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Detection of Floc-Producing Sugars by a Protein Dye-Binding Method

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Trace amounts of protein have been shown to be major contributors to the ability of granulated cane sugar to cause floc in carbonated beverages. This research was designed to develop a protein dye-binding technique for determining the quantitative presence of the proteins in suspected floc-causing sugars. The procedure was standardized for variables of reagent concentrations, time, colors, and volumes. The results showed a significant correlation between the protein levels determined by the dye-binding method and the Kjeldahl procedure. Floc-positive sugars showed protein concentrations ranging from 0.3 to 0.4% whereas the floc-negative samples ranged from 0.004 to 0.006%. On the basis of these results, it can be concluded that this dye-binding method can serve as a reliable and rapid procedure for predicting the ability of a sugar to produce floc.

The occurrence of floc in acidified carbonated beverages, bottler's concentrates, and pharmaceutical syrups has been a major production problem for many years. Eis et al. (1952) identified the causative agent for floc production in beet sugar as a saponin. However, this was not the case when floc-producing cane sugar was analyzed. Efforts to identify the floc-causing substance (FCS) in cane sugar has led to the analyses and identification of several impurities in the sugar: namely, starch, wax, ash constituents, decolorizing carbon, protein, and silicon dioxide (Roberts and Carpenter, 1974; Stansbury and Hoffpauir, 1959).

Research to detect the FCS has narrowed the possibilities to protein and an amylose derivative (Cohen et al., 1970; Liuzzo and Hsu, 1975). Liuzzo et al. (1977) reported that the amino acid content of floc was directly related to the flocking occurrences of sugar. These workers concluded that the FCS in granulated cane sugar was due to an amylose-related substance which can complex a number of other compounds to enhance the floc formation, especially trace protein.

The removal of the FCS from the tons of cane sugar usually purchased by a commercial firm for use in production does not presently seem to be economically feasible. A more practical approach to the problem would be to detect the FCS in a shipment by a rapid and reliable method in order to divert the sugar to an industry whose products are not affected by the sugar tendency to floc.

Protein dye-binding techniques applied to detection of protein in several food products have been used since they were first reported by Fraenkel-Conrat and Cooper (1944). The purpose of this research was to develop a rapid dye-binding technique for detection of the protein responsible for flocculation, thus providing a screening procedure for flocking sugars.

EXPERIMENTAL SECTION

Samples. All sugar samples used were granulated cane sugars. Three floc-positive sugars and a floc-negative sample were obtained from the American Society of Soft Drink Technologists. Three floc-negative sugars were purchased from local supermarkets. Chemically pure sucrose was used for comparative purposes.

Protein Separation. The amount of protein in refined sugars is extremely small. Therefore, this presents a problem in its separation from the sugars. Several methods were attempted, but a modification of the Sevag method (Staub, 1965) proved most suitable.

Three hundred grams of cane sugar was dissolved in 300 mL of distilled water and filtered through a coarse-porosity fritted glass funnel to remove gross foreign materials. The solution was placed in centrifuge bottles, and chloroform at 20% of the water volume of the sugar solution was added. This was followed by 1-butanol at 20% of the chloroform volume. The contents were mixed on a rotary shaker (Lab-Line Junior Orbit Shaker) set a 200 rpm to facilitate the denaturation of protein in the chloroform emulsion. After 30 min, the mixture was centrifuged at 16300 g (Sorvall type GSA high-speed centrifuge rotor) for 5 min. A gellike layer appeared in the water–chloroform interface. The aqueous phase was separated in a separatory funnel, and the chloroform phase was washed with water to remove all the gellike interface which contained the trace protein.

Protein Dye Binding. The sugar impurities in the gellike interface were collected in a test tube, and 0.02 mL was diluted to 0.27 mL with distilled water. The diluted sample was placed in an 8 × 60 mM test tube to which was added 0.03 mL of 1 M Tris-HCl at pH 7.5 containing 1% sodium dodecyl sulfate (Long, 1961).

Trichloroacetic acid (0.06 mL of 60% was added to each tube to give a final concentration of 10%. The samples were mixed on a Vortex mixer for 2 min. The contents of each tube was spot filtered under suction through a Millipore membrane of 0.22-μm pore size. The tube was rinsed with about 0.3 mL of 6% Cl_3AcOH. The entire filter area was flooded twice with 2 mL of the Cl_3AcOH solution.

The membrane was stained with 0.05% Amido Black 10B for 15 min. The dye solution was prepared by dissolving the Amido Black in a mixture of methanol, glacial acetic acid, and distilled water (45:10:45 vol %). Acid Orange 12, Orange G, and Coomassie Blue were not rec-

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Table I. Comparison of the Dye-Binding and the Kjeldahl Methods[a]

sample[b]	% protein[c] dye method	Kjeldahl method
A	0.366	0.205
B	0.370	0.509
C	0.004	0.002
D	0.303	0.253
E	0.004	0.004
F	0.006	0.018
G	0.004	0.002

[a] Correlation $r = 0.908$ ($p < 0.01$); standard error = 0.08. [b] Samples A, B, and D were floc-positive sugars, and samples C and E-G were nonflocculating sugars. [c] Values were averages of triplicate determinations.

ommended for this determination. The former two dyes are optically less sensitive to protein content than Amido Black since they have only one azo chromophore; the latter forms a weak absorptive complex with protein. The membrane was rinsed with distilled water followed by washing under agitation with a destaining solution (methanol–glacial acetic acid–distilled water, 90:2:8 vol %) until the washings were colorless. At this stage, the sample precipitates appeared as blue spots on an almost colorless background. The destaining solution was washed off by rinsing with distilled water for 1–2 min.

Excess membrane was trimmed from around the blue spot, and the portion of membrane containing the spot was transferred to a test tube. Six-tenths of a milliliter of the eluant solution (25 mM NaOH and 0.05 mM EDTA in 50 vol % aqueous ethanol) was added to the stained membrane, and the mixture was mixed in a Vortex mixer for several minutes to extract the bound dye. The absorbance of the eluate was measured against the blank eluant solution at 630 nm (Beckman Model 25 spectrophotometer) 15 min after the addition of the eluting solution.

A standard curve was prepared consisting of 1% chemically pure egg albumin dissolved in 100 mL Tris-HCl (pH 7.5). The egg albumin solution was diluted to contain from 1000 to 8000 μg/mL and 0 to 10 μg per mL. The egg albumin solutions were treated as were the samples from protein separation through the reading step in the spectrophotometer.

A Study of Variables. For determination of the optimum conditions necessary to obtain the best results, several variables were studied: namely, concentration of Cl_3AcOH, pore size of the membrane, volume of eluant solution, volume of sample solution, color stability, dye concentration, and staining time. The concentrations, sizes, staining times, and volumes found most suitable were those used in this study.

Kjeldahl Method for Protein Determination. The Kjeldahl method for nitrogen evaluation (AOAC, 1975) was used to evaluate the protein concentration in the floc-positive and floc-negative cane sugars by using 10-g samples. Results obtained by this method were compared to those obtained by the dye-binding technique.

RESULTS AND DISCUSSION

Comparison of Protein Determinations by the Dye-Binding Technique and the Kjeldahl Method. Results shown in Table I indicate that protein values determined by the dye-binding technique and the Kjeldahl method compare favorably. This fact was strengthened by a correlation (r) value of 0.908 with a standard error of 0.08 and a coefficient of 0.838. An analysis of variance

Table II. Replication Efficiency of the Dye-Binding Method[a]

sample[b]	I	II	III	mean[c]
A	0.692	0.569	0.563	0.607
B	0.560	0.687	0.772	0.673
C	0.016	0.012	0.020	0.017
D	0.494	0.571	0.558	0.541
E	0.016	0.024	0.011	0.017
F	0.027	0.054	0.039	0.040
G	0.014	0.021	0.025	0.020

[a] Shown as optical density values recorded at 630 nm. [b] Samples A, B, and D were floc-positive sugars, and samples C and E-G were nonflocculating sugars. [c] No significant difference between replications ($p < 0.05$).

and the Student's t test of the data showed that no significant difference existed between results obtained by both methods at the 5% level of probability. The results further showed that floc-positive sugars contained from 0.3 to 0.4% protein while the commercial nonfloccers had a concentration of 0.004–0.006%. The difference between these concentrations, coupled with the statistical correlation of the two methods used for protein evaluation, emphasizes the feasibility of the dye-binding technique for predicting the flocculating capabilities of cane sugar.

Standard Curve Development. Linear relationships were obtained for both standard curves determined with egg albumin at the two ranges tested (1000–8000 and 0–10 μg/mL) when concentration was plotted against optical density of the dye-bound protein.

Reproducibility of Dye-Binding Technique. Values shown in Table II suggest that the dye-binding method was capable of reproducible results on the sugar samples which were analyzed. An analysis of variance showed an F value of 0.37 for the variable of replications, indicating no significant differences in replicates at the 5% level of probability (3.88 needed for significance).

The dye-binding method herein described is proposed as a rapid, reliable, and reproducible procedure for the determination of trace amounts of protein which are responsible for flocculations of cane sugar solutions. This technique could easily be used as a quality control procedure to assure the good quality of procured cane sugar in the production of sugar-containing beverages, pharmaceuticals, and food products.

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