CHAPTER 2

Haze in Beverages

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Contents

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Abstract Beverages such as beer, wine, clear fruit juices, teas, and formulated products with similar ingredients are generally expected by consumers to be clear (free of turbidity) and to remain so during the normal shelf life of the product. Hazy products are often regarded as defective and perhaps even potentially harmful. Since consumers are usually more certain of what they perceive visually than of what they taste or smell, the development of haze in a clear product can reduce the likelihood of repeat purchasing of a product and can have serious economic consequences to a producer. Hazes are caused by suspended insoluble particles of colloidal or larger size that can be perceived visually or by instruments. Hazes in clear beverages can arise from a number of causes, but are most often due to protein–polyphenol interaction. The nature of protein–polyphenol interaction and its effect on haze particles, analysis of haze constituents, and stabilization of beverages against haze formation are reviewed.

I. THE PHYSICS OF HAZE

The phenomenon of haze or turbidity in beverages occurs when light passing through a sample is deflected or scattered by suspended particulate matter. An observer perceives the scattered light and, as a result, the sample appears turbid. While particles larger than colloidal size can scatter light, these usually settle out and do not form stable systems. Stable systems must either have particles of a density similar to the suspending liquid or have particles that are sufficiently small for the ambient thermal energy to keep them suspended indefinitely. The latter are called colloids, which by most definitions range between 1 and 1000 nm (largest dimension). Ambient temperatures provide energy that results in Brownian motion. This produces random collisions of solvent molecules with particles that are sufficient to keep small particles with somewhat greater density than a solvent in suspension indefinitely.

While light scattering can be observed with a photometer (an instrument in which a light source, a sample, and a detector are in a straight line; see Fig. 2.1), light that fails to reach the detector could either have been scattered or absorbed. For this reason, scattering is typically observed with an instrument in which the detector is placed at some angle to the incident light beam (see Fig. 2.2). The two most frequently

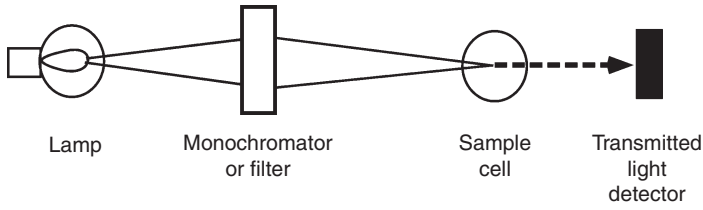

FIGURE 2.1 The light path in a photometer.

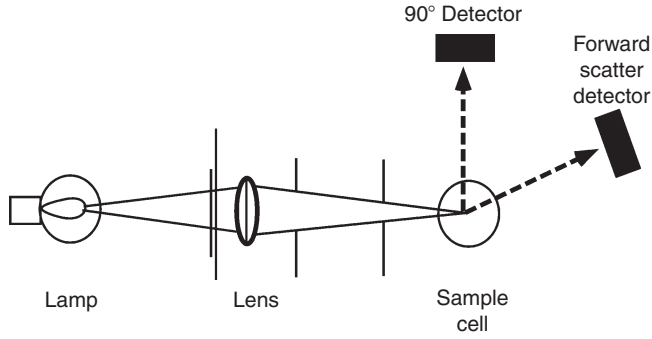

FIGURE 2.2 The light path in a turbidimeter.

used types of static light scattering instruments have detectors at a narrow angle (generally in the 11–25° range) or at 90°. The latter are also called nephelometers. In the case of instruments designed to measure very high turbidities, there may also be a backscatter detector; however, the levels of turbidity typically found problematic in clear beverages are considerably lower than this level. With most light scattering instruments, the amount of light scattered in colored solutions is underestimated because some of the scattered light is absorbed. In order to make color-corrected haze measurements or haze-corrected color measurements, an instrument that simultaneously measures scattering and transmission is required. A ratio between the two observations is then used to produce the desired result.

Turbidimeters can employ white light, light passed through an optical filter, or monochromatic (laser) light. Because the relationship between the wavelength of light and the size of the particles affects scattering, instruments that use different light sources (e.g., white light vs. white light passed through a green filter) inevitably give different results. Most photometric instruments employ filters or monochrometers to select light of a narrow wavelength range; this certainly impacts the results.

The intensity of scattered light depends on a number of parameters including the size, shape, and concentration of the suspended particles,

the angle at which the scattering is observed, the wavelength of light in relation to the particle diameter, and the refractive indices of the particle and the solvent. The physics of this for spheres much smaller than the wavelength of light was described by Strutt (who later inherited the title of Lord Rayleigh) in 1871 (Strutt, 1871). Rayleigh later developed the form shown in Eqs. (1) and (2), which express the scattered light intensity at any angle relative to the incident beam, where I_θ is the intensity of the scattered light at angle θ, r is the particle radius, and λ' is the wavelength of light used (Thorne and Svendsen, 1962).

$$I_\theta = \left[\frac{3}{z^3}(\sin z - z\cos z)\right]^2 \tag{1}$$

$$z = \frac{4\pi r}{\lambda'}\sin\left(\frac{\theta}{2}\right) \tag{2}$$

The Mie theory (actually Mie's solution to Maxwell's equations for spheres) can be applied to spherical particles that are smaller than, similar in size to, and larger than the wavelength of light used (Mie, 1908). With particles much larger than the wavelength, the Mie theory can be simplified to the Fraunhofer theory. The mathematics of scattering is complicated for other than spherical shapes, and that is why the assumption that particles are spherical is often made.

Many anecdotal accounts state that turbidity measured with narrow-angle scattering is oversensitive to large particles, while that measured at 90° is oversensitive to small particles (Siebert, 2008). This can lead to ''invisible hazes'' that are perceptible visually but not with a turbidimeter, and vice versa. According to both the Rayleigh and Mie theories, light scattering intensity is very strongly influenced by the relationship of particle size to the wavelength of light used, with larger particles scattering light much more intensely than small particles at narrow angles (Gales, 2000; Siebert, 2008). With 90° scattering, small particles scatter substantially more intensely than larger ones. Light scattering results are highly influenced by the method of instrument calibration (Gales, 2000; Siebert, 2008). This is frequently done using formazin, which is very different in size and shape from yeast (often the largest particles encountered in a sample) or fine-particle haze (the smallest). Commercial culture yeasts are usually polyploid and larger than diploid strains, with oval shapes and mean diameters on the order of 10 μm. Fine particle hazes in filtered beverages are typically below 1 μm. Process samples or those that develop haze in package can have intermediate-sized particles. Calibration of a turbidimeter with particles of the same size as those measured should give correct results at any angle. However, the sizes of particles found in beverage samples are frequently not known in advance and may be bi- or

even trimodal. For example, both yeast and fine-particle haze are present in samples of fermented beverages during processing.

For a fixed number of colloidal-size spherical particles, 90° scattering intensity appears to be essentially proportional to the particle radius squared (Siebert, 2000); this was attributed to the particle cross-sectional area.

Temperature can affect haze in several ways. Lowering temperature can result in reduced solubility of marginally soluble substances and may lead to a higher concentration of particles. This is responsible for the phenomenon known as ''chill haze.'' Typically, warming a sample will dispel most of the turbidity provoked by chilling. On the other hand, elevated temperatures can speed interactions between substances that form insoluble particles, leading to more rapid haze development.

II. VISUAL PERCEPTION OF HAZE

As with instrumental turbidity measurements, the conditions under which humans observe light scattering also impact results. As expected, the geometry of the viewing system (the angle between the light beam and the observation) has a large influence (Gales, 2000). Visual observations are nearly always made with white light, but differences in the light source (e.g., incandescent, photoflood, or fluorescent lamps) presumably have some effect on the results.

It has been observed that the particle size and concentration as well as the illumination intensity, solution color, and viewing background influence visual perception of turbidity. Studies were carried out with a sensory panel using polymer spheres with a number of diameters in the range 0.15–10.3 μm, each suspended in different colored solutions. Thresholds were determined using the Ascending Method of Limits. When expressed as weight or number concentration, the thresholds varied greatly, but when expressed as turbidity measured at 90° they were quite similar, regardless of the particle size or solution color (Carrasco and Siebert, 1999; Fleet and Siebert, 2005). With bright illumination, thresholds ranged from 0.21 to 2.19 nephelometric turbidity units (NTU). Surprisingly, reducing illumination intensity led to generally lower thresholds (greater sensitivity) up to a point, but further reductions produced higher thresholds (Fleet and Siebert, 2005). It appears that lower illumination results in less reflection from the sample container, making it easier to perceive the scattered light. Using light-colored rather than black viewing backgrounds led to much higher thresholds (Fleet and Siebert, 2006). This appears to be due to the difficulty in seeing scattered white light against a light-colored background.

Suprathreshold particle suspensions were evaluated using Magnitude Estimation (ME) and Sensory Descriptive Analysis (Carrasco and Siebert,

1999). Equations predicting ME or instrumental turbidity as a function of sample characteristics were developed. Principal Components Analysis was applied to the Descriptive Analysis results and this indicated that panelists responded to only two fundamental properties, degree of cloudiness and homogeneity/nonhomogeneity. Only the larger particles studied caused much change on the second axis, leading to the conclusion that when modest numbers of large particles are present, samples take on a nonuniform appearance (Carrasco and Siebert, 1999; Siebert, 2008).

III. CAUSES OF HAZES IN BEVERAGES

Hazes in clear beverages (beer, wine, clear fruit juices, tea, etc.) can be caused by a variety of phenomena. Processing problems can lead to particles from filter media (such as diatomaceous earth) or adsorbents. These are not normal occurrences and can usually be readily discovered and their cause addressed.

Grape juice and wine can contain tartrate particles that arise from tartaric acid in grapes forming salts with various cations. Often, this leads to regular crystals. Cool storage and additions of seed crystals or salts facilitates settling out of tartrate precipitates during processing (Jackson, 1994).

Grains typically contain oxalates. As a result, grain-based beverages (such as beer) can develop oxalate crystals (mainly calcium salts). The standard practice of adding gypsum ($CaSO_4$) to brewing water (for a number of reasons) leads to crystallization and precipitation of calcium oxalate during processing (Rehberger and Luther, 1999).

Fragments of plant raw materials (e.g., grape skins or fruit pulp) can in some cases pass through a process and enter the final product. If this occurs due to a processing problem, it is generally transitory and can be addressed by refiltering the product.

Microorganisms (yeast or bacteria), which may be either culture organisms added intentionally or contaminants, if not removed by filtration or sedimentation can lead to turbidity. The organisms themselves, in sufficient numbers, scatter light. The growth of some organisms alters product chemistry and may cause formation of unsightly hazes, ropes, or strings.

In some cases, microbial cell fragments may arise and can be particularly problematic. For example, the disc centrifuges often used to remove yeast after brewery fermentations are known to produce shearing forces that break off yeast cell wall fragments (Siebert *et al.*, 1987). Agitation of yeast by other means is also problematic (Lewis and Poerwantaro, 1991; Stoupis *et al.*, 2003). In beer, the resulting particles resist sedimentation and impair filtration.

Sucrose syrups from either beet or cane origin are used in some formulated products or added to coffee beverages at the point of sale

(in the form of flavored sugar syrups). Occasionally, these products develop flocs (large gauzy-appearing structures that float in the product). A number of causes have been associated with these, but most authors have attributed this to associations between positively charged proteins and negatively charged polysaccharides that form either under acidic conditions or in products containing ethanol (Clarke *et al.*, 1978; Foong *et al.*, 2002; Morel du Boil, 1997).

Polysaccharide–protein interaction has also been reported in apple juice, between arabinogalactan and protein (Brillouet *et al.*, 1996).

A number of polysaccharides have been associated with beverage hazes or flocs. These include arabinans in red wine (Belleville *et al.*, 1993), starch and mannan in wheat beers (Delvaux *et al.*, 2000), beta-glucans in beer (Jackson and Bamforth, 1983), and retrograded starch in apple juice (Beveridge, 1997).

Polyphenols have been implicated in hazes of many beverages including white wine (Somers and Ziemelis, 1985), apple juice (Beveridge, 1997; Van Buren and Way, 1978), and beer (Gramshaw, 1969; Steiner and Stocker, 1969).

Proteins have been associated with hazes in beer (Asano *et al.*, 1982; de Clerck, 1969), red and white wine (Dizy and Bisson, 1999; Hsu *et al.*, 1989; Pocock and Rankine, 1973; Sitters and Rankine, 1980; Waters *et al.*, 1995), apple juice (Beveridge *et al.*, 1998; Hsu *et al.*, 1989), grape juice (Hsu and Heatherbell, 1987; Hsu *et al.*, 1987), pear juice (Hsu *et al.*, 1990), and kiwifruit juice (Wilson and Burns, 1983).

While most of the previously mentioned causes of haze can create product defects, they do not normally occur if a process is carried out properly. The most frequent cause of haze in clear beverages is protein–polyphenol interaction (Bamforth, 1999; Siebert, 1999). This occurs normally and even when a beverage is properly stabilized, protein–polyphenol haze usually develops eventually. The objective is to delay its onset so that any haze produced is imperceptible until after a product's intended shelf life.

IV. DIAGNOSING HAZE PROBLEMS

A. Microscopy

Light microscopy can be used to detect particles with regular shapes (e.g., crystals) and microbes like yeast and bacterial cells (Glenister, 1971). Microscopy can also detect some irregular particles such as diatomaceous earth or adsorbents (Glenister, 1974). It is much less informative with amorphous particle hazes. The use of specific stains can, however, provide useful information. An excellent book by Glenister (unfortunately no

longer in print) describes stains and microscopic techniques that are useful for characterizing beer hazes of various origins (Glenister, 1975).

A fluorescent tag (fluorescein isothiocyanate) attached to the lectin Concanavalin A is useful in staining yeast cell wall fragments (Siebert *et al.*, 1981). Concanavalin A specifically binds to mannan, which is prominent in yeast cell walls.

B. Chemical analysis

Chemical analysis of haze materials isolated from a beverage must be interpreted with caution because composition is often not well-related to cause. For example, beer hazes typically contain a high proportion of carbohydrate, with a modest amount of protein, and little polyphenol (Belleau and Dadic, 1981; Siebert *et al.*, 1981). In order to prevent or delay haze formation, however, it is not necessary or helpful to remove carbohydrate. Reducing the amount of either protein or polyphenol typically has that effect. As a result, it appears that the large amount of carbohydrate found in the haze was coagulated with or adhered in some way to the protein–polyphenol haze backbone.

C. Enzyme treatment

Treatment with specific enzymes has sometimes been used to diagnose haze problems (Siebert *et al.*, 1981). Conclusions of the effects must be tempered by considering that enzyme preparations may have small amounts of unspecified enzyme contaminants.

V. PROTEIN–POLYPHENOL HAZE

A. Nature of haze-active (HA) protein

Only proteins that contain proline bind polyphenols. Asano *et al*. (1982) demonstrated that the haze-forming activity of a protein is roughly proportional to the mole percentage of proline it contains (see Fig. 2.3). DNA has codes for exactly 20 amino acids. If each of these were equally present in a protein, there would be 5 mol% of each one. In fact, most proteins have much less proline than this. There are a few exceptions. Casein has about 8 mol% proline and the grain prolamins (proline-rich, alcohol-soluble proteins) are even higher. Hordein, the barley prolamin, contains about 20 mol% proline. As a result, it readily forms haze with polyphenols and is the main beer haze-active (HA) protein. Hordein contains even more glutamine (Q) than proline (P), and often these amino acids are adjacent in the protein (see Fig. 2.4). In fact, the sequence P-Q-Q-P occurs

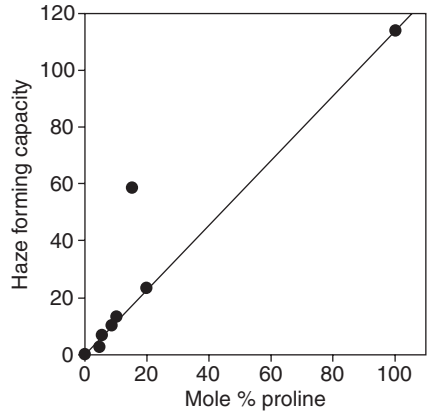

FIGURE 2.3 The relationship between the proline content of a polypeptide and its haze-forming activity with catechin based on data from Asano *et al.* (1982).

Q Q Q P F P Q Q P I P Q Q P Q P Y P Q-
Q P Q P Y P Q Q P F P P Q Q P F P Q Q-
P V P Q Q P Q P Y P Q Q P F P P Q Q P-
F P Q Q P P F W Q Q K P F P Q Q P P F-
G L Q Q P I L S Q Q Q PC T P Q Q T P L-
P Q -

FIGURE 2.4 Partial amino acid sequence of barley hordein (source of haze-active protein in beer); P = proline and Q = glutamine.

repeatedly. The adjacent location of proline and glutamine appears to provide unusually strong polyphenol binding. Some relatively proline-rich proteins (PRPs) have been found in apple juice (5 mol%) (Wu and Siebert, 2002) and grape seeds (9.5 mol% proline) (Wu and Lu, 2004).

Even higher proline contents have been reported in salivary PRPs; these can contain 40–45 mol% proline and also have a substantial amount of glutamine. This protein binds ingested polyphenols, which precipitates the PRPs and removes the lubrication these normally provide. The result is the sensation of astringency (Green, 1993; Haslam and Lilley, 1988).

The number of different amino acids actually found in proteins is greater than the 20 in the DNA code. This is because some of them are produced by posttranslational modification. Hydroxyproline, for example, is not coded in DNA. Proline is inserted in the peptide chain and the hydroxy group is later added to the peptidically liked proline to form hydroxyproline. Although hydroxyproline is found in some proteins that are known to be haze active, such as gelatin, polyhydroxyproline (the synthetic homopolymer of hydroxyproline) forms no haze with

polyphenols (Siebert *et al.*, 1996c). The polyphenol binding of gelatin (and similar proteins) appears to be entirely due to the proline they contain. Free proline does not form haze with polyphenols (Siebert *et al.*, 1996a); it appears that only peptidically linked proline can do that.

Proline differs from all the other coded amino acids in having a secondary amine group that participates in a peptide bond (see Figs. 2.5 and 2.6). Because of its ring system, proline is more rigid than most amino acids and cannot form an alpha-helix structure. As a result, it leads to a more open, less compact protein, which provides better access to polyphenol-binding sites than a more compact protein structure (Hagerman and Butler, 1981). Only one other amino acid (but not one of the coded 20), when peptidically linked, has been shown to bind polyphenols. This is sarcosine (*N*-methyl glycine), which has a secondary amine like proline (see Fig. 2.7), but lacks a ring system. Polysarcosine has been shown to bind to polyphenols and produce haze (Hagerman and Butler, 1981; Siebert and Lynn, 2008). So it appears that the essential

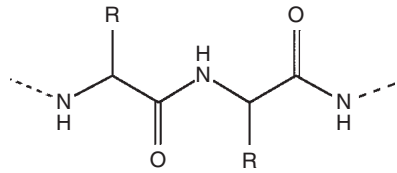

FIGURE 2.5 Segment of a peptide composed of alpha-amino acids.

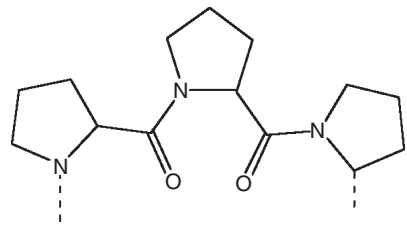

FIGURE 2.6 Segment of a peptide composed of proline.

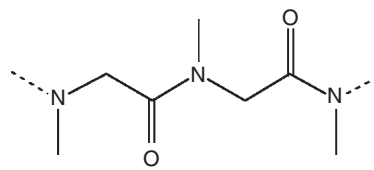

FIGURE 2.7 Segment of a peptide composed of sarcosine.

feature for polyphenol binding is a peptidically linked secondary amine rather than a nitrogen-containing ring system.

B. Nature of HA polyphenols

The polyphenol molecular features that lead to attachment to proteins are generally understood. Simple phenols, that is, phenols containing a single hydroxy group on an aromatic ring, essentially do not bind to proteins (Eastmond and Gardner, 1974). In experiments in which various polyphenols were combined with bovine serum albumin, the energy released upon binding was observed (McManus *et al.*, 1985). The binding energy was weak with *m*-diphenol, moderate with *o*-diphenol, and strong with the vicinal triphenol. So, two or more hydroxy groups on an aromatic ring are required and the binding is stronger when they are adjacent and when there are more hydroxy groups.

One aromatic ring with two or more hydroxy groups constitutes one binding moiety. In order to cross-link two protein molecules, a polyphenol needs to have two such binding groups. ''Single-ended'' polyphenols (with only one binding moiety) can bind to proteins and have been shown to compete with HA polyphenols for binding sites in proteins under some conditions, inhibiting haze formation (Siebert and Lynn, 1998).

The polyphenols in beer, fruit juices, and tea are typically members of the flavan-3-ols (see Fig. 2.8) and the proanthocyanidins constructed from them.

Each of the flavan-3-ols has two asymmetric centers, at positions 2 and 3 (on the C ring). The more naturally prominent members of the flavan-3-ols are (+)-catechin and (−)-epicatechin (see Fig. 2.9). These two molecules are remarkably similar, differing only in the orientation of the hydroxy group at position 3; both are expected to have one moderately strong binding end (the B ring) and one weak binding end (the A ring).

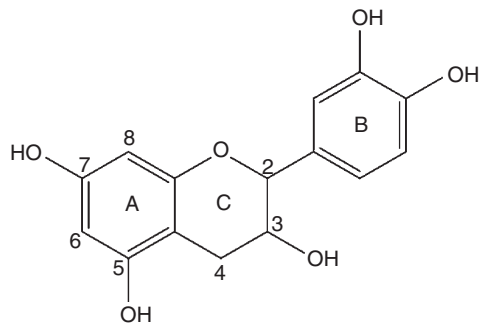

FIGURE 2.8 The basic flavan-3-ol structure.

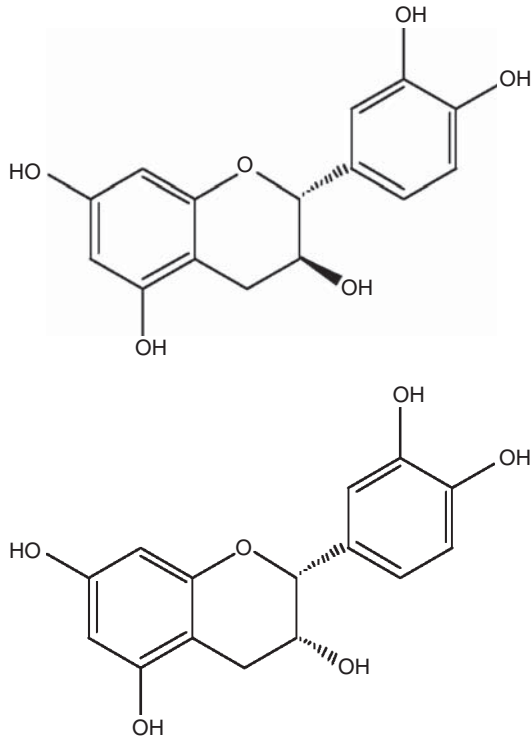

FIGURE 2.9 The structures of (+)-catechin (top) and (−)-epicatechin (bottom).

However, one study showed substantial differences between them, with (+)-catechin producing more haze than (−)-epicatechin when combined with polyproline (the synthetic homopolymer of proline) at 25 °C (Siebert and Lynn, 1998). Also prominent in some beverages are gallocatechin and epigallocatechin; in these compounds, an additional hydroxy group is located on the B-ring vicinal to the two already there (see Fig. 2.10). These molecules are expected to have one strongly binding end (the B ring with three vicinal hydroxy groups) and one weakly binding end.

Proanthocyanidins are formed from flavan-3-ol ''building blocks''; although not truly polymers, it is convenient to refer to these as dimers, trimers, etc., indicating the number of flavan-3-ol ''monomers'' they contain. As the size and complexity of proanthocyanidins increase, their haze-forming activity increases (Asano *et al.*, 1984; Hagerman and Butler, 1981; Mulkay and Jerumanis, 1983) and their solubility and likelihood of surviving processing decrease (Asano *et al.*, 1984). As a result, beer and clear fruit juices have significant amounts of monomers and dimers but little of trimers (and higher). The prominent proanthocyanidin

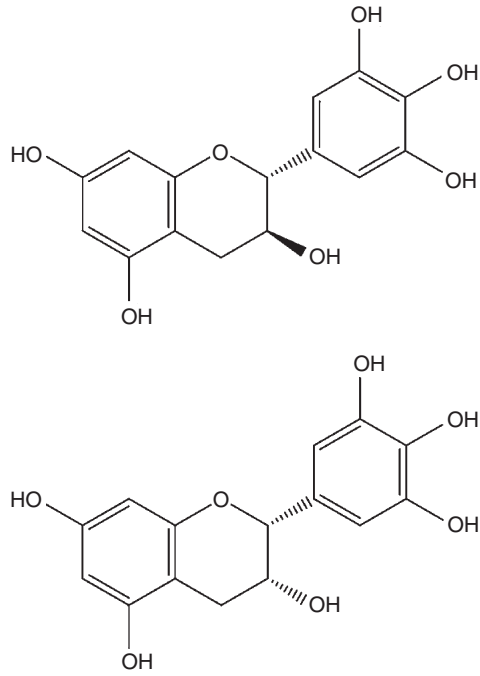

FIGURE 2.10 The structures of gallocatechin (top) and epigallocatechin (bottom).

''dimers'' found in most fruit juices and beer are formed of two monomers connected from position 8 (on the A ring) of one monomer to position 4 (on the C ring) of the other. The prominent dimer in grape juice and wine is procyanidin B1 (catechin–epicatechin; see Fig. 2.11). In apple juice, procyanidin B2 (two epicatechins) predominates. The prominent dimers in beer are procyanidin B3 (two catechins joined together) and prodelphinidin B3 (a catechin and a gallocatechin joined together; see Fig. 2.11). Because of the additional hydroxy group in prodelphinidin B3, this compound is expected to be somewhat more haze active than procyanidin B3, and that has been shown to be the case (Mulkay and Jerumanis, 1983).

C. Nature of protein–polyphenol interaction

The basic mechanism is that a polyphenol molecule with at least two binding sites attaches to two proteins and bridges them together. Additional polyphenol molecules attach this structure to additional protein molecules and eventually the complex grows so large that it is no longer soluble. At this point, it becomes a colloidal particle and scatters light. The

particle may continue to grow until it is so large that Brownian motion can no longer suspend it; at that point it starts to sediment.

Because warming can often disperse protein–polyphenol hazes, it is clear that covalent bonding is not involved in their formation. Asano *et al.* demonstrated that protein–polyphenol haze formation is inhibited by the nonpolar solvent dioxane and the hydrogen bond acceptor dimethylformamide (DMF), but not by a solution of sodium chloride (Asano *et al.*, 1982).

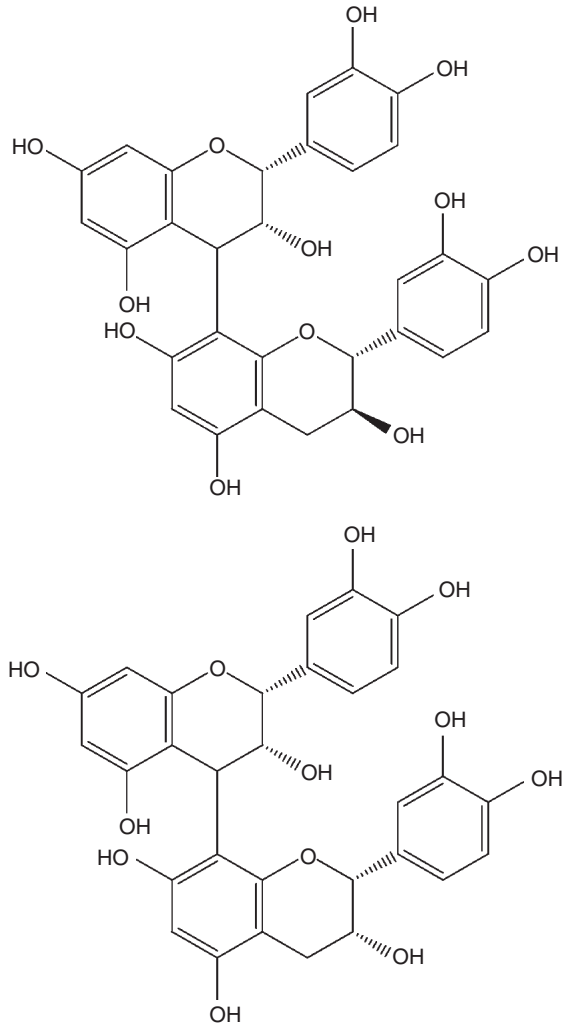

FIGURE 2.11 Continued

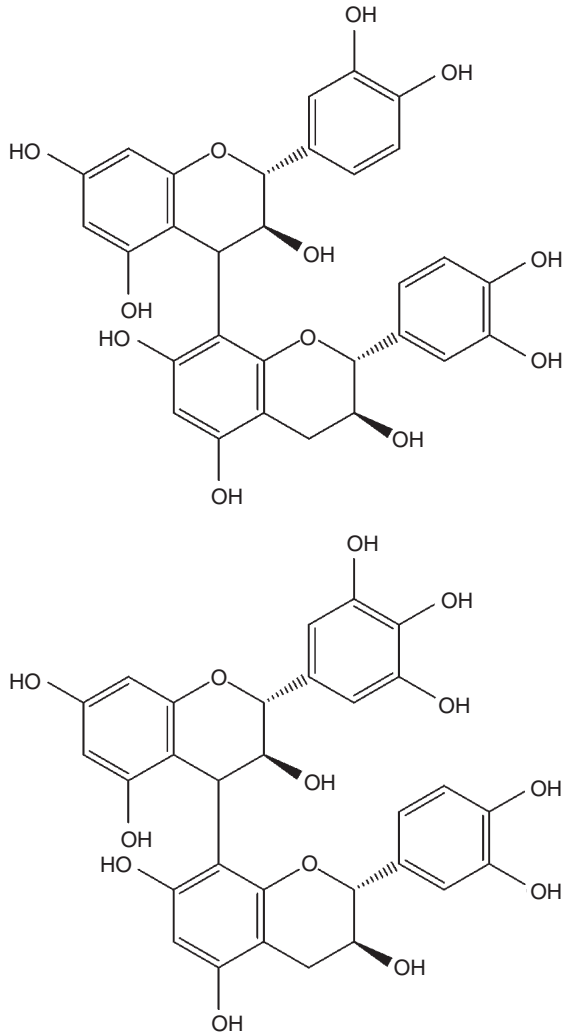

FIGURE 2.11 The structures of the procyanidin "dimers" prominent in (from top to bottom) grape juice (procyanidin B1), apple juice (procyanidin B2), and beer (procyanidin B3 and prodelphinidin B3).

They concluded that the interaction involves hydrophobic and hydrogen bonding, but not ionic bonding. Siebert *et al.* demonstrated that preformed haze could be dispelled by adding dioxane or DMF, but adding sodium chloride solution actually increased the haze (Siebert and Lynn, 2008). Increased binding strength with increased ionic strength is a known effect

of hydrophobic bonding (Oh *et al.*, 1980). Hagerman and coworkers carried out a study in which two quite different types of polyphenols were each combined with bovine serum albumin; in one of these cases, the interaction was attributed to hydrogen bonding (Hagerman *et al.*, 1998). Bianco *et al.* (1997) used NMR to measure the energy involved in polyphenol interactions with caffeine, a surrogate compound for peptidically linked proline. Based on the binding energy observed, the authors suggested that π-bonding occurs (in the case of peptidically linked proline, this would be manifested as stacking of the flat aromatic ring of the polyphenol with the relatively flat proline ring).

A response surface model of the effects of HA protein concentration (gliadin, the wheat prolamin), HA polyphenol concentration (tannic acid, TA), alcohol, and pH on the amount of haze formed was constructed using a buffer model system (Siebert *et al.*, 1996a). Figure 2.12 shows the effects of protein and polyphenol on haze predicted by the model at fixed levels of pH and alcohol. The model indicates that as protein increases at fixed polyphenol levels, the haze rises to a point and then starts to decline. Similarly, when polyphenol increases at a fixed protein level, the haze increases to a maximum and then declines.

A conceptual model that accounted for this behavior was proposed (see Fig. 2.13) (Siebert *et al.*, 1996c). It is assumed that there are a fixed number of polyphenol-binding sites in an HA protein, presumably related to the proline content, and an HA polyphenol molecule can attach to at the most two proteins (this is likely for steric reasons, even if a

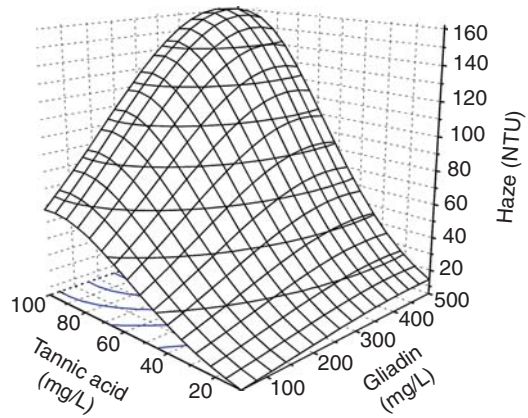

FIGURE 2.12 Response surface model predictions of the effects of HA protein (gliadin) and HA polyphenol (TA) on the haze intensity in a model system at fixed levels of pH and alcohol. Reprinted with permission from Siebert *et al.* (1996a). Copyright 1996 American Chemical Society.

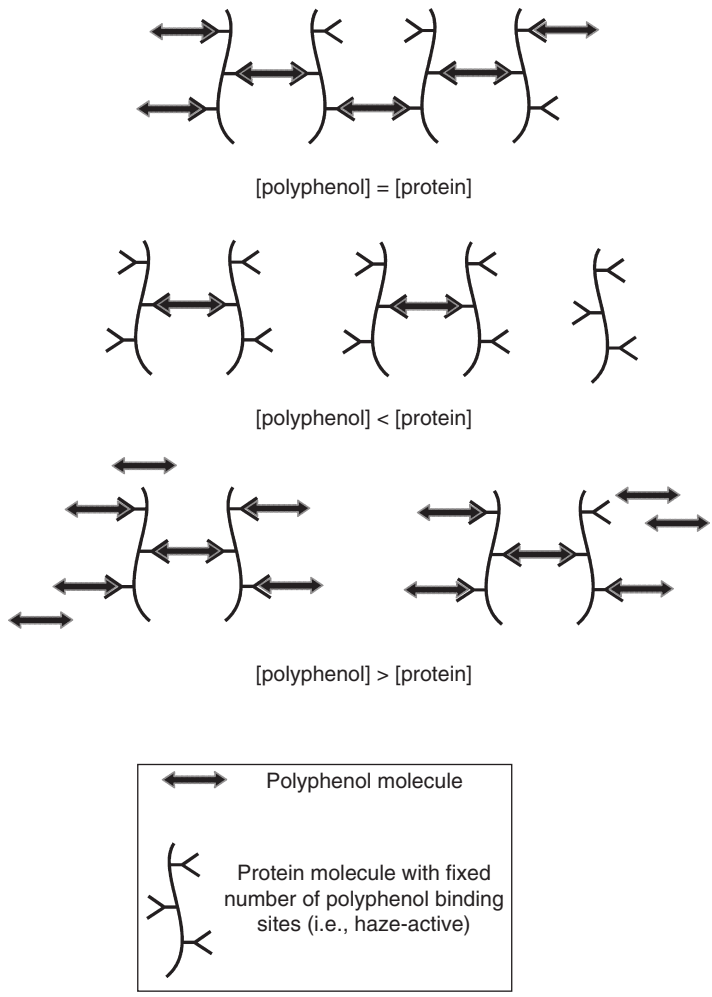

FIGURE 2.13 Concept of influence of protein–polyphenol proportion on particle size and haze. Reprinted with permission from Siebert *et al.* (1996c). Copyright 1996 American Chemical Society.

polyphenol has more than two parts of the molecule that can bind to protein). In some cases, a polyphenol may form an intramolecular bridge between two parts of a protein molecule, but this would not lead to haze. When there are similar numbers of polyphenol-binding sites in proteins and polyphenol-binding ends present in a system, a large network will form, corresponding to large particles and a lot of light scattering. When there is a high proportion of protein to polyphenol, polyphenols will have

no difficulty finding sites in proteins to attach to and can readily join two protein molecules together. However, there will be few additional polyphenols available to join these protein ''dimers'' or ''sandwiches'' together. This will produce small particles with relatively little light scattering. With a polyphenol-rich system, most of the attachment sites in the proteins will be occupied by one end of an HA polyphenol molecule; however, few sites in other proteins will be available for the other end of the polyphenol to attach to. Small particles will again result, with little light scattering.

This conceptual model was later verified with particle size analysis (Siebert and Lynn, 2000) (see Fig. 2.14). When each of the several concentrations of gliadin was combined with a fixed amount of TA in a model system, the particle sizes changed, and the largest particles were seen with a gliadin-to-TA concentration ratio (by weight) of 5:1, with smaller particles at higher and lower ratios. A similar pattern was seen when a fixed amount of gliadin was combined with various levels of TA. Once again, the largest particles were seen with intermediate ratios. The changes were striking in that they were not gradual shifts of a monomodal distribution. Rather, particles of one or two discrete sizes were present, depending on the protein-to-polyphenol ratio.

D. Effects of conditions on particle size and haze intensity

A more detailed study was carried out with many more levels of protein and polyphenol than were used to construct the initial response surface model (Siebert and Lynn, 2000). The results (see Fig. 2.15) indicated that

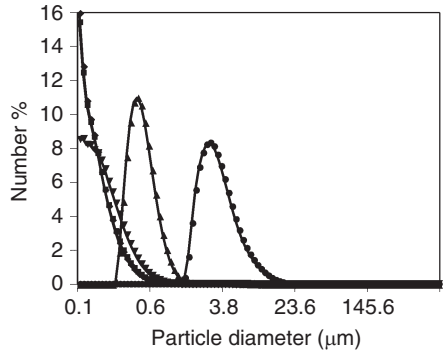

FIGURE 2.14 Particle sizes measured with 100 (■), 200 (●), 300 (▲), 400 (♦), and 500 (▼) mg/L protein (gliadin) concentrations added to 40 mg/L TA. Reprinted with permission from Siebert and Lynn (2000). Copyright 2000 American Society of Brewing Chemists.

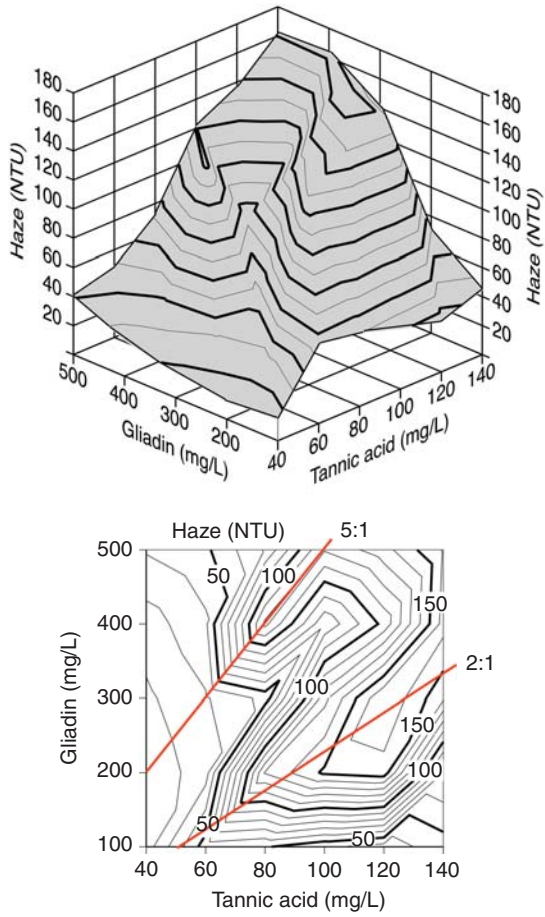

FIGURE 2.15 Response surface predictions from haze intensity observations made with 30 combinations of gliadin and TA at pH 4.5. Reprinted with permission from Siebert and Lynn (2000). Copyright 2000 American Society of Brewing Chemists.

the basic concept of the relationship of protein-to-polyphenol ratio to haze intensity was correct, but that there was fine structure. Ridges of greater haze intensity were seen at 2:1 and 5:1 concentration weight ratios of gliadin to TA; these correspond to TA:gliadin molar ratios of 15:1 and 6:1, respectively. These ratios coincided with the larger size particles observed with particle size analysis. This indicates remarkably quantized behavior. With 30 different protein:polyphenol ratios, particles of only a few sizes were seen and changes were in the proportions of particles of different size.

E. Particle size effects on sedimentation and filtration operations

The dramatic changes in haze particle size seen with alterations in protein-to-polyphenol ratio in a model system, would, if this also occurs in real beverages, have profound effects on both sedimentation (e.g., cold maturation in a tank or centrifugation) and filtration operations.

F. The effects of pH and alcohol on haze

The previously described response surface model was used to predict the effects of ethanol and pH on haze intensity at fixed levels of protein and polyphenol (Siebert *et al.*, 1996a) (see Fig. 2.16). Changing the ethanol concentration at the pH of grape juice and wine (near 3) would appear to have little effect on haze intensity. Slightly above pH 4, increasing ethanol first decreased haze intensity and then increased it slightly. This is within the pH range of apple juice and beer. This effect was attributed to the known suppression of protein–polyphenol interaction caused by nonpolar solvents. Ethanol, which is semipolar, has been shown to decrease protein–polyphenol precipitation (Hagerman *et al.*, 1998). At higher ethanol levels, haze tends to increase. This could be due to the well-known effect of ethanol in reducing protein solubility by competing for water.

The effect of pH on haze intensity was striking (Siebert *et al.*, 1996a) (see Fig. 2.16). When the pH rose from near 3 to slightly above 4, the haze intensity increased by a factor of 7 with the same amounts of protein and polyphenol. At higher pH, the haze intensity declined. While proteins

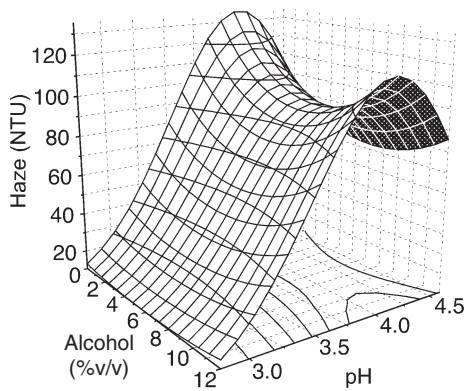

FIGURE 2.16 Response surface model predictions of the effects of pH and ethanol on haze intensity in a model system at fixed levels of protein and polyphenol. Reprinted with permission from Siebert *et al.* (1996a). Copyright 1996 American Chemical Society.

often have least solubility near their isoelectric points, the calculated pI of gliadin is near 8 (Siebert, 2006) and the pKa of polyphenols is also high, typically of the order 9 or 10. The interaction of saliva protein and polyphenol also demonstrates a sharp maximum slightly above pH 4 (Siebert and Chassy, 2003). So presumably the behavior has something fundamental to do with the nature of the protein–polyphenol interaction.

G. Time course of haze formation

The time course of protein–polyphenol haze development in many packaged clear beverages has a two-phase pattern (see, for example, Fig. 2.17). At first no observable change occurs for some time. After this, haze formation begins and follows an essentially linear development rate. This phenomenon has been reported in beer (McMurrough *et al.*, 1992) as well as apple juice, grape juice, and cranberry juice cocktail (Siebert, 1999, 2006).

While it is possible that soluble protein–polyphenol complexes could be formed during the initial stage and that these only grow large enough to become insoluble particles (which scatter light) during the second phase, the pattern has typically been attributed to changes in the polyphenol component that affect the development of the protein–polyphenol haze (see Fig. 2.18). Various authors have proposed that oxidation or polymerization of polyphenols enhances their combination with proteins and thus their haze-forming activity. The evidence here, however, is somewhat contradictory. Oxygen 18 added to beer reportedly ended up in the polyphenol fraction (Owades and Jakovac, 1966); this indicates that

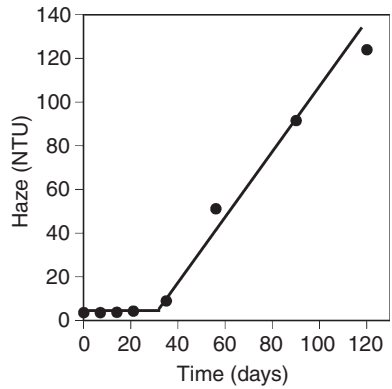

FIGURE 2.17 The haze development pattern in cranberry juice cocktail stored at 37 °C. Reprinted with permission from Siebert (1999). Copyright 1999 American Chemical Society.

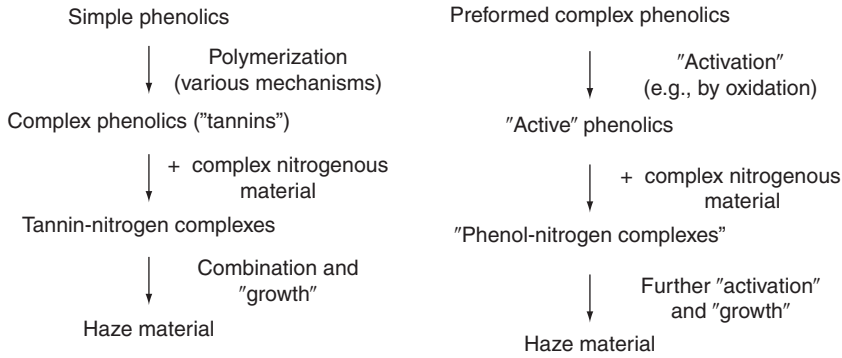

FIGURE 2.18 Possible mechanisms of polyphenol polymerization or activation leading to haze development based on concepts from Gardner and McGuinness (1977).

polyphenol oxidation is occurring. It has been reported, however, that dimeric proanthocyanidins depolymerized rather than polymerized in wort and beer (Derdelinckx and Jerumanis, 1987). Radiolabeled epicatechin did not polymerize in beer to form dimers or trimers; however, labeled dimeric catechin was readily incorporated into beer haze (McGuinness *et al.*, 1975). Some authors who looked for, but failed to find evidence of polymerization, attributed it instead to ''activation'' of some sort (Gardner and McGuinness, 1977). McMurrough *et al.* (1992) showed that reducing polyphenol concentration by treatment with polyvinylpolypyrrolidone (PVPP), a well-known polyphenol adsorbent (see later), led to a longer time before the start of haze formation and to a lower haze development rate once the process began. It is quite clear that some change in the polyphenols occurs that leads to the increase in the rate of haze formation.

H. Beverage differences

In general, beer tends to be rich in HA protein and poor in HA polyphenol, while apple juice tends to have the opposite pattern (Siebert *et al.*, 1996a). Grape juice is fairly low in HA protein and variable in HA polyphenol.

White wines were uniformly low in HA protein, while red wines were quite variable (Siebert *et al.*, 1996b). *Vitis vinifera* white wines had very low levels of HA polyphenols, while *Vitis labrusca* white wines had higher and *vinifera–labrusca* hybrids had intermediate levels (Siebert *et al.*, 1996b). All red wines had high levels of HA polyphenols, and most had low levels of HA protein; the two exceptions were both hybrids.

VI. ANALYSES RELATED TO PROTEIN–POLYPHENOL HAZE FORMATION

A. Predictive haze tests

Most producers of clear beverages employ forcing tests in which packages of the beverage are stored at elevated temperature for some time, often with agitation or periods of chilling (Bamforth, 1999; Berg, 1991). After the storage period, samples are withdrawn and their hazes are measured, either as is or after chilling. The test conditions are usually designed to produce results similar to those expected after much longer periods in the trade. A more rapid predictive test for beer is based on the addition of alcohol followed by chilling and then measuring haze (Chapon, 1973); this has generated useful results (McCarthy *et al.*, 2005; Moll *et al.*, 1976).

B. HA protein

A number of approaches have been used to determine the amount of HA protein in a sample. The most successful of these is based on adding a fixed amount of TA to a sample (Thompson and Forward, 1969); after incubation, the turbidity is measured and the increase in turbidity observed is presumed to be proportional to the amount of HA protein in the sample. This method has the advantage that only substances able to form haze with polyphenols respond. The saturated ammonium sulfate precipitation limit (SAPL) method has also been widely used, but is far inferior in providing useful information (Berg *et al.*, 2007; Siebert *et al.*, 2005).

C. HA polyphenol

A variation on the Thompson and Forward method was developed in which a HA peptide or peptide-like material (e.g., gelatin, gliadin, polyproline, or soluble polyvinylpyrrolidone) is added to a sample to induce haze in proportion to the amount of HA polyphenol it contains (Siebert *et al.*, 1996a). This gives little response in beer, which contains very little HA polyphenol, and causes much larger haze increases in fruit juices and wine.

Both of the tests based on provoking haze with a single addition have the disadvantage that the endogenous amounts of the complementary material influence results. For example, when adding TA to induce haze with HA protein, the amount of endogenous HA polyphenol will influence results. And similarly, when adding a protein-like material to provoke a response with HA polyphenols, the endogenous HA protein will affect results. The effects are small when measuring high concentrations of one species in the presence of small amounts of the other. In most beers, for example, which are high in HA protein and low in HA polyphenol, the

results of a single-addition method are fairly accurate for HA protein and inaccurate for HA polyphenol. Titration provides an approach to measurement that is less affected by endogenous amounts of HA species. Methods that carry out titration manually or automatically have been widely used. The Tannometer instrument developed by Chapon (1993), which carries out automated turbidimetric titrations, has been widely used. The P-T Standard instrument of Schneider has been applied to beer (Schneider and Raske, 1997).

VII. PREVENTING OR DELAYING HAZE DEVELOPMENT

It is normal to employ beverage-processing steps that lead to a reduction in the likelihood of haze formation, or at least a delay the onset of haze development beyond the intended shelf life of a product.

A. Cold maturation

A traditional approach in many cases is prolonged cold storage followed by a sharp filtration, also carried out cold. Cold storage encourages formation and settling out of insoluble complexes. Reduced temperature decreases the solubility of some potential haze material and also reduces the energy from ambient heat that keeps particles suspended. As a result, some of the haze material is precipitated and left on the floor of the storage tank or taken out by the filter.

The effect of cold maturation can be enhanced by the use of fining agents. These facilitate the formation of haze and precipitation of substances that, if not removed, could later give rise to haze. A number of substances have been used as fining agents, including the HA proteins gelatin and isinglass (Harding, 1979; Hickman *et al.*, 2000), HA polyphenols such as TA, and some fine particles such as bentonite (Duncan, 1992) and colloidal silica (Hahn and Possmann, 1977) or silica sol (Goertges and Haubrich, 1992). Gelatin is often used to fine fruit juices (Bannach, 1984) and wine (Baldwin, 1992). Also often used for fruit juices and wine are bentonite or silica. Mixtures of two or more fining agents are frequently used. Gelatin, isinglass, TA, and colloidal silica are used in beer fining.

Both isinglass and gelatin are derived from collagen proteins. Gelatin is largely from bovine or porcine skins, while isinglass is from the swim bladders of certain tropical fishes. Collagen is rich in both proline and hydroxyproline. Gelatin is generally thought to contain about 12% proline and a similar amount of hydroxyproline. While hydroxyproline does not participate in binding polyphenols (see earlier), it does facilitate a very open molecular structure, and this presumably aids access to the polyphenol-binding sites in the protein.

TA is not a single well-defined compound, but rather a family of related compounds with some common structural features (Haslam, 1974). All TAs have some number of gallic acid (3,4,5-trihydroxybenzoic acid) moieties attached to a glucose molecule by ester linkages. Some of the galloyl groups can be attached to other galloyl groups (also by ester linkage) rather than the glucose. The structure of a particular TA (number and location of galloyl groups) depends on its natural source. Because galloyl groups have three vicinal hydroxy groups on an aromatic ring, they bind very strongly to HA proteins. It was shown that the strength of TA binding to proteins is a function of the number of terminal galloyl groups; that is, those with all three hydroxy groups available (Siebert, 1999). Apparently interior galloyls (those with one of the hydroxy groups occupied in an ester linkage) are not available to bind to proteins, probably for steric reasons. TA is a very strong HA polyphenol. This has benefits for use both as a reagent to measure HA protein and as a fining agent in beverage stabilization.

B. Ultrafiltration

The removal of macromolecules by ultrafiltration has often been used in the production of clear fruit juices and wine (Girard and Fukumoto, 2000). This treatment removes both proteins and polysaccharides. Ultrafiltration through a 10,000 Da cut-off membrane has been shown to stabilize wines against haze formation (Flores, 1990).

Because proteins are involved in beer (Evans and Sheehan, 2002) and champagne foams (Senée *et al.*, 1999), and these are desirable properties, ultrafiltration is not a suitable treatment for these products. Adsorbents that indiscriminately remove protein are unsuitable for the same reason.

C. Adsorbents

Adsorbents that remove proteins or polyphenols are used to treat a number of beverages to delay the onset of haze formation. Protein adsorbents include bentonite and silica. Bentonite removes protein nonspecifically (see Fig. 2.19) and so is unsuitable for stabilizing beverages where foam is desirable (beer and champagne). Silica, on the other hand, has remarkable specificity for HA proteins while virtually sparing foam-active proteins in beer (Siebert and Lynn, 1997b) (see Fig. 2.20). Silica removes approximately 80% of the HA protein from unstabilized beer, while leaving foam-active protein nearly untouched at commercial treatment levels.

This was shown to occur because silica binds to the same features in polypeptides that polyphenols do (peptidically linked proline; Siebert and Lynn, 1997b) (see the concept in Fig. 2.21). In contrast, in unstabilized apple juice, silica removes only on the order of 20% of the HA protein

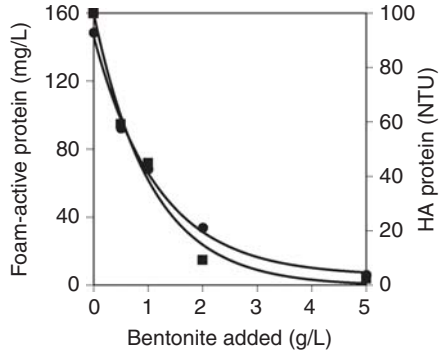

FIGURE 2.19 The effects on foam active (■) and HA (●) protein of treating unstabilized beer with bentonite. Reprinted with permission from Siebert and Lynn (1997b). Copyright 1997 American Society of Brewing Chemists.

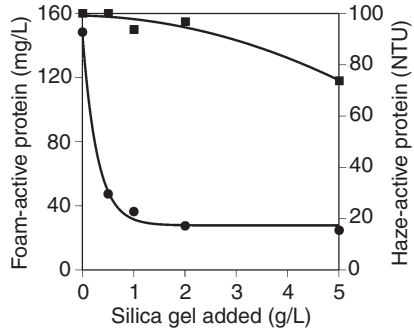

FIGURE 2.20 The effects on foam active (■) and HA (●) protein of treating unstabilized beer with silica hydrogel. Reprinted with permission from Siebert and Lynn (1997b). Copyright 1997 American Society of Brewing Chemists.

even with very high treatment levels (Siebert and Lynn, 1997a). This difference is accounted for in Fig. 2.22; silica has limited effectiveness in polyphenol-rich beverages (especially fruit juices), where most of the polyphenol-binding sites in proteins are occupied by polyphenols, leaving few for silica to attach to (Siebert and Lynn, 1997a).

Polyphenol adsorbents are mainly polyamides (Dadic, 1973). At one time various nylons were used, but PVPP is most frequently used today (McMurrough *et al.*, 1997). The structure of PVPP (see Fig. 2.23) resembles that of polyproline (Fig. 2.6); both have five-membered, saturated, nitrogen-containing rings and amide bonds.

As a result, it appears likely that PVPP binds to polyphenols in a similar manner to that of HA proteins. As with gliadin–TA binding,

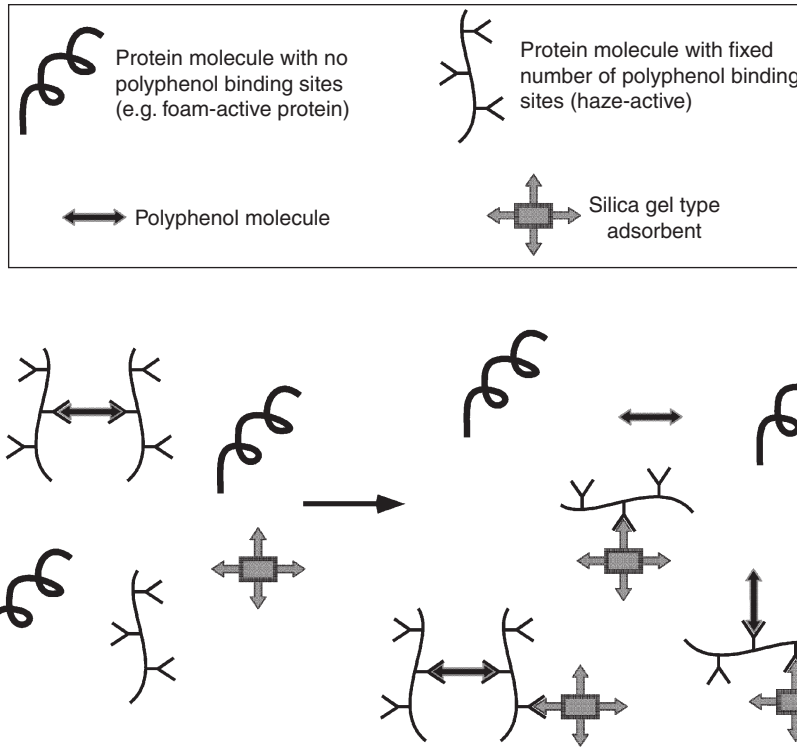

FIGURE 2.21 Concept of silica binding to HA protein in beer. Reprinted with permission from Siebert and Lynn (1997b). Copyright 1997 American Society of Brewing Chemists.

DMF and dioxane both impede polyphenol binding to PVPP, while NaCl enhances it (Siebert and Lynn, 2008). So, both hydrogen and hydrophobic bonding appear to be involved, but not ionic bonding. While PVPP works even in protein-rich beverages, it is far less effective than in polyphenol-rich beverages. PVPP removed at most half of the HA polyphenol from unstabilized beer (Siebert and Lynn, 1997b), but at high doses it took out 100% of the HA polyphenol from unstabilized apple juice (Siebert and Lynn, 1997a). This appears to be because much of the HA polyphenol in protein-rich beverages is attached to proteins at both ends and inaccessible to PVPP (see Fig. 2.24). In order for PVPP to bind to the polyphenol, the complex with protein must come apart, at least at one end. If PVPP binds to one end of a polyphenol molecule that is attached to protein at the other end, then the PVPP treatment could remove some HA protein and there is some evidence that this occurs (Siebert and Lynn, 1997b). In high-polyphenol, low-protein beverages, the vast majority of the HA polyphenol is readily accessible (see Fig. 2.25).

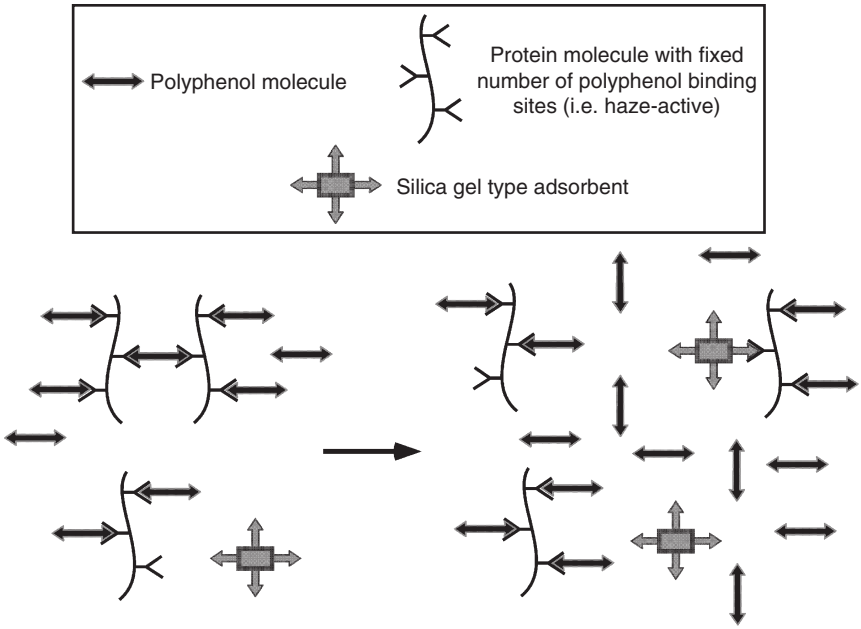

FIGURE 2.22 Concept of silica action in a polyphenol-rich beverage. Reprinted with permission from Siebert and Lynn (1997a). Copyright 1997 American Chemical Society.

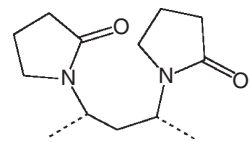

FIGURE 2.23 Structure of a segment of polyvinylpyrrolidone or PVPP.

PVPP is commonly used to remove undesirable brown or pink pigments from wine (Jackson, 1994). However, because much of the color of red wine is due to polyphenolic compounds, treatment with PVPP or other polyamides can diminish the red color and so must be carefully controlled. Additions of gelatin or egg white (egg albumin has about 3.6 mol% proline) have traditionally been used to more gently remove some polyphenol from red wines to ''soften'' astringency.

D. Enzymes

At one time, broad spectrum proteolytic enzymes (mainly papain and bromelain) were widely used to delay or minimize haze formation in beer (de Clerck, 1969). The enzymes cleaved protein chains, that when

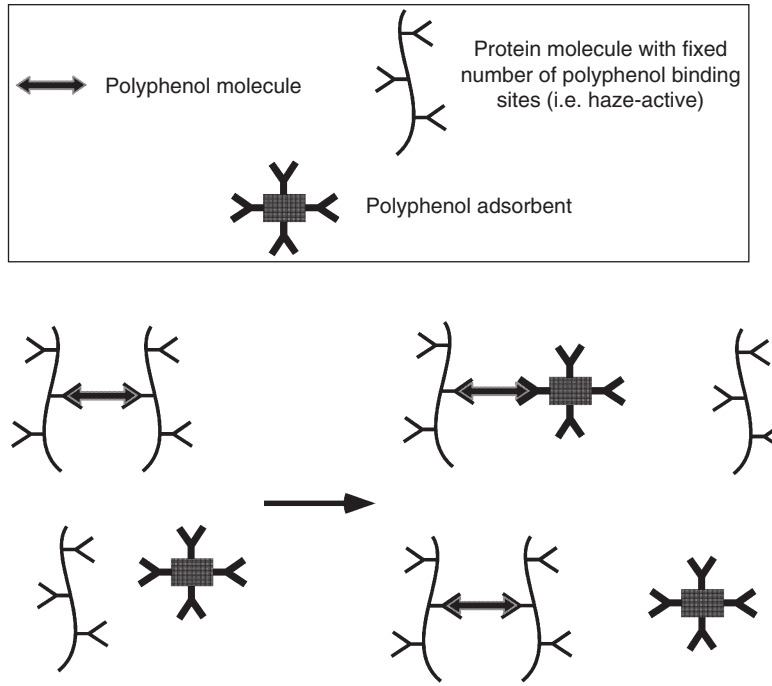

FIGURE 2.24 Concept of PVPP action in beer. Reprinted with permission from Siebert and Lynn (1997b). Copyright 1997 American Society of Brewing Chemists.

cross-linked by polyphenols, led to smaller and more soluble complexes that resulted in less haze. These enzymes were inexpensive and effective in stabilizing beer against haze formation. Unfortunately, the enzymes also attacked foam proteins, seriously impairing beer foam. This often led to the use of a foam stabilizer (typically propylene glycol alginate) to at least partially offset the damage. Most major brewers replaced enzyme (and foam stabilizer) use with adsorbent treatments.

Recently, proteolytic enzymes that cleave peptide bonds only adjacent to proline were introduced (Lopez and Edens, 2005). Since proline is involved in the polyphenol-binding sites and there is little proline in the foam-active proteins, these enzymes are specific for haze proteins and do little damage to foam proteins.

VIII. SUMMARY

This review has summarized knowledge of the phenomena of haze development in clear beverages. The most frequent cause of haze is from the interaction of PRPs with polyphenols that have at least two binding

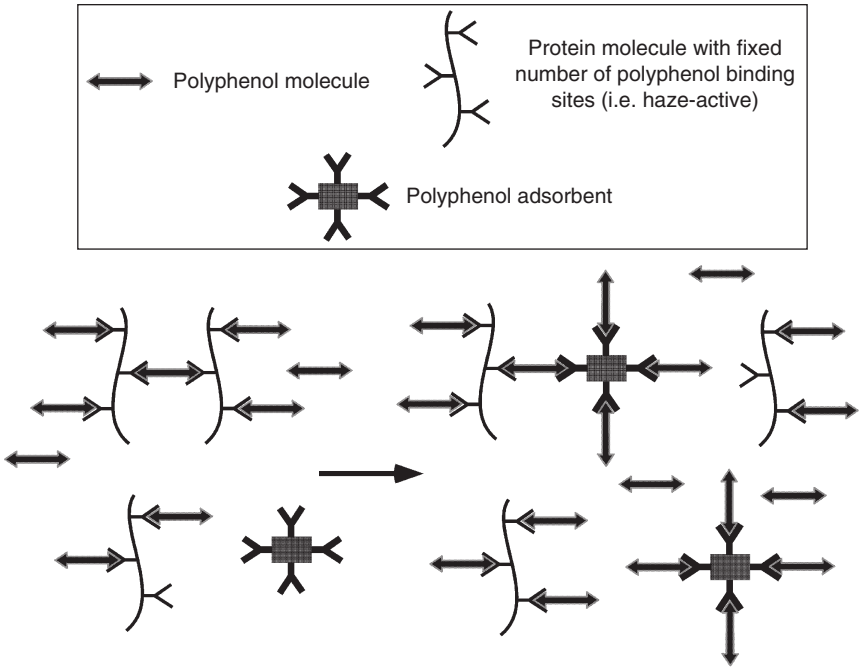

FIGURE 2.25 Concept of PVPP action in a polyphenol-rich beverage. Reprinted with permission from Siebert and Lynn (1997a). Copyright 1997 American Chemical Society.

locations. Beverages are generally stabilized against haze formation with fining agents or adsorbents that remove one or another of the HA species, or with enzymes that attack the HA proteins. The nature of a beverage (protein-rich or polyphenol-rich) impacts the effectiveness of particular protein and polyphenol adsorbents.

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