken together with the results in Tables 2–5, the results indicate that commercial beers are at one end of the spectrum, containing a considerable amount of haze-active protein and almost no haze-active polyphenol, while commercial apple juices are just the opposite. Grape juices and wines are somewhat intermediate, but generally resemble apple juice more than beer. Note that the largest hazes in many cases are not found with the highest gelatin additions. This is because the ratio of haze-active protein to haze-active polyphenol has a strong effect on haze formation (Siebert et al., 1996).

Table 7. Haze (NTU) Resulting from Addition of Gelatin to Commercial Grape Juices and Incubation at 25 °C

gelatin	white			red	
(g/L)	1	2	blush 1	1	2
0	4.4	0.9	1.3	4.0	0.4
1	6.3	3.5	3.8	88.7	6.0
2	413	5.1	5.2	59.3	5.3
4	387	6.6	6.7	50.0	6.0
5	228	7.8	8.0	37.2	7.2

Table 8. Haze (NTU) Resulting from Addition of Gelatin to Wines and Incubation at 25 °C

gelatin	white			red	
(g/L)	1	2	3	1	2
0.0	4.8	0.4	0.7	1.3	0.6
0.2	5.9	0.5	1.0	43	244
0.4	6.7	0.6	1.0	206	287
0.6	6.5	0.8	1.1	60	385
0.8	6.3	0.9	1.2	34	176
1.0	6.1	0.8	1.3	19	106
2.0	6.5	1.3	1.8	8	116

Table 9. Haze (NTU) Resulting from Addition of Catechin and Incubation at 25 °C

catechin		apple juice		clarified	beer	
(g/L)	control	1	2	cider	1	2
0.0	0.2	17	0.6	34	4.4	2.8
0.50	0.9	16	1.0	36	5.8	5.5
1.25	2.3	17	1.5	40	12	12
2.50	5.6	33	1.9	41	27	31

Table 10. Haze (NTU) Resulting from Addition of Catechin and Incubation at 80 °

catechin		apple juice		clarified	beer	
(g/L)	control	1	2	cider	1	2
0.0	0.2	17	0.7	43	3.7	3.1
0.50	1.2	14	4.0	42	5.8	12
1.25	2.8	14	1.5	47		33
2.50	4.7	17	1.8	53	158	97

It was previously shown that the response pattern of several haze-active proteins was quite different with tannic acid and catechin (Siebert et al., 1996). Asano and co-workers showed that the haze-forming activity of catechin correlated with that of polyphenols isolated from beer (Asano et al., 1982); results for tannic acid were not reported in this study. An experiment in which catechin was added to the samples and haze was developed in 30 min at 25 and 80 °C was carried out (see Tables 9 and 10). The results for the apple juices were similar to those obtained with TA (see Table 2), although the hazes were generally lower. Interpretation of the results is difficult because the control showed that the catechin solution by itself contributed hazes of magnitudes similar to the increases seen when it was added to some of the beverages. The beers showed a response, but it was much smaller than that produced with tannic acid. Unlike the case with TA, with

Table 11. Comparison of Products of Maximum Tannic Acid-Induced and Gelatin-Induced Haze for Various Beverages

sample	max haze (NTU)		TA × gelatin product
	TA	gelatin	
apple juice 1	7	312	2184
apple juice 2	2.4	60	144
clarified cider	15	196	2940
beer 1	5298	1.1	5828
beer 2	4242	0.7	2969
white grape 1	2.2	409	900
white grape 2	2.7	6.9	19
blush grape	2.8	6.7	19
red grape 1	4.4	85	374
red grape 2	3.4	6.8	23
white wine 1	1.9	1.9	3.6
red wine 1	14.8	205	3034
red wine 2	4.6	384	1766

catechin the beer haze was greater with 80 °C than with 25 °C incubation.

Continuum or Discontinuous Behavior? The large difference in the protein/polyphenol ratios in beer and the other beverages calls into question whether it is valid to consider these systems comparable. If the proteins and polyphenols in the two products are similar, then the system might be expected to behave as would other systems of limited solubility, where the following type of relationship usually applies:

$$K_{sp} = k[\text{protein}][\text{polyphenol}] \quad (1)$$

Here the solubility product constant is equal to a constant times the product of the concentrations of the two components of a poorly soluble product. If this relationship applies to protein−polyphenol haze, the products of the haze-active protein concentration and haze-active polyphenol concentration should be similar in products near the solubility limit. This idea was tested for the apple juice, grape juice, and beer results using the maximum hazes produced by tannic acid or gelatin addition in Tables 2, 4, and 5−8 as estimates of the amounts of haze-active protein and haze-active polyphenol in each beverage. Their products are shown in Table 11. Apple juice 2 has been extremely stable in all our work and is likely far removed from the equilibrium where haze is easily produced. The products from the other apple juice, cider, the two beers, and the two red wines, however, fall within a factor of ∼3 range. That suggests that the these systems may behave similarly. The grape juice and white wine results, however, are much lower. This indicates that a continuum model for all these beverages based solely on the solubility limit is apparently not satisfactory.

The Nature of Beverage Haze-Active Protein. Chromatographic profiles of cider (clarified unstabilized apple juice) protein before and after induction of haze by addition of tannic acid showed only minor differences (data not shown); this result suggests that only a small proportion of the total protein is involved in haze formation. It also suggests that analyzing collected haze may be a useful approach to characterize fruit juice haze-active protein. In apple juice hazes in which hydrolysis and amino acid analysis were carried out, a significant amount of proline (ranging from 4.6% to 15.9%) was found (Johnson et al., 1968). This is particularly interesting since the amount of free proline in apple juice is quite low compared to grape juice and beer (Wallrauch and Faethe, 1988). Finding proline in apple juice haze protein is not surprising, however, since model system results show that proline is apparently required for a peptide to demonstrate haze-forming activity (Asano et al., 1982; Siebert et al., 1996). This also fits with the fact that PVPP, whose structure resembles polyproline (both have saturated five-member, nitrogen-containing rings and amide bonds) and which must also operate by binding polyphenols, is effective in stabilizing juice. The similarities between effective fruit juice and beer stabilization treatments also suggest that the nature of the haze-active proteins may be similar. The wheat protein gliadin is, like barley hordein, a prolamin that is rich (∼15 mol %) in proline (Asano et al., 1982). Gliadin is commercially available and quite haze-active; as such it appears to be a good model for beverage haze-active protein.

The Nature of Beverage Haze-Active Polyphenol. The literature clearly implicates proanthocyanidins, particularly the dimers, in the formation of haze in beer, wine, grape juice, and apple juice. Unfortunately, these compounds are not commercially available and are difficult to synthesize. Tannic acid and catechin are readily available and can be used as substitutes for the proanthocyanidins in model system studies or for additions to beverages. A study of the ability of various polyphenols to bind β-glucosidase led to the conclusion that an *o*-dihydroxybenzene group is needed for each attachment to the protein (Haslam, 1974). Molecules with one *o*-diphenol group can attach to protein but not cross-link. The precipitating ability of polyphenols increases as the number of *o*-diphenol groups in the molecule increases. Digalloyl glucose was roughly equivalent to procyanidin B_2 (dimer) in precipitating ability while trigalloyl glucose and procyanidin C_1 (trimer) were similar and 3−4 times more effective. Although the haze-forming activity of isolated beer polyphenols correlated with that of catechin (Asano et al., 1982), catechin has one *o*-diphenol group and one *m*-diphenol group and would be expected to be much less haze-active than tannic acid or proanthocyanidin dimers or trimers (Asano et al., 1983). As a result, tannic acid appears to be a better choice for model system studies. This likely accounts for the much lower hazes seen with catechin than with tannic acid in the results reported here.

The Influence of Other Beverage Components on Haze Formation. Beverages contain a number of constituents that may exert an influence on protein−polyphenol haze formation. These include alcohol, hydrogen ion (pH), amino acids, metal ions, and carbohydrates.

(a) Alcohol. Alcohol was found to reduce the amount of haze induced in apple juice by tannic acid. The effect, however, was relatively small even with ethanol levels as high as 95%. The haze-suppressing action of ethanol is probably due to the slightly less polar (than water) nature of ethanol as a solvent. Haze formation in a model system was inhibited by 25% dioxane, a nonpolar solvent, and it was concluded that dioxane interferes with hydrophobic bonding between proteins and polyphenols (Asano et al., 1982). Dioxane also dissolves freshly formed model system haze to a significant extent (Siebert et al., 1996). It is likely that ethanol, which is intermediate in polarity between water and dioxane, can reduce haze formation to some extent.

(b) pH. The pH of a solution often influences how proteins behave because of the effect on the net charge of the molecules. This can lead to more or less ionization, with a change in ionic attraction or repulsion, and

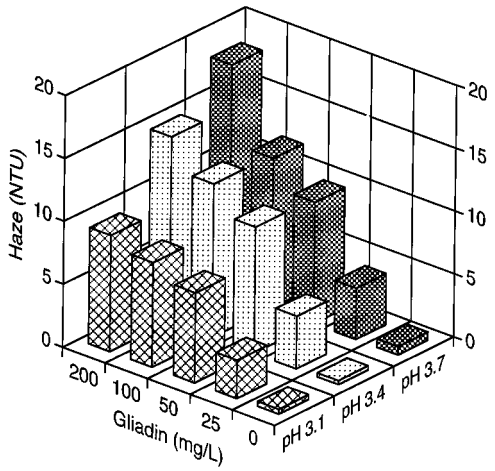

Figure 1. Haze (NTU) formed in buffer with 600 mg/L catechin at various pH's and gliadin concentrations.

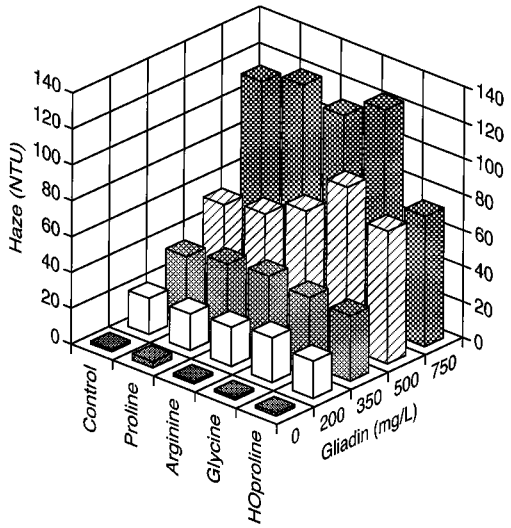

Figure 2. Effect of 200 mg/L of the indicated free amino acids on haze formation when 500 mg/L catechin was combined with the indicated level of gliadin in pH 4.2 buffer. HOproline, hydroxyproline.

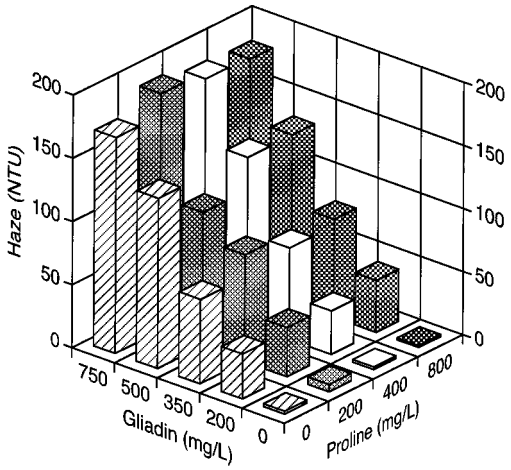

Figure 3. Effect of various levels of free proline on haze formation when 500 mg/L catechin was combined with the indicated levels of gliadin in pH 4.2 buffer.

may also alter the protein solubility or the molecular conformation, which in turn can influence the accessibility of sites on the protein to which polyphenols can bind. The interactions between proteins and polyphenols appear to be mainly hydrophobic with perhaps some contribution from hydrogen bonding (Asano et al., 1982; Hagerman and Butler, 1981; Siebert et al., 1996), so any net charge on the molecule might be expected to reduce haze formation. Within the fairly narrow range of pH 3.7−4.2, the effect of pH on haze in a model system was found to be modest. Wine and grape juice, however, are significantly lower in pH, and a study of the effect of pH in the range 3.1−3.7 was carried out using gliadin and catechin in phosphate buffer with 30 min haze development at 80 °C (see Figure 1). Clearly the hazes formed under these conditions are modest and pH has a strong effect, with the lowest haze formation at the lowest pH. This result may account for the relatively low level of haze-forming activity seen with grape juice.

(c) Free Amino Acids. Since proline is an important component of proteins that bind to polyphenols, it appears that free proline might compete with the haze-active protein in binding to polyphenols. Free amino acid−polyphenol complexes would be much smaller and are more likely to be soluble than protein−polyphenol complexes. This might account for the difference in the protein/polyphenol balance between beer (high in protein) and apple juice (high in polyphenol) since beer typically has ∼150 mg/L proline (Hough et al., 1982) and apple juice has almost none (Wallrauch and Faethe, 1988). Grape juice ranges from about 150 to 1000 mg/L proline, while orange, grapefruit, and passion fruit juices range even higher (Wallrauch and Faethe, 1988). A model system experiment was performed in which gelatin was added to each of several free amino acids (proline, hydroxyproline, arginine, glycine) in pH 4.2 phosphate buffer. Tannic acid was then added, the mixtures were held 30 min at 25 °C, and the hazes were measured. No consistent effects on haze were seen with any of the amino acids.

Since gelatin and tannic acid are both very strong haze formers, it is possible that their complexes are held together by multiple interactions that are difficult to disrupt. A similar experiment was carried out with gliadin and catechin, which form haze somewhat less readily than gelatin and tannic acid. In this case, the four amino acids were each added at 200 mg/L. These mixtures were held at 80 °C to develop haze and then measured (see Figure 2). The results show a small degree of variation with glycine and hydroxyproline at one gliadin concentration each but no consistent trend with any amino acid. Proline in particular gave results almost identical to the control.

Proline was added at several concentrations up to 800 mg/L to the gliadin−catechin model system, and the mixtures were held at 80 °C to develop haze and then measured. The results are shown in Figure 3. It appears that neither free proline nor other free amino acids have an effect on the amount of haze formed. There is thus no indication that free amino acids compete with protein binding sites for polyphenol molecules.

(d) Metals. Some literature reports have implicated metal ions in haze formation. Mostly their action has been explained as catalytic, presumably in oxidizing or polymerizing polyphenols that then react with protein. In the main, the effect of metals seems to be a problem that normally is not seen when good practices (use of stainless steel or nonmetallic equipment) are employed.

(e) Carbohydrates. There is some evidence that carbohydrates may be involved in producing or stabilizing cloudy fruit juices, and starch systems are used to

Table 12. Effects of Several Carbohydrates on the Haze (NTU) Produced by Catechin and Gliadin in pH 4.2 Phosphate Buffer

carbohydrate (mg/L)	sucrose	poly(ethylene glycol)	starch	pectin
0	14.5	14.5	14.5	14.5
2		14.9	14.1	14.6
50		13.1	15.3	77.3
100		12.7	13.8	63.5
200	10.7	14.0	13.8	71.5
400		12.0	14.2	
1000		10.7	17.1	
2000	11.5	9.4	16.7	
10000	11.7	9.1		
20000	10.8			

Table 13. Effects of Arabinogalactan (AG) and Poly(galacturonic acid) (PG) on the Haze (NTU) Produced by Catechin and Gliadin in pH 4.2 Phosphate Buffer

carbohydrate (mg/L)	AG + buffer	AG + catechin + gliadin	PG + buffer	PG + catechin + gliadin
0	0.1	29.1	0.1	29.1
2	0.2	33.6	0.5	123
10	0.1	36.1	2.0	88
50	0.2	44.1	9.6	540
100	0.2	44.8	19.7	390
200	0.3	66.0	40.9	391
400	0.4	93.8	62.4	433
1000	1.3	59.6	84.7	381

produce synthetic stable clouds in some beverages. As noted previously, collected beverage hazes are often found to be rich in carbohydrate in spite of the fact that the useful means of colloidal stabilization rely on removing either protein or polyphenol and not carbohydrate. Polysaccharides can interact with colloidal substances and are thought to function as "protective colloids" that slow or prevent haze formation or precipitation (Jackson, 1994). In this role they presumably compete for either protein or polyphenol binding sites or sterically block access to them. The effects of a number of carbohydrates present in beverages on haze formation in a model system were studied. The test carbohydrates were added at a range of concentrations to catechin and gliadin in pH 4.2 buffer. The samples were held at 80 °C for 30 min, and the hazes were then measured. The results are shown in Table 12. Except for pectin, which produced a sudden haze increase at 50 mg/L and may have exceeded its solubility limit, the effects of all the carbohydrates were quite small. Sucrose appeared to decrease haze slightly, as did poly(ethylene glycol); these compounds may be slightly protective. Starch appeared to cause a slight decrease in haze followed by an increase.

A similar experiment was performed with two polysaccharides that occur in fruit juices, arabinogalactan (AG), and poly(galacturonic acid) (PG). In this case the carbohydrates were added in pH 4.2 phosphate buffer with or without 600 mg/L catechin and 250 mg/L gliadin (see Table 13). Both carbohydrates resulted in significantly increased haze compared with catechin and gliadin alone, although PG alone at the higher concentrations produced considerable haze, which may indicate proximity to its solubility limit. It appears that these polysaccharides interact with protein and polyphenol to form more haze. What is not clear is whether the levels of the carbohydrates normally present in the beverages of interest are sufficient to exert an influence on the haze produced.

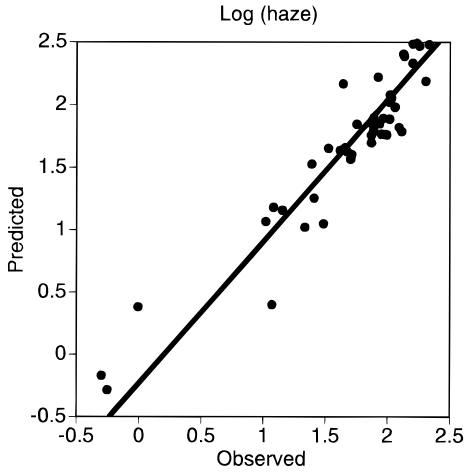

Figure 4. Plot of the observed (measured) haze versus the haze value predicted (by the model equation) for the same set of conditions when the model fit was made to the logarithm of measured haze.

Multivariate Model of Effects on Haze. From the discussion above it is apparent that four components normally influence the haze in the beverages of interest: haze-active protein, haze-active polyphenol, pH, and alcohol. The interactions of these factors on the amount of haze formed in a phosphate buffer model system was studied using a statistical experiment design. A 24-experiment face-centered central composite design with four replications of the center point was used to examine the results of various combinations of the four variables:

component	low level	high level
protein (gliadin, mg/L)	50	500
polyphenol (tannic acid, mg/L)	10	100
pH	2.8	4.6
alcohol (v/v % of 95% EtOH)	0	12

A high proportion of the initial combinations resulted in low hazes due to the system behavior. Additional experiments (24 more) were added to the design, with several of these replicating conditions in the first 24 experiments. The replicate points produced results in reasonably good agreement with the first set. It was considered appropriate to add the second group of results to the original data set. Multiple linear regression was applied to the expanded set including all possible squared and two-way interaction terms produced from the four independent variables. The plot of predicted (from the model) versus observed values showed curvature and only a fair fit to linear form ($R^2 = 0.544$). This nonlinearity probably resulted, at least in part, from variations in the haze particle sizes produced under different conditions (Siebert et al., 1996). Variations in particle size are known to result in nonlinear light scattering behavior (Thorne, 1963). The logarithm of haze rather than the measured haze was then modeled; this linearized the relationship and improved the fit to $R^2 = 0.882$ (see Figure 4). Backward stepwise regression was used to eliminate terms that contributed little to the model. Three of the terms were eliminated and R^2 remained at 0.88. The formula

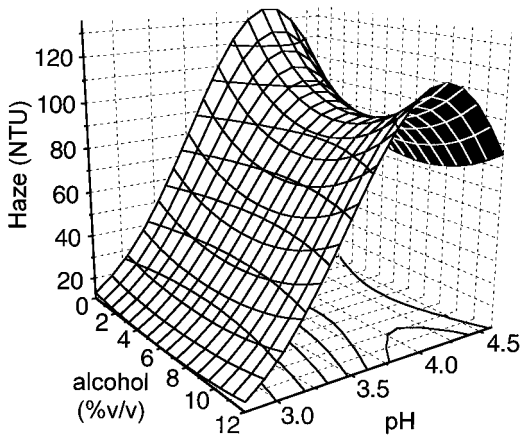

Figure 5. Effects of alcohol and pH on haze predicted by the response surface model at 275 mg/L gliadin and 55 mg/L tannic acid.

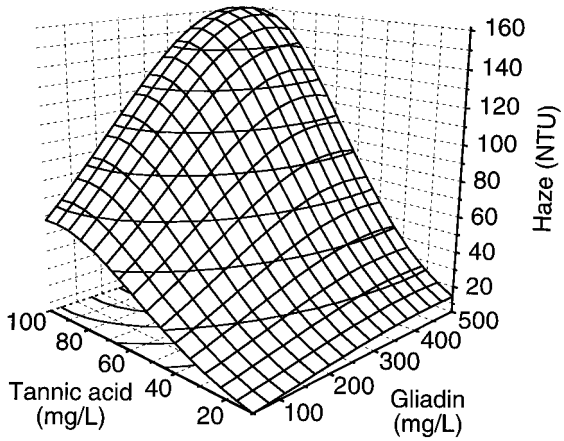

Figure 6. Effects of gliadin and tannic acid concentration on haze predicted by the response surface model at 6% (v/v) alcohol and pH 3.7.

defined by the model is

$$\log(\text{haze}) = -10.097 - (0.017)\text{AL} + (4.879)\text{pH} + (7.804 \times 10^{-4})\text{GL} + (0.057)\text{TA} + (3.202 \times 10^{-3})\text{Al}^2 - (0.548)\text{pH}^2 - (2.816 \times 10^{-6})\text{GL}^2 - (1.431 \times 10^{-4})\text{TA}^2 - (0.006)\text{AL pH} + (4.711 \times 10^{-4})\text{pH GL} - (0.008)\text{pH TA} \quad (2)$$

where AL is the percent alcohol (v/v), TA is tannic acid (in mg/L), and GL is gliadin (in mg/L).

Figure 5 shows the predicted hazes at various combinations of pH and alcohol at the middle levels of gliadin and tannic acid used in the study. It can be seen that there is a ridge of haze at approximately pH 4.1−4.2. This is in the region of many beers and apple juices. The haze is lower at higher pH's and much lower at lower pH's, particularly in the range of grape juices and wines (near pH 3). Near pH 4 the effect of increasing alcohol is at first a decline in haze, presumably because alcohol interferes with hydrophobic bonding, as discussed above. At higher alcohol levels, however, haze increases, probably because the alcohol begins to desolubilize protein (as seen in the alcohol cooling test for beer colloidal stability). Note that the effect of increasing alcohol is predicted to be much smaller, and essentially insignificant at the pH of wine and grape juice. While gliadin is similar in solubility and amino acid composition to barley hordein, the source of the haze-active protein in beer, and is therefore likely to exhibit a similar pH response, relatively little is known about the nature of haze-active protein in fruit juices.

The effects of varying gliadin and tannic acid concentrations at the center point levels of alcohol and pH (see Figure 6) are seen to be very similar to those previously reported in the gelatin−tannic acid and gliadin−catechin systems (Siebert et al., 1996). As protein (gliadin) increases at a fixed level of tannic acid, the haze at first increases, then plateaus, and finally declines somewhat. Similar results are seen as polyphenol (here tannic acid) concentration increases at a fixed protein level. These results are consistent with the model proposed previously (Siebert et al., 1996).

The regions of greatest interest are naturally those combinations of alcohol and pH occupied by beer, wine, and apple and grape juices. These are depicted in Figure 7, which shows the same information as Figure 5 but as a contour plot. The haze predicted in the beer and apple juice pH region is ~7 times greater than for equivalent protein and polyphenol levels at the pH of grape juice and wine. Apple juice is typically in the region where the highest haze response would be expected if the protein and polyphenol concentrations were the same in all products. This would also be the case for nonalcoholic beers. Moving from low-alcohol beer to light beer to regular beer to malt liquors would increase alcohol and move toward somewhat lower haze. The model predicts almost no alcohol effect, however, at the lower pH of grape juice and wine. Separate contour plots for haze as a function of gliadin and tannic acid concentrations at the pH and alcohol region of various beverages were constructed. The pattern for regular beer was very similar to that for apple juice or nonalcoholic beer (see Figure 8) except for a lowering of expected haze due to the alcohol effect. Results for grape juice and wine (Figure 9) were quite similar to each other and demonstrate less haze and less curvature with increasing tannic acid than beer.

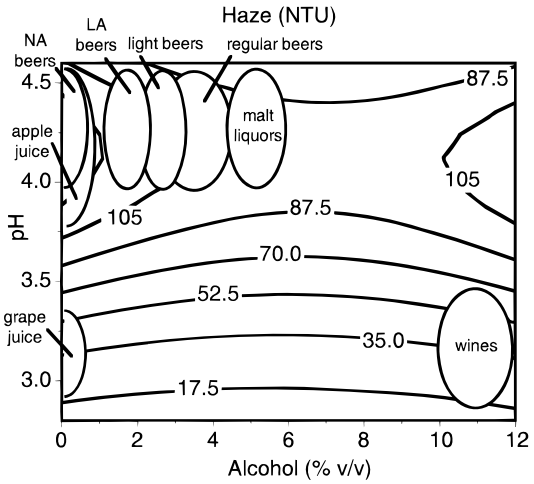

Figure 7. Haze behavior (NTU) of the model system predicted from the response surface equation in the pH and alcohol regions occupied by various beverages for 275 mg/L gliadin and 55 mg/L tannic acid. NA beer, nonalcoholic beer; LA beer, low-alcohol beer.

Implications of the Results. The results described here, taken together with the model for haze formation advanced previously (Siebert et al., 1996), explain many of the differences seen in haze formation between beverages. It is apparent that compositional differences

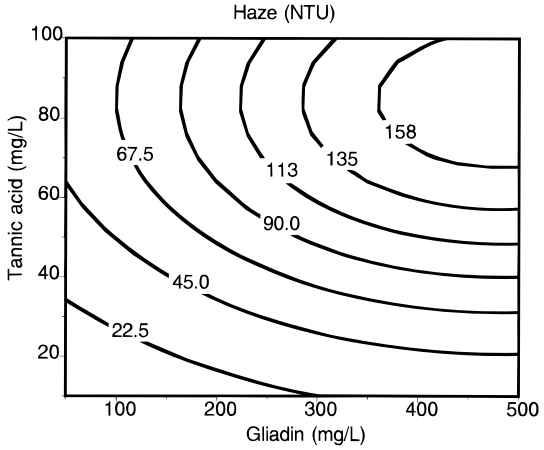

Figure 8. Predicted haze response to variations in protein and polyphenol concentration at the alcohol (0% v/v) and pH (4.2) range of apple juice.

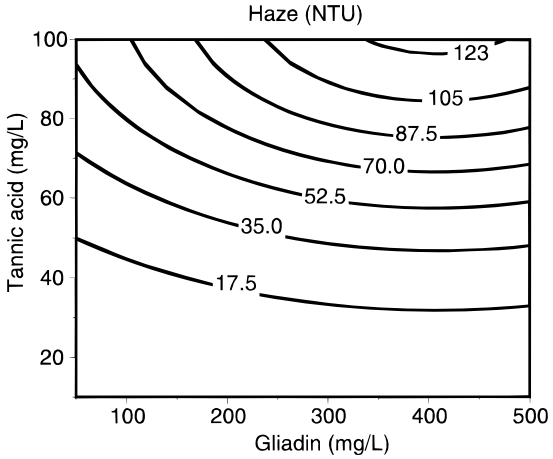

Figure 9. Predicted haze response to variations in protein and polyphenol concentration at the alcohol (12% v/v) and pH (3.2) range of wine.

between beverages result in different responses when a haze-active polyphenol or protein is added, either as a fining agent or to provoke haze in a turbidimetric analytical method. It can also be seen that the form of the haze-active materials varies across the range of beverages, and this likely has an influence on the efficacy of stabilization treatments. In beer nearly all the haze-active polyphenol present is likely bound to proteins in which the majority of the polyphenol binding sites are unoccupied, while in apple juice most of the polyphenol is presumably free and the vast majority of the polyphenol binding sites on the haze-active proteins will be occupied. As a result, the nature of the complexes that fining agents or adsorbents contact is quite different in different beverages.

Conclusions. Beer has much higher levels of haze-active protein and much lower levels of haze-active polyphenols than apple and grape juices and wine. A solubility product relationship does not hold for these species, at least in part because the ratio of haze-active protein to haze-active polyphenol exerts a strong influence on the amount of haze formed. Free amino acids did not affect haze formation in the model system. Several carbohydrates (sucrose, starch, poly(ethylene glycol)) had little or no effect on haze formation in the model system, but three fruit polysaccharides (pectin, arabinogalactan, poly(galacturonic acid)) increased haze. The effects of alcohol and pH on haze formation are complex. Maximum haze for a given protein and polyphenol concentration is produced at pH ~4.2, with much less haze at lower pH and somewhat less at higher pH. Alcohol had no effect on haze near pH 3 but at the pH of apple juice and beer resulted in first a decline and later a rise in haze. It is likely that the haze-active proteins in apple and grape juice and wine contain, as in the case of beer, a significant percentage of proline. It appears that there are great similarities in the haze formation mechanisms in these products, and a single model can explain the observed behavior. The results reported here have important implications for analytical methods designed to measure haze-active proteins and polyphenols, for prediction of colloidal stability in beverages, and for methods used to achieve stabilization.

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