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**Formation of Polyphenol-Denatured Protein Flocs in Alcohol Beverages
Sweetened with Refined Cane Sugars**

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ABSTRACT: The sporadic appearance of floc from refined, white cane sugars in alcohol beverages remains a technical problem for both beverage manufacturers and sugar refiners. Cane invert sugars mixed with 60% pure alcohol and water increased light scattering by up to ~1000-fold. Insoluble and soluble starch, fat, inorganic ash, oligosaccharides, Brix, and pH were *not* involved in the prevailing floc formation mechanism. Strong polynomial correlations existed between the haze floc and indicator values I.V. (color at 420 nm pH 9.0/color at pH 4.0 - an indirect measure of polyphenolic and flavonoid colorants) ($R^2=0.815$) and protein ($R^2=0.819$) content of the invert sugars. Ethanol induced denaturation of the protein exposed hydrophobic polyphenol binding sites that were further exposed when heated to 80 °C. A tentative mechanism for floc formation was advanced by molecular probing with a haze (floc) active protein and polyphenol as well as polar, non-polar, and ionic solvents.

KEYWORDS: alcohol beverage floc; refined cane sugar; haze active protein; haze active polyphenol; denatured protein;

INTRODUCTION

The sporadic appearance of unattractive floc from refined, cane (white) sugars in alcoholic beverages is a concern to both beverage manufacturers and sugar refiners. Although floc is a quality parameter that will not impact health and nutritional status of the beverage, the consumer expects the beverage to be clear and will most likely be deterred from purchasing the beverage. Furthermore, floc formation can limit the shelf-life of the beverage. The visible defect may be particulate and sedimentary or “woolly” and suspended in the beverage or precipitated.¹ Cohen et al.² reported that, while all acid beverage floc (ABF) forming sugars also develop a floc on the addition of ethanol, not all alcohol haze forming sugars develop ABF. With the declining use of high fructose corn syrup as a beverage sweetener in recent years, floc from either beet or cane sugars, inverted or non-inverted, remains a technical problem that is not easily managed. Typically, refined beet sugars are less prone to cause haze and the primary cause has been attributed to saponins.^{1,3} For refined cane sugars, however, no definitive cause has been attributed to any one quality parameter. Silica, polysaccharides,⁴ protein,⁴⁻⁵ and fats/waxes have all been implicated (see reviews^{1,6}). Polysaccharides in cane sugar can include dextran formed from microbial deterioration (levan and sarkaran polysaccharides are also formed from specific microbial deteriorations in a few parts of the world, particularly dry areas), starch, indigenous cane polysaccharide ISP (arabinoglucan), and small amounts of soluble cellulose and pectin.¹ Clarke et al.⁴ put forward a mechanism for acid beverage floc that incorporated a coacervate of a positively charged protein interacting with a negatively charged ISP. It has been more recently reported by McKee et al.⁷ for acid beverage floc formation only, that when a high (>3.2) color indicator value I.V. (color absorbance ratio at 420 nm of the sugar solution at pH 9.0 to pH 3.0) is present in cane refined sugar there is a strong likelihood that acid beverage floc will form, but

no explanation has been put forward. As high I.V. values are indicators of polyphenolic and flavonoid colorants that naturally occur in sugarcane,⁸⁻⁹ these may be involved with floc formation or they may be indirectly involved. Polyphenolic-protein haze flocs are known to form in beer, wine, cider, and fruit juices.¹⁰ Iciek et al.,³ however, reported that the use of I.V. values to predict acid beverage floc formation from cane sugars has not been totally reliable as outliers have been found and stated further studies were required.

Lemos et al.⁶ studied twenty-one refined cane sugars from Brazil for their formation of alcohol flocs at 55-89% v/v ethanol, and reported that total starch and dextran positively correlated with alcohol floc formation. They used a starch method (ICUMSA GS1-16) that is now known to only measure soluble starch and provides no information of the amount of insoluble starch present, and the Haze dextran method used to determine dextran is not specific to dextran and can measure other haze forming compounds as well. Because insoluble starch has not been previously detected before by commonly used starch methods, its contribution to beverage floc formation has never been realized. Fortunately, a new USDA research method has now been developed by Cole et al.¹¹ to measure total, insoluble and soluble starch, utilizing microwave-assisted probe sonication.

Alcohol beverages are high value-added products with an enormous international market. Such products are often sweetened with white, refined sugars sourced from around the world; the sugars can be sucrose or invert sugars. Alcohol beverage manufacturers can occasionally encounter batches of sugars that cause unwanted floc in their alcoholic products. Unfortunately, there are no rigorous specifications for the prediction of floc from refined cane sugars and, in particular, unwanted false negative results. This may be because there are multiple causes and/or because the underlying reasons for floc formation are still not completely known. At this present

time, some alcohol beverage manufacturers estimate floc formation from batches of sugar by creating a 60% ABV (alcohol beverage volume) sugar base, which is simply invert sugar (or other sugar) mixed with pure alcohol and water to a 60% ABV target. If after 30 min the fortified sugar turns hazy ($\text{NTU} \geq 2.0$) it is considered to be unstable. This fortified sugar must stand for 24 h before it is filtered to remove flocs. Once flocs are removed the now clear, fortified sugar solution can be used to make flavored beverage products.

This study was undertaken to improve understanding of the root cause or causes of floc formation in alcoholic beverages sweetened with refined cane sugars. Another long-term goal of the study is to underpin the development of rigorous specifications for the measurement of alcohol haze formation.

MATERIALS AND METHODS

Chemicals. All chemicals were analytical grade. Dimethylformamide (DMF), 1,4-dioxane, tannic acid, gelatin (Type B) from bovine skin, tannic acid, triethanolamine, dioxane, and dimethylformamide (DMF) were obtained from Sigma-Aldrich (St. Louis, MO, USA). De-ionized water of <18 ohm resistance) was used in sample preparation. Absolute ethanol was from Koptec (King of Prussia, PA, USA). Sodium chloride was from J. T. Baker (Phillipsburg, NJ, USA). Dextranase sourced from *Chaetomium erraticum* was produced by Amano Enzymes, Inc. (Gifu, Japan) and obtained from Alma sugarcane factory (Lakeland, LA, USA), and had an activity of 52,000 DU/mL. An intermediate-temperature (IT) stable amylase from *Bacillus amyloliquefaciens* was produced by and obtained from Amano Enzymes, Inc. (Gifu, Japan) (activity of 447 KRAU/g). A high temperature (HT) stable amylase produced by Novozymes

(Franklinton, NC, USA) was sourced from *Bacillus licheniformis* and obtained from Brenntag (Houston, TX, USA), (activity of 400 KRAU/g).

Beverages. Commercial invert sugars (~75 Brix syrup) from various refinery sources (denoted by upper case letters), and 60% ABV unfiltered and filtered sugar bases produced from the invert sugars, were obtained from a large North American alcohol beverage manufacturer. The 60% ABV sugar base (1 L) was created by adding invert sugar syrup (260 g) to 780 mL of 77% ethanol plus 30 mL of de-ionized water. This 60% ABV sugar base (unfiltered) was left for 24 h by the beverage manufacturer to approximately estimate if any floc will form. The unfiltered 60% ABV was then pressure filtered through diatomaceous earth to form the filtered 60% ABV sugar base.

Stock Solutions. Tannic acid stock solution (5,000 ppm) was prepared by dissolving tannic acid in ethanol (20% of final stock solution volume) and making to the volume with de-ionized water. Gelatin stock solution was prepared daily by dissolving protein (5,000 ppm) in de-ionized water.

Floc Measurements by Haze. Light scattering measurements were undertaken with a Hach Model 2000N nephelometer (Hach, Loveland, CO). Samples containing flocs were gently shaken first before measurement. Results were expressed in nephelometric turbidity units (NTU).

Particle Size Analysis. This was conducted on a Malvern Zetasizer Nano ZS90 (Worcestershire, UK). Samples were inverted gently to ensure even mixing. Unfiltered 60% ABV samples (1 to 1.5 mL) were analyzed. The parameters of the Zetasizer software (ver. 7.11)

were established for the refractive index of ethanol. Triplicate samples were run, and eleven scans were run per replicate.

Effect of a Haze-Active Protein and Polyphenol. Floc was produced by adding gelatin (5,000 ppm; haze-forming protein) or tannic acid (5,000 ppm; haze-forming polyphenol) to the test 60% ABV solution (20 mL in a 100 mL beaker). The samples were incubated for 30 min at 25 °C.

Heating Experiments. The unfiltered 60% ABV samples were incubated for 30 min in a water bath at 80 °C; the 80 °C samples were cooled to 25 °C before measuring the haze. The haze of the samples at room temperature (25 °C) were also measured and compared.

Enzyme Protein Experiments. The protein enzyme (20 mL) directly from the container was heated to 80 °C or 100 °C for 30 min, and then allowed to cool to 25 °C. The treated protein enzyme at 0.1, 0.25, 0.50, 0.75, or 1.0 mL was added to tannic acid (500 ppm; 5 mL) in 20 mL of de-ionized water in a 60 mL test-tube and gently mixed. Care was also taken to gently shake the treated enzyme before adding it to the final solution. The samples were incubated for 30 min at 25 °C before turbidity was measured. Blank samples were also prepared that did not contain any tannic acid solution, to take into account the Haze created by the denatured protein alone. The final Haze (NTU) measurement was $NTU_{\text{sample}} - NTU_{\text{blank}}$.

Effect of Solvent Experiments. To 20 mL of unfiltered 60% ABV samples, 0, 2, 4, and 8 mL of either de-ionized water, absolute ethanol, dioxane, DMF, or 20% NaCl solution were added and gently mixed. This resulted in solutions containing 0, 10, 20, and 40% solvent. The haze of the resulting solution left at 25 °C (room temperature) for 30 min was then measured.

Insoluble and Soluble Starch. Samples were analyzed for total, soluble, and insoluble starch using the microwave-assisted sonication/iodometric USDA Research method.¹¹ Invert syrups were first adjusted to ~15 Brix before analyses and starch was assayed in triplicate; concentrations are quoted as average ppm on a Brix basis.

Brix (% dissolved refractometric solids), pH, Ash, and Fat. Brix was measured using an Index Instruments (Kissimmee, FL) TCR 15-30 temperature controlled refractometer accurate to ± 0.01 Brix, and results expressed as an average of triplicates. The pH was measured on a Metrohm Brinkman 716 DMS Titrino (Riverview, FL, USA) with a Mettler Toledo (Columbus, OH, USA) xerolyte electrode. Inorganic ash was measured according to method AOAC 900.12; the weighed samples were ashed at 600 °C. Fat analysis followed MWL FO 08 method based on AOAC 922.06 method. The homogenized sample was treated with HCl and washed twice with petroleum ether and diethyl ether. Both ash and fat were expressed as % on a wet weight basis and results are averages of triplicate analysis.

Color and Indicator Values. Color was measured as the absorbance at 420 nm and calculated according to the official ICUMSA method GS2/3-9 (1994) with slight modifications. The invert syrup samples and 60% ABV unfiltered and filtered samples were not initially Brix adjusted. Samples (~5 mL) were diluted in triethanolamine/hydrochloric acid buffer (5 mL; pH 7) and filtered through a 0.45 μ m filter. Color was also measured at pH 3.0, 4.0, and 9.0 by first adjusting the buffer with HCl and NaOH solutions, respectively. The Indicator Value (I.V.) was measured as both the ratio of color at pH 9.0/color at pH 4.0 as well as color at pH 9.0/color at pH 3.0. Results are expressed as an average of triplicates.

Protein. The presence of protein was confirmed qualitatively by scanning the invert sugar in a Beckman DU-64 UV/Vis Spectrophotometer between 220 – 350 nm. Protein was present as a

peak at 280 nm due to the presence of select amino-acids (tyrosine and particularly tryptophan and phenylalanine). Once protein presence was confirmed it was assayed using a Bio-Rad protein colorimetric assay (Bio-Rad Laboratories, Hercules, CA, USA) based on the Bradford dye-binding method.¹² Bovine serum albumen (BSA) standard was used to create the standard curve at 595 nm absorption. This method is based on the dye binding primarily to basic (particularly arginine) and aromatic amino-acids. Results are expressed as an average of duplicates.

Dextran. The Rapid Dextran Test (Ecolab, KS, USA) was used to measure monoclonal antibody dextran. A conversion factor was calculated for each batch of antibody used. Results are expressed as an average of duplicates.

Oligosaccharides measured using ion chromatography with integrated pulsed amperometric detection (IC-IPAD). An oligosaccharide fingerprint chromatogram (up to 12 DP) of each sample was obtained by using a strong NaOH/NaOAc gradient over 40 min,¹³ using CarboPac PA1 analytical and guard columns (Dionex Corp., Sunnyvale, CA, USA) at 25 °C. All samples were adjusted to 7.0 Brix prior to comparison.

Statistics. Pearson correlation coefficients were calculated to investigate relationships among the different chemical and physical parameters using Microsoft Excel 2013TM. Statistical differences between parameters at the 5% probability level were calculated by the Students t-test also using Microsoft Excel 2013TM.

RESULTS AND DISCUSSION

Floc Formation by Cane Invert Sugars. Four invert sugars (A to D) produced from sugarcane refined sugars and known to cause alcohol floc formation, were first studied. Floc (haze) formation was measured by light scattering nephelometry, which is used routinely in the quality control of beverages, including alcohol beverages such as ciders and beers.¹⁰ The haze of the invert sugars was very low, i.e., between 0.018 to 0.024 NTU, and bore no relationship to the floc formed on the addition of ethanol (Table 1). Flocs formed in the unfiltered 60% ABV samples (invert sugar mixed with pure alcohol and water to a 60% ABV target) increased light scattering by up to ~1000-fold, i.e., 11.3 to 20.5 NTU greater than the initial invert sugars. The flocs formed were white and fluffy and eventually precipitated to the bottom of the bottle. As expected, when the 60% ABV samples were filtered the NTU values markedly decreased (Table 1).

The acidic pH values of the initial invert sugars varied from 3.85 to 5.77 (Table 1). The alcohol manufacturer who provided the samples does not adjust the pH of the sugar when producing beverages, but many alcohol beverages are between pH 3.0 and 4.0. As seen in Table 1, the addition of ethanol to the sugars increased the pH most likely because of the diluting out of acids, whereas the filtering of these solutions to remove the flocs had negligible effect on the pH. The initial pH of the invert sugars strongly affected the pH of the final filtered ABV solutions ($R^2=0.975$; $y=0.484x + 3.424$). Neither the pH nor the Brix values were found to have any relationship on floc formation.

Chemical Composition Analyses. At the beginning of this study, the cause of floc formation in alcohol beverages sweetened with refined cane sugars was not known so initial research focused on a few possibilities, including starch, dextran, oligosaccharides, fat, ash, protein, color, etc. In this study, none of the invert sugars contained any soluble or insoluble

219 starch (Table 2), which strongly indicated that no starch physical forms are involved in the floc
220 formation mechanism. Nevertheless, this does not preclude that starch molecules could not be
221 entrained or co-precipitated with the precipitated floc. Lemos et al.⁶ showed that soluble starch
222 can cause the formation of flocs in ethanol solution although dextran had a greater effect. In this
223 study, high molecular weight dextran was measured in the samples using a specific monoclonal
224 antibody method, and results are listed in Tables 2 and 3. All the invert sugars contained dextran
225 but there was not a strong relationship between dextran and haze floc formation. Nevertheless,
226 the dextran content was markedly reduced in the 60% ABV samples suggesting it became
227 insoluble in the ethanol and may have associated with the floc, although it was not involved in
228 the initial mechanism of floc formation. Fat concentrations of 0.3 to 0.5% were measured in the
229 invert sugars which may reflect wax from the surface of sugarcane stalks. The fat concentrations
230 were reduced to negligible levels (<0.1%) in the unfiltered 60% ABV samples, which are less
231 than the 0.1% limit of the method of analysis used. Thus fat was dissolved in the ethanol and not
232 involved in floc formation (Table 2). Additionally, inorganic ash concentrations were found to
233 have no relationship with floc formation (Table 2). Fingerprint oligosaccharide chromatograms
234 of the unfiltered 60% ABV samples are illustrated in Figure 1. The samples contained very few
235 oligosaccharides which were not related to floc formation. The chromatograms of the initial
236 invert sugars and filtered samples (not shown) were very similar to the unfiltered samples.

237 The color of the initial four cane invert samples A to D as well as three more (E to G) sent
238 from the alcohol manufacturer was measured at pH 3.0, 4.0, 7.0, and 9.0, and the color Indicator
239 Value I.V. was calculated as the ratio of color at pH 9.0/color at pH 4.0 as well as pH 9.0
240 color/color at pH 3.0. As expected, colors at pH 4.0 and 7.0 were lower than at pH 9.0, because
241 color intensity depends on the solution pH due to changes in molecular structure, ionization, and

association-dissociation equilibria¹⁵; the changes are not linear and color intensity changes most steeply in the pH range 6.0 to 8.0 (Tables 2 and 3).

None of the individual colors measured at the various pHs exhibited a strong relationship with floc formation (NTU), although a moderate correlation ($R^2=0.679$) existed for color at pH 9.0. It is well known that cane derived colorants, e.g., flavonoids and polyphenols, are sensitive to the higher pH values while process derived colorants are not.¹⁴ In contrast, a strong polynomial relationship ($R^2=0.815$) existed between the I.V. (color ratio at pH 9.0/pH 4.0) values of the invert sugars with haze turbidity formation (Figure 2). Thus, floc formation became progressively worse with increased values of I.V. Since the two I.V. values, i.e., color ratio at pH 9.0/pH 4.0 and pH 9.0/pH 3.0, were strongly related ($R^2=0.952$; $y=1.1161x-0.1425$), most likely both could be used to indicate floc formation. These results confirmed the findings of McKee et al.⁷ for acid beverage flocs. It must be noted, however, that the I.V. (pH 9.0/pH 3.0) values which correlated strongly with floc formation in this study, i.e., 1.27 to 1.54, were not in the range reported to predict acid beverage floc formation with white, refined cane sugars.⁷ Moreover, McKee et al.⁷ observed that I.V. values <2.1 indicated a low floc potential for acid beverage flocs. This suggests that much lower concentrations of polyphenolic colorants are involved in the formation of alcohol beverage flocs most likely because of the addition of ethanol. It is well known that sugarcane juice contains a complex mixture of polyphenolic and phenolic flavonoid colorants that are monomeric and sensitive to pH. The major polyphenols in sugarcane are caffeic, chlorogenic, and coumaric acids.⁹ The predominant flavonoids are tricetin, naringenin, luteolin, and apigenin.⁸⁻⁹ Flavonoids are water soluble and at acid pH are non-ionized.

To qualitatively determine the presence of protein in all the cane invert sugars A to G and their corresponding 60% ABV unfiltered solutions, they were first scanned between 220 and 350 nm, and all showed a protein peak at 280 nm. As a consequence, they were all subsequently quantitatively analyzed for protein using a dye-binding method,¹² and results are also listed in Tables 2 and 3. The protein levels in the invert sugars were very low ranging from 0.11 to 0.60 $\mu\text{g/mL}$ (ppm). Naidoo and Simpson⁵ reported that higher levels, i.e., $>2.00 \mu\text{g/mL}$, of protein in refined cane sugars assayed with a protein method also based on the Bradford dye-binding method, predicted that acid beverage flocs would form. Similar to the I.V. values (Figure 2), the protein had a polynomial relationship ($R^2=0.819$) with haze (floc) formation, indicating that floc formation became progressively worse with increased in protein concentrations and that protein was somehow involved in floc formation (Figure 3).

Small amounts of protein naturally occur in sugarcane juice⁴ including invertase and diastase enzymes.¹⁶ In addition, proteins as industrial enzymes are applied during sugar manufacture especially at the sugarcane factory. Amylases are often routinely added to degrade starch and dextranase is occasionally added to degrade dextran when it is present from the deterioration of sugarcane.¹³ Floc formation in other alcohol beverages, i.e., beer, has also been reported to be affected by proteins such as proteases added during beer processing.¹⁰ Not all proteins, however, are haze-active and contribute to floc formation. Proteins that readily interact and precipitate with polyphenols are generally large, have high proline (a non-polar, hydrophobic amino acid) content, and lack secondary or tertiary structure, i.e., are partially or fully denatured.¹⁷ Prolines are known to be involved with non-specific interactions primarily via ring stacking with their prolyl residues and with a preference for Pro-Pro repeats.¹⁸ It must also be noted that different

polyphenols and tannins have been reported to show variations in interaction with a given protein.¹⁹

The Bradford protein assay used in this study is based on dye-binding primarily to basic, particularly arginine, and aromatic amino-acids, which means it is not as sensitive to proline. Arginine, however, has been reported to interact with polyphenol flavonoids, but to a lesser extent than proline.¹⁸ Furthermore, the protein assay was calibrated using the globular protein bovine serum albumen (BSA) which is known to have specific poly-phenol binding cavities.²⁰ A protein assay which is more sensitive to proline may have provided protein concentrations in the cane invert sugars that had an even stronger relationship with floc formation than what was found in this study (Table 2 and Figure 3). To the best knowledge of the authors no such proline-sensitive protein assay has been developed yet.

As shown in Tables 2 and 3, the protein concentrations increased markedly in the unfiltered 60% ABV solutions when compared to the initial invert sugars. This can be explained by the Bradford dye-binding protein assay used. Ethanol at high concentrations such as the 60% in this studies' ABV solutions, is known to induce and accelerate the denaturation of proteins.²¹ Partial or full denaturation of the protein will have exposed additional polyphenol binding sites including proline and to a lesser extent other amino acids such as phenylalanine, tyrosine, arginine that have also been suggested to interact with polyphenol flavonoids.¹⁸ Denaturation of the protein by ethanol may also have reduced the proximity of bulky amino acids that were near to potential polyphenol binding sites and, therefore, increased accessibility.²² As a consequence, since the Bradford dye-binding assay is based on binding to arginine and aromatic amino acids more protein was detected.

Downstream sugar factory and refinery processes, e.g., evaporation and crystallization, are operated at elevated temperatures and are expected to partially or fully denature proteins. Furthermore, many sugar refinery processes have been reported⁴ to reduce or remove the amount of protein in processing streams. Nevertheless, protein can still be detected in refined sugars⁴ albeit at very low concentrations, and high temperature stable amylases have been known to cause carry-over (residual) amylase activity in refined sugars.²³ Clarke et al.,⁴ reported that sugarcane protein in refined sugars contained proline (4%). Proline is also one of the major amino acid constituents (7%) of dextranase enzymes sourced from *Chaetomium gracile* and *Chaetomium erraticum* fungi²⁴ which are added at factories. Proline (3%) is also contained in amylase proteins produced from *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus licheniformis* bacteria²⁵ which are added at the factory. Furthermore, Clarke et al.⁴ reported that “it has been observed, in attempts to treat floccing sugars with enzymes, that the addition of any protein, e.g., amylase, pectinase, dextranase, to a sugar solution enhanced floc formation”. To confirm that dextranase and amylase proteins could be involved with polyphenol-protein interactions, they were reacted with tannic acid (TA), a very haze-active polyphenol that is known to combine with haze-active protein to form a floc that can be measured by light scattering nephelometry¹⁴ (Table 4). At very low volumes, dextranase (0.281% protein) formed floc with TA as did IT stable amylase (*Bacillus amyloliquefaciens*; 2.235% protein) and especially HT stable amylase (*Bacillus licheniformis*; 1.541% protein).

Relative Levels of Haze-Active Proteins and Polyphenols in Cane Invert Sugars. Since the compositional analyses results suggested polyphenolic I.V. colorants and proteins may be involved in floc formation these were further investigated. Estimates of the relative amounts of haze-active proteins in the 60% ABV samples were made by adding tannic acid (TA).

Conversely estimates of the relative amounts of “sensitive” or haze-active polyphenolics in the 60% ABV samples were made by adding bovine gelatin, a very haze-active protein²⁶ that contains 16% proline,²⁷ and known to combine with haze-active polyphenolics. No pH adjustments were undertaken in these experiments which were conducted at 25 °C. The results for the invert sugars are listed in Table 5. A beet invert sugar (control) was included in this study which did not form any haze, but showed a very small increase in haze as a result of the addition of both tannic acid and particularly protein (Table 5). Thus the beet invert sugar must contain very small amounts of haze-active protein and slightly more haze-active polyphenols. The initial I.V. value of the beet invert was 1.13 which was low compared to values for cane sugars (see Table 3), but as the value was greater than 1.0 it confirmed that the beet invert sugar contained a very small amount of polyphenol colorants. Such natural colorants most likely came from the beet leaves and tuberous root. The beet invert sugar also contained a very small amount of protein (0.04 µg/mL) detected by the Bradford dye-binding protein assay,¹² which confirmed that haze-active protein was present.

Results listed in Table 5 also showed that cane invert sugar D contained some haze-active protein since haze was formed on the addition of tannic acid. The reduced value at 500 ppm of tannic acid is due to dilution because the additive was added by volume. In comparison, the addition of gelatin to sugar D caused a decrease in floc formation indicating this sugar had negligible amounts of polyphenols or that the polyphenols were all already bound to sites on the sugar’s protein. Invert sugars A and C did not seem to contain any available haze-active protein that could bind with tannic acid because the addition of this haze-active polyphenol had no effect (Table 5). Cane invert sugar C had increased haze formation on the addition of gelatin (the reduced value at 500 ppm of gelatin is due to dilution because the additive was added by

volume) indicating not all of its polyphenolic colorants were bound. Beers are known to contain a considerable amount of haze-active protein and very little haze-active polyphenol, whereas commercial apple juices are just the opposite.⁸ However, the highest hazes in alcohol beverages have been reported to depend on the ratio of haze-active protein to haze-active polyphenol.²⁶ Moreover, different polyphenols and tannins can also show variations in interactions with a given protein.¹⁹

Nature of the Floc Interactions. The nature of polyphenolic-protein interactions has been the subject of many studies because they are involved in floc formation in other alcohol beverages^{10,26} and also because phenolic compounds in foods are of great interest for their health and dietary benefits.²⁸ For example, proteins are being used in foods to complex with bitter and astringent flavonoids.¹⁸ It is generally accepted that the polyphenol-protein interaction in solution is a reversible interaction leading to an equilibrium between the soluble protein/polyphenol complexes and the reactants.¹⁹ These soluble complexes may reach a size where they are no longer soluble and this creates a floc. In turn, the flocs then may aggregate or they may undergo changes resulting in precipitation/sedimentation.¹⁹

Heating of unfiltered 60% ABV samples recreated in the laboratory from the initial invert sugars was undertaken at 80 °C, to evaluate if hydrophobic interactions were involved in floc formation. As shown in Table 6, there was a small but definite increase ($P < 0.05$) in the turbidity of the A to D samples on heating, which indicated that heat-induced partial or full denaturation of the protein exposed additional polyphenol binding sites. This was confirmed by the corresponding slight increase in concentrations of dye-binding protein on heating (Table 6). Although this protein increase was not always significantly different, the trend was always to increase protein on heating. Heating to increase floc formation is also consistent with a greater

role for hydrophobic rather than hydrogen bonding.²⁶ This is because hydrophobic groups, i.e., from hydrophobic proline, tend to be concentrated in the interior of water-soluble proteins, it is likely that more hydrophobic (polyphenol) bonding sites were exposed by the heating as protein hydrogen bonds are broken with the heat.²⁶ Heating has similarly been shown to increase the hydrophobicity of ovalbumin and lysozyme proteins.²⁹ In this study, the heating of the flocs (60% ABV samples) to 80 °C not only consistently increased floc formation but also tended to create some larger floc particles with an example shown in Figure 4. This is further strong evidence that protein is involved with the formation and aggregation of flocs. During sugar processing at both the factory and refinery, proteins have been exposed to temperatures >80 °C for prolonged periods and have been presumed to be thoroughly denatured. On the other hand, high temperature (HT) stable amylases added in the factory to control starch have been found to still be active in refined sugars indicating full denaturation does not always occur.²³ The re-heating dextranase to 80 and 100 °C for 30 min created up to ~800-fold more floc compared to the non-heated dextranase (Table 4) although heating at 100 °C was worse than 80 °C. Thus >80 °C temperatures caused too much heat denaturation and destroyed some of the polyphenol binding sites. Heating the IT amylase to 80 °C, however, created less binding to polyphenols and was even worse at 100 °C (Table 4). Unlike the IT Amylase, the HT amylase generally increased bounding to TA when it was pre-heated at 80 °C but this was reduced at 100 °C. Therefore, the heat-stability of the amylase protein affects alcohol floc formation. Overall, the results in Table 6, further suggest proteins in refined sugars are not always completely denatured as previously considered, but further studies are needed to confirm this.

The type of bonding involved in the floc formation was further probed by investigating the effect of adding polar, non-polar, and ionic solvents (Table 7). Since the initial protein-

polyphenol interaction is considered reversible²⁶ it should be possible to dissolve already-formed floc in DMF (a hydrogen bond acceptor) and dioxane (non-polar) solvents. As shown in Table 7, all the flocs in the initial 60% ABV samples were progressively dissolved by dioxane and particularly DMF, although not all the flocs were dissolved in 40% of the solvent. Dissolution in DMF suggests that hydrogen bonds play some role in floc formation. Bennick¹⁹ observed that hydrogen bonds as well as hydrophobic effects are involved in polyphenolic/protein interactions.

Water aliquots added in the same proportions of the other solvents also caused a decline in haze most likely because of dilution as the flocs dissolved in this polar solvent. Thus the components of the floc are water soluble. The solubility of proteins in aqueous solutions depends on the distribution of hydrophilic and hydrophobic amino acid residues on the protein surface. As stated earlier, hydrophobic residues predominantly occur in the globular protein core.

In comparison to the solvent water, all the cane invert sugars dissolved even further in the ionic sodium chloride solution. Hagerman and Butler³⁰ previously reported that ionic interaction between proteins and polyphenols is considered to be of little or no importance and Bennick¹⁹ reported there was little evidence that covalent bonding is involved. Furthermore, Asano et al. (1982) reported that, in acidic conditions, such as pH 4.5 in alcoholic drinks sweetened with sugar, hydroxyl groups of polyphenols have no charge and, therefore, ionic bonding is not involved in the floc formation mechanism. Therefore, the sodium chloride most likely allowed more floc to dissolve because it altered the dielectric constant of the solvent. In strong contrast, ethanol, a miscible solvent, is known to decrease the dielectric constant of water which in turn allows proteins to come close together and eventually precipitate, i.e., desolubilize the protein. Moreover, at high concentrations such as in this study, ethanol accelerates the denaturation of

protein by disrupting hydrophobic interactions between amino acids. These reasons most likely explain why the cane invert sugars produced even more haze on the addition of 10 to 40% ethanol (Table 7). This was also further evidenced by cane invert sugar A which contained more protein than the other sugars, exhibiting the greatest increase in haze with ethanol addition (see Table 2).

The solution pH is also known to often influence how proteins behave by affecting the net charge of the protein molecules. This can lead to more or less ionization, with a change in ionic attraction or repulsion which may also alter the protein solubility or the molecular conformation.¹⁰ This in turn may influence the accessibility of the sites on the protein to which the polyphenols can bind. Many alcoholic beverages are between pH 3.0 and 4.0. At such acidic pH, the proteins in the cane samples will be positively charged.⁴ Generally, it has been found that the binding affinity of proteins for polyphenols and the precipitation of polyphenol complexes is pH dependent, with the greatest precipitation occurring near the isoelectric point of the protein due to the decreased electrostatic repulsion of protein molecules at this pH.^{19,30} The cane invert sugars provided by the beverage manufacturer were acidic (see Table 2) but not adjusted for pH. At such acidic pH, the proteins in the cane sugars will be positively charged,⁴ thus more studies are now warranted on the effects of pH.

Critical Concentrations and Ratios of Components for Floc Formation. Polyphenols are thought to be multivalent ligands and may form crosslinks with two or more protein molecules. They can cause structural and conformational changes of the protein. De Freitas and Mateus³¹ showed that the protein binding affinity depends on the molecular size of the polyphenol molecule, with the higher the molecular size the greater the tendency to interact with proteins. McMurrough et al.³² showed that the concentration of polyphenol proanthocyanidin dimers of

catechin and/or epicatechin were closely related to the rate of chill haze formation in beets. Thus the stoichiometry and size of the polyphenol-protein complexes depends on the concentrations as well as the reactants and protein/polyphenol ratio.²⁶ As the beet invert sugar (control) in this study which formed no floc still contained very low amounts of protein (0.041 $\mu\text{g/mL}$) and polyphenols (I.V. 1.05) (see Table 3), there are most likely critical concentrations of protein and polyphenols both individually and in relation to one another. An excess of one component may change the interaction, but further studies are needed on this.

Mechanism of Floc Formation. The results presented in this paper have allowed a tentative mechanism for the formation of floc in alcohol beverages sweetened with cane sugars to be advanced, which is illustrated in Figure 5. If critical concentrations of polyphenolic I.V. color compounds and denatured protein with polyphenol binding sites are available, they interact to form a floc of colloidal size that causes light scattering. Additional heating of the floc can cause aggregation. Eventually the floc particle reaches such a large size that it precipitates and may co-precipitate with other colloidal species such as dextran and, if present, other polysaccharides rendered insoluble in the ethanol solvent. These other colloidal species may be entrained on the floc molecules. Overall the initial floc interaction is dependent on hydrophobic and hydrogen bonding.

Practical Solutions to Floc Problems. Dunsmore et al.³³ reported that acid beverage floc forming compounds found in refined sugars could be removed with select refining processes, but they were unable to explain why they worked. Results in this study can now explain these differences. Phosphatation clarification of refinery liquors incorporates the addition of ionic (cationic) flocculants. As results in this study have shown that the floc forming interaction is not based on ionic bonding but on hydrophobic and hydrogen bonding, the ionic flocculants would

not have been able to remove the floc components. Carbonatation clarification incorporates lesser amounts of ionic flocculants and also has a more inherent filtration system compared to phosphatation clarification and thus was more likely able to remove the protein floc component. Dunsmore et al.³³ also reported that refinery liquors filtered through membranes of 0.60, 0.80, and 1.20 μm pore sizes at temperatures $<60\text{ }^{\circ}\text{C}$ rendered them floc-less (floc negative) whereas those filtered at $80\text{ }^{\circ}\text{C}$ still formed flocs. Liquors filtered through a $0.45\text{ }\mu\text{m}$ membrane were floc-less even at $80\text{ }^{\circ}\text{C}$. The results presented in this study can also explain these temperature results. As shown in Table 6, on heating floc solutions to $80\text{ }^{\circ}\text{C}$ there was a definite increase in floc (haze) formation because heating exposes hydrophobic groups concentrated in the interior of the water-soluble protein which are polyphenol bonding sites, causing more flocs to form.²⁶ Thus, at $80\text{ }^{\circ}\text{C}$ - a common temperature in a sugar refinery, protein was further denatured and floc formed. Because the small $0.45\text{ }\mu\text{m}$ membrane rendered the liquor floc-less this gave an indication of the physical size of the protein, however, membranes on an industrial scale are not widely used as they become clogged easily.

An alternative practical solution to removing floc components is the use of active carbon. Eggleston et al.³⁴ undertook laboratory studies with powdered activated carbon (PAC) and demonstrated that powdered activated carbon removed amylase protein from refinery liquors after phosphatation clarification, and the PAC also preferentially removed polyphenolic and flavonoid colorants. This confirmed the research by Paton and Smith,³⁵ who used chromatography profiles to show that granular activated carbon was capable of removing all polyphenolic and flavonoid colorants from refinery liquor whereas ion exchange resins and bone char were not. Therefore, granular activated carbon, used in many sugar refineries as a

491 decolorizing process, or powdered activated carbon could be very useful in removing the
492 components involved in floc formation, and more studies are now warranted.

493

494 **ABBREVIATIONS USED**

495 **I.V.** Indicator value (ratio of color at pH 9.0/color at pH 4.0)

496 **ABF** Acid beverage floc

497 **ISP** Indigenous cane polysaccharide

498 **ICUMSA** International Commission for Uniform Methods in Sugar Analyses

499 **ABV** Alcohol beverage volume

500 **NTU** Nephelometer turbidity units

501 **DMF** Dimethyl formamide

502 **IC-IPAD** Ion chromatography with integrated pulsed amperometric detection

503 **Pro** Proline

504 **BSA** Bovine serum albumen

505 **TA** Tannic acid

506 **PAC** Powdered activated carbon

507

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FIGURE CAPTIONS

Figure 1. IC-IPAD oligosaccharide chromatograms of unfiltered 60% ABV solutions A to D.

Figure 2. Relationship between floc formation (haze) and the color indicator values (color at pH 9.0/color at pH 4.0) for initial invert sugars A to G (see Tables 2 and 3).

Figure 3. Relationship between floc formation (haze) and the protein concentrations for initial unfiltered 60% ABV solutions produced from invert sugars A to G (see Tables 2 and 3). Not enough sample from invert sugar B was available for protein analyses.

Figure 4. Particle size distribution graphs for unfiltered 60% ABV solutions produced with cane invert sugar A at (upper graph) 25 °C and (lower graph) 80 °C for 30 min.

Figure 5. Tentative mechanism for floc formation in alcohol beverages sweetened with refined cane sugar. Most of the interactions are based on hydrophobic and hydrogen bonding. Colloidal species such as dextran, starch, and silica may be entrained or co-precipitated with the floc. Precipitation of floc also occurs without heating the floc.

Table 1. Haze (NTU), Brix (% dissolved refractometric solids), and pH of initial invert sugars, non-filtered (floc formed) alcohol solutions, and filtered alcohol solutions.*

Sample	Flocs Present	Brix %	pH	Turbidity (NTU)
Invert Sugar A	x	77.36	3.85	0.020
Invert Sugar B	x	72.22	3.55	0.018
Invert Sugar C	x	71.80	5.77	0.020
Invert Sugar D	x	76.47	4.93	0.024
Unfiltered 60% ABV Solution A	✓	33.42	5.33	20.50
Unfiltered 60% ABV Solution B	✓	32.64	5.09	19.30
Unfiltered 60% ABV Solution C	✓	32.40	6.28	14.84
Unfiltered 60% ABV Solution D	✓	33.22	5.60	11.34
Filtered 60% ABV Solution A	x	33.92	5.34	0.024
Filtered 60% ABV Solution B	x	32.52	5.14	0.030
Filtered 60% ABV Solution C	x	32.41	6.28	0.023
Filtered 60% ABV Solution D	x	33.96	5.70	0.024

*All of these samples were provided by the North American alcoholic beverage manufacturer

Table 2. Compositional analyses of initial invert sugars, non-filtered (floc formed) alcohol solutions, and filtered alcohol solutions.*

Sample	Insoluble Starch (ppm/Brix)	Soluble Starch (ppm/Brix)	Dextran (ppm/Brix)	Ash %	Protein % [□]	Fat %	Color at pH 3.0 (ICU)	Color at pH 4.0 (ICU)	Color at pH 7.0 (ICU)	Color at pH 9.0 (ICU)	I.V. 9.0/3.0	I.V. 9.0/4.0
Invert Sugar A	0	0	165.6	<0.1	0.60	0.5	244.4	248.7	277.3	370.7	1.52	1.50
Invert Sugar B	0	0	n.d. [†]	<0.1	n.d. [†]	0.5	220.9	222.6	255.7	322.5	1.46	1.45
Invert Sugar C	0	0	186.7	0.3	0.11	0.3	238.8	237.5	252.7	300.9	1.26	1.27
Invert Sugar D	0	0	102.9	<0.1	0.13	0.4	224.7	229.3	247.9	290.2	1.27	1.27
Unfiltered 60% ABV Solution A	0	0	5.2	<0.1	29.67	<0.1	306.1	308.3	309.9	350.2	1.14	1.14
Unfiltered 60% ABV Solution B	0	0	n.d. [†]	<0.1	n.d. [†]	<0.1	297.4	300.0	310.6	335.8	1.13	1.12
Unfiltered 60% ABV Solution C	0	0	4.5	<0.1	29.73	<0.1	319.4	317.7	325.3	349.5	1.09	1.10
Unfiltered 60% ABV Solution D	0	0	0	<0.1	25.79	<0.1	290.2	296.4	300.5	301.2	1.08	1.07
Filtered 60% ABV Solution A	0	0	n.d.	<0.1	n.d.	<0.1	298.7	299.8	306.6	341.3	1.14	1.14
Filtered 60% ABV Solution B	0	0	n.d.	<0.1	n.d.	<0.1	296.7	295.2	307.8	331.5	1.12	1.12
Filtered 60% ABV Solution C	0	0	n.d.	<0.1	n.d.	<0.1	319.8	n.d. [‡]	350.8	358.6	1.12	n.d. [‡]
Filtered 60% ABV Solution D	0	0	n.d.	<0.1	n.d.	<0.1	321.4	299.2	297.9	319.9	1.07	1.07

*All of these samples were provided by the North American alcoholic beverage manufacturer
[□] All the invert sugars A to D and corresponding unfiltered 60%ABV solutions were shown to qualitatively contain protein
n.d.[†]= not determined because of insufficient sample
[‡]n.d.=not determined as anomalous

Table 3. Compositional analyses of initial invert sugars E to G and beet invert sugar (control) as well as their corresponding unfiltered (floc formed) alcohol solutions.* Invert sugars E, F, and G, formed flocs of 16.8, 10.4, and 14.4 NTU, respectively, in the unfiltered 60% ABV solutions. The beet invert sugar did not form visible floc (0.059 NTU) when in the unfiltered 60% ABV solution.

Sample	Insoluble Starch (ppm/Brix)	Soluble Starch (ppm/Brix)	Dextran (ppm/Brix)	Qual. Protein	Quant. Protein ($\mu\text{g/mL}$)	Color at pH 3.0 (ICU)	Color at pH 4.0 (ICU)	Color at pH 7.0 (ICU)	Color at pH 9.0 (ICU)	I.V. 9/3	I.V. 9/4
Invert Sugar E	0	0	136.6	✓	0.250	236.9	246.9	271.7	360.6	1.52	1.46
Invert Sugar F	0	0	116.0	✓	0.150	239.9	241.8	255.2	289	1.20	1.20
Invert Sugar G	0	0	129.5	✓	0.330	239.2	240.8	249.8	294.6	1.23	1.22
Beet Invert Sugar (control)	0	0	27.3	✓	0.041	159.9	167.5	167.5	175.5	1.10	1.05
Unfiltered 60% ABV Solution E	0	0	0	✓	34.80	301.0	296.2	316.7	342.0	1.4	1.15
Unfiltered 60% ABV Solution F	0	0	0	✓	32.29	300.8	298.4	309.0	316.0	1.05	1.09
Unfiltered 60% ABV Solution G	0	0	1.2	✓	30.48	304.5	295.9	313.1	320.4	1.05	1.05
Beet Invert Sugar (control)	0	0	1.3	✓	25.69	291.5	297.1	302.4	302.0	1.04	1.02

*All invert samples were provided by the North American alcoholic beverage manufacturer and the 60% ABV solutions were created in the laboratory †n.d.=not determined

Table 4. Model solutions to show the effect of enzyme proteins, non pre-heated and pre-heated, on Haze (NTU) formation (average values shown) with tannic acid, after incubation at 25 °C for 30 min.‡

%	Dextranase [□]	Dextranase [□]	Dextranase [□]	Intermed.	Intermed.	Intermed.	High	High	High
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Final Volume Added	Non Pre-heated	Pre-heated to 80 °C	Pre-heated to 100 °C	Temp. Stable Amylase* Non Pre-heated	Temp. Stable Amylase* Pre-heated to 80 °C	Temp. Stable Amylase* Pre-heated to 100 °C	Temp. Stable Amylase† Non Pre-heated	Temp. Stable Amylase† Pre-heated to 80 °C	Temp. Stable Amylase† Pre-heated to 100 °C
0	0.026	0.026	0.026	0.023	0.023	0.023	0.026	0.026	0.026
0.1	0.097	25.9	21.4	19.6	8.2	2.8	37.6	58.4	36.0
0.25	0.095	63.4	65.4	82.6	13.7	2.5	132.0	209.9	83.8
0.5	0.114	101.0	57.0	175.0	116.0	10.4	343.0	363.0	308.0
0.75	1.830	168.0	127.0	199.0	81.0	21.2	313.0	373.3	320.0
1.0	4.120	145.0	128.0	194.0	21.0	21.0	349.0	274.7	308.0

‡ The initial protein content of the non-heated enzymes were 0.281, 2.235, and 1.541% for dextranase, intermediate temperature stable amylase, and high temperature stable amylase, respectively

□ Dextranase enzyme is most active at ~50 °C (Eggleston et al., 2007) and sourced from *Chaetomium erraticum*.

*Sourced from *Bacillus amyloliquefaciens*

† Sourced from *Bacillus licheniformis*

Table 5. Haze (NTU) resulting from the addition of haze-active gelatin protein or haze active poly-phenolic tannic acid on unfiltered alcohol samples incubated at 25° C for 30 min.*

Additive concentration (ppm)	De-ionized Water	Canada (A)	Mischprobe (C)	USA (D)	Beet Invert Sugar
Gelatin B					
0	0.032 [□]	16.9	13.6	9.8	0.101
25	0.041	16.5	15.7	8.5	0.125
100	0.035	16.3	17.4	8.2	0.125
500	1.61	13.4	11.3	7.3	0.116
Tannic Acid[†]					
0	nd [‡]	16.9	13.6	7.6	0.068
25	nd	15.4	12.8	9.2	0.098
100	nd	14.7	12.7	9.2	1.210
500	nd	12.2	10.9	7.9	0.089

*The A, C, and D 60% ABV samples were re-created in the USDA laboratory from the original invert sugars

[□] Haze (NTU) values among the samples and additive concentrations were statistically different at the 5% probability level for all samples

[†] The stock solution of tannic acid (5000 ppm) used to create the solutions was 0.063 NTU

[‡] nd=not determined

Table 6. Haze (NTU) resulting from the incubation of 60% ABV samples at 25 and 80 °C and associated protein contents.

Unfiltered alcohol solution	Haze at 25 °C NTU	Haze at 80 °C NTU	Protein at 25 °C (µg/mL) [†]	Protein at 80 °C (µg/mL)
A*	12.3 [‡]	14.2	29.67a [□]	29.93a
B*	13.6	16.7	32.61a	31.58a
C*	10.7	11.5	30.40a	31.31a
D*	7.16	7.90	25.79b	29.17a
Beet invert sugar (control)	0.059	0.066	25.69b	30.75a
Analytical grade sucrose (control)	8.49	8.26	26.9a	27.64a

*The A to D 60% ABV samples were re-created in the USDA laboratory from the original invert sugars. Initial Haze (NTU) values are lower because they had not been left as long to form as the original samples (see Table 1).

[†] Based on the Bradford dye-binding protein assay

[‡] Haze (NTU) values at 25 and 80 °C were statistically different at the 5% probability level for all samples

[□] Values followed by the same lower case letter were not significantly different at the 5% probability level between the two samples at the two temperatures

Table 7. Effect of various concentrations of polar and non-polar solvents on Haze (NTU) formation or dissolution on unfiltered 60% ABV samples incubated at 25° C for 30 min.*

% Final Volume Added	Water	Ethanol	Dioxane	DMF	20% Sodium Chloride Solution
Polarity:	Very Polar	Low polar & low non-polar	Non-polar	Polar (hydrogen bond acceptor)	Ionic
Canada (A)					
0	16.2 [□]	16.2	16.2	16.2	16.2
10	11.3	16.7	14.4	13.0	13.2
20	11.5	21.1	13.9	12.5	9.8
40	7.2	21.1	12.9	9.8	5.3
Mischprobe (C)					
0	12.6	12.6	12.6	12.6	12.6
10	10.2	12.2	11.2	11.3	10.0
20	9.4	12.5	10.7	10.2	9.3
40	5.9	12.9	9.2	8.4	5.1
USA (D)					
0	7.4	7.4	7.4	7.4	7.4
10	6.2	7.4	6.8	6.2	4.6
20	4.8	8.0	6.3	5.1	4.4
40	3.7	8.4	6.0	5.0	2.7
Beet invert sugar					
0	0.072	0.072	0.072	0.072	0.072
10	0.065	0.086	0.068	0.075	0.087
20	0.021	0.091	0.087	0.093	0.088
40	0.022	0.116	0.082	0.088	0.070
Analytical grade sucrose					
0	7.4	7.4	7.4	7.4	7.4
10	6.9	7.6	6.9	6.5	6.3
20	5.8	7.6	6.2	6.2	5.5
40	5.8	9.8	6.7	5.6	4.2

*The A, C, and D 60% ABV samples were re-created in the USDA laboratory from the original invert sugars. Initial Haze (NTU) values are lower because they had not been left as long to form as the original samples (see Table 1).

[□] Haze (NTU) values among the samples and additive concentrations were statistically different at the 5% probability level

Figure 1.

Figure 2.

Figure 3.

Figure 4.

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Tentative Mechanism for Floc Formation
(mostly hydrophobic and hydrogen bond interactions)

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759 **Figure 5.**

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