BEET SUGARS

Acid-Insoluble Constituents in Selected Samples

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Acidified aqueous concentrates from refined beet sugars sometimes produce undesirable precipitates on prolonged standing. The present investigation indicates that in addition to sugar beet saponin and its derivatives, the flocculent precipitate contains fat and various adsorbed colloidal impurities from the refined sugar. Paper chromatography and ionophoresis were found to be useful supplementary tools for investigating the minute amounts of floc obtained from laboratory scale preparation.

REFINED SUGAR, from both beet and cane, occasionally contains a few parts per million of material which will gradually precipitate from an acidified aqueous solution of the sugar. This so-called "floc" is undesirable in bottlers' concentrates and acidic pharmaceutical sirups. From a study at this laboratory, it appears that the composition of the floc from refined beet sugar is somewhat more complicated than the sugar beet saponin and its derivatives indicated by Eis, Clark, McGinnis, and Alston (*4*). In view of the interest in the problem throughout the beet sugar industry, the results of this investigation are presented here to supplement those reported by Eis and coworkers.

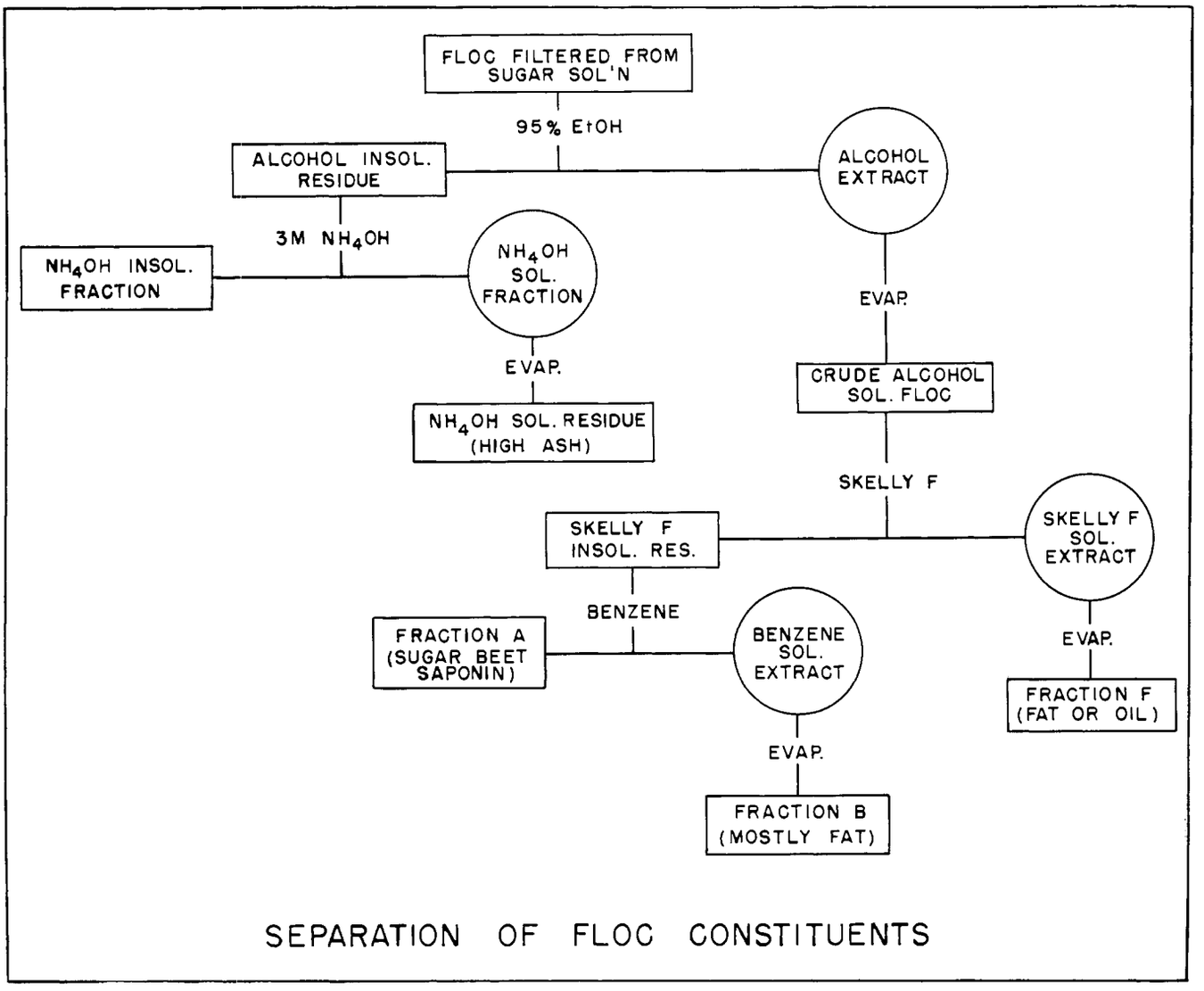
Isolation of Floc

The floc is produced when a solution of floc-containing refined sugar is acidified (pH 1 to 2.5 for fairly rapid formation). Although the formation of the floc is independent of the acid used, neutral salts at moderate ionic strength fail to give any precipitate on long standing. The floc particles in a sirup are readily redispersed by any motion within the solution. Centrifugation, even at the high speeds obtainable in a supercentrifuge, fails to sediment the particles adequately in sirups over a wide density range. The floc can be coprecipitated with aluminum hydroxide, and then the mixture can be centrifuged and washed, and the alumina hydrate removed with acid. Adsorption methods using calcium carbonate, magnesia, or charcoal failed to be adaptable to preparative purposes. Hot 95% ethyl alcohol extracts the floc former, but offers disadvantages in obtaining and maintaining large quantities of pure solvent, and it may extract materials other than those contributing to the formation of floc. Filtration (*4*) offers the simplest method for laboratory scale floc isolation after acidification of a sirup. In attempting to isolate a few parts per million of floc, it is obvious that special care must be observed to exclude extraneous contamination. All solvents and filter papers used were carefully checked, and purified, if necessary, so that no significant nonvolatile material was introduced. The filtration and isolation procedure finally used is as follows:

About 45 gallons of a sugar solution (25 to 35% by weight in distilled water) in a clean stainless steel kettle was heated to 170° to 180° F. After 80 ml. of concentrated sulfuric acid in 1 liter of water was added while stirring, the solution was allowed to cool for 24 to 48 hours. At the end of this time, large floc particles were clearly visible. A 4-liter Büchner funnel with suction hose attached was lowered gently into the kettle for the filtration. The floc was collected on Whatman No. 5 filter paper. This paper was changed when the flow rate became too slow, but not more than once during a run. Siliceous filter aid was not added because it caused high-ash products. Similar high-ash products resulted when tap water was used to dissolve the refined sugar.

Fractionation and Characterization Of Floc Materials

The fractionation procedure is shown schematically in the flow sheet. After filtration of the solution, the filter papers were placed in a Soxhlet thimble and washed slowly at room temperature with water until no acid or reducing substance could be detected in the wash water after a tenfold concentration. The papers were then extracted in the Soxhlet for at least 24 hours with 95% ethyl alcohol. This extract was evaporated to give the alcohol-soluble crude floc fraction. A considerable amount of dark residue remained on the filter papers after the alcohol extraction, so that the papers were treated portionwise in a beaker with hot, aqueous 3 *M* ammonium hydroxide. Each portion of solution was decanted and filtered through Whatman No. 43 paper. The combined filtrates were evaporated to give an alcohol-insoluble, ammonia-soluble fraction. An undetermined amount of dark material which failed to dissolve in any of the common solvents was left on the papers. The results of a number of such experiments are shown in Table I. The values recorded are



for actual recoveries and quantitatively probably only indicate order of magnitude.

Table I. Amounts of Floc Isolated from Refined Beet Sugars

Sample	Crude Alcohol-Sol. Fraction, P.P.M.	NH_3-Sol. Fraction, P.P.M.
A	8.7	1.1
	8.5	[a]
	9.7	[a]
	12.1	2.1
B	5.2	1.7[b]
C	3.3	[a]

[a] Not determined.
[b] Much decolorizing carbon left on filter paper after extraction.

The crude alcohol-soluble fraction was a gummy, brownish, intractable material with a characteristic odor of slightly rancid fat. No crystalline products could be obtained from any solvent either before or after acid hydrolysis. A partial separation of the dried mixture could be achieved by a 2-hour extraction in a Soxhlet thimble with Skellysolve F or by batch extraction with the same solvent in the beaker used for the initial isolation. Although only small quantities of this fraction (designated F) were available for analysis, samples were saponified (*11*), giving reasonable saponification numbers for a fat or oil (186.0 and 186.5), with very low free acid values. Saponification products were completely soluble in water above pH 7, and a water-insoluble material which was soluble in fat solvents was produced on acidification. Such alkali-solubility characteristics are not expected of waxes. At least a portion of the hydrolytic products reacted with urea in methanol to form addition compounds (*1*, *9*). Decomposition of the addition compounds and extraction of the hydrocarbon components gave material with an equivalent weight of 267, indicating a mixture of C_{16} and C_{18} aliphatic acids. Although the samples had obviously started to rancidify, the refractive index of fraction F was close to that reported for some natural oils (*6*). On the basis of these findings this fraction is considered to consist of a natural fat or oil.

The moderately tractable residue left after removal of fraction F was washed with dilute acid in a centrifuge tube until the washings (concentrated 50- to 100-fold) gave no test for reducing substance when spotted on paper and sprayed with 3,5-dinitrosalicylic acid reagent (*12*). The washed residue was placed in a Soxhlet and extracted with benzene, giving a soluble fraction B and a residual fraction A. Fraction B had many of the characteristics of fraction F. It could be saponified, giving somewhat higher saponification numbers (232) and free acid values

(34.1). On prolonged extraction of the thimble residue with benzene, some of the difficultly soluble fraction A was extracted into fraction B. Fractions F and B both seem to be real, since the material obtained by extraction of the initial alcohol-soluble crude floc with benzene is not completely soluble in Skellysolve F.

The solid, noncrystalline residue left after removal of the F and B fractions was soluble in ethyl alcohol (fraction A), and contained no appreciable ash. It appeared to be mainly the known sugar beet saponin reported by Eis *et al.* (*4*). Oleanolic acid and glucuronic acid were the main hydrolysis products that were obtained as follows:

Fraction A (83.2 mg.) was dissolved in 20 ml. of hot ethyl alcohol and filtered through a fine sintered-glass funnel (2 mg. recovered). After the filter had been washed with 5 ml. more of ethyl alcohol, 7 ml. of concentrated hydrochloric acid and 18 ml. of water were added and the mixture was refluxed for 7 hours. The solution was evaporated to dryness at room temperature. Water was then added and the insoluble material filtered and washed.

When the carbohydrate-containing filtrate was analyzed by paper chromatographic methods, glucuronic acid was identified as the major constituent. Total reducing substance in the sample showed 12.75 mg. calculated as glucose (*2*), or a maximum of about 17.5 mg. of glucuronic acid on the basis of an experimentally determined conversion factor. Analysis of a portion of the sample for glucuronic acid by an adaptation of the carbazole method for anhydrogalacturonic acid (*8*) indicated 14.4 mg. of glucuronic acid.

The water-insoluble portion of the hydrolyzate was dissolved in alcohol, evaporated, and dried in a vacuum desiccator over phosphorus pentoxide, yielding 44.3 mg. of dried material. It could not be crystallized from any solvent, but paper chromatography showed no saponin material remaining. It was suspended in a large volume of ether and filtered to remove a small amount of insoluble material. Evaporation of the ether solution left a residue which became partly crystalline on recrystallization from an acetone-water mixture. As treatment of a sample of this material with charcoal failed to give completely crystallized product, a small sample was chromatographed on a silica-Celite (3 to 1 mixture) column and developed with chloroform, and then with chloroform-butanol mixtures. Although various fractions were collected, most of them seemed to be similar and were not completely crystalline after precipitation from acetone-water. The main eluate fraction was dried and submitted for x-ray analysis, along with an authentic sample of oleanolic acid similarly recrystallized from acetone-water and dried. The x-ray patterns were identical, but it is of interest to note that crystals of oleanolic acid from acetone-water are polymorphic with those from ethyl alcohol–water. The optical rotation of the partially crystalline aglycone sample not fractionated on silica was $[\alpha]_D^{26} = 61.8°$, $c = 1.04$, in chloroform. Comparison of this value with that for pure oleanolic acid, $[\alpha]_D = 79.8$ (*5*), indicates that the sample contained impurity other than uncrystallized oleanolic acid.

The alcohol-insoluble, ammonia (aqueous or ethanolic)-soluble fraction obtained after separation of the crude alcohol-soluble floc has not been characterized extensively. One sample submitted for analysis indicated about 37% ash, 94% of which was silica. This dark-brown material fails to give qualitative tests for carbohydrate, enol, triterpene, or sterol. The ammoniacal solution does not give any appreciable precipitate when acidified. The colored material can be precipitated on barium carbonate, but after solution of the barium carbonate in hydrochloric acid and prolonged dialysis, the organic material still contains a large amount of ash. Further work on this fraction has been postponed until a larger quantity is available.

Table II shows the relative amounts of fractions A, B, and F (material soluble in organic solvents) in flocs from refined sugars from three different sources, representing three different beet-growing areas. From Tables I and II it can be seen that the amount of saponin material (fraction A) recovered from each of the refined sugars is around 2 p.p.m.

Table II. Composition of Crude Alcohol-Soluble Floc

Sample	Fraction F (Fat), %	Fraction B (Mixture), %	Fraction A (Saponin), %
A	39.5	40.3	19.8
	35.5	48.6	15.3
B	46.0	11.9	42.5
C	33.4	14.1	52.4

Paper Chromatographic and Ionophoretic Examination of Floc Constituents

Paper chromatography was used to investigate the nature and homogeneity of fraction A and its derived products. The carbohydrate moiety obtained by hydrolysis was examined by known methods (*12*), and found to consist mainly of glucuronic acid. In addition, however, the chromatograms of the carbohydrate constituents from a number of different floc preparations always indicated the presence of small amounts of both glucose and xylose, neither of which has previously been reported in sugar beet saponin. No ketose could be detected. A sample of glucuronic acid (lactone) treated like saponin revealed no decomposition leading to xylose under these conditions.

By using a spray reagent of 10% antimony pentachloride in chloroform (*10*) it was possible to detect minute amounts of saponin, sapogenin, and fatty material on paper. Both oleanolic acid and saponin give characteristic pink spots without heating, turning to blue on standing or when moistened. As little as 5 microliters of 0.01% solution of saponin and sapogenin will give a visible spot. The fatty fractions give brownish spots with the spray reagent. Separation of saponin and sapogenin is readily achieved using benzene or ether as a solvent. Unfortunately the sapogenin moves as fast as the solvent front ($Rf = 1.00$), and the saponin fails to move at all ($Rf = 0$). The fatty material moves to the same place as the sapogenin. When alcoholic developing solvents (and aqueous mixtures) are used, the saponin moves completely and the sapogenin fails to move. After about 60 trials using various solvents recommended for saponins and similar compounds and including reversed phase chromatography, an ethyl alcohol–ethyl acetate–water mixture (120, 80, and 160 volumes, respectively) was found which resolves the oleanolic acid and saponin with *Rf*'s of about 0.2 and 0.8, respectively. It is not completely satisfactory because, if the concentration of the saponin-sapogenin mixture is much greater than 0.1%, the resolution of the spots is impaired. Experiments showed the presence of no, or only trace amounts of, oleanolic acid in the F, B, and A fractions from refined sugar, and it is conceivable that these small amounts could have come from hydrolysis of the saponin during the isolation.

Paper ionophoresis of fraction A in pH 10 borate buffer was attempted, using essentially the apparatus and techniques suggested for proteins and carbohydrates (*3, 7*). Antimony pentachloride in chloroform was employed as a spray reagent for the detection of triterpenes. The saponin migrates toward the anode, and under the conditions of these experiments, the sapogenin remains adsorbed at the origin. The glycosidic material in fraction A migrates at the same rate as the known sugar beet saponin. In some of the saponin preparations, the crude ash-free glycosidic material appears to consist of two components (*4, 5*). In these samples the main component migrates like the known saponin, with an indication of a small amount of a faster moving component. The two spots are not due to the glucuronic acid–lactone equilibrium, because at pH 10 glucuronic acid migrates as one component only. It has

not been determined whether the difference in migration rate is due to variation in the carbohydrate or aglycone moiety.

Ionophoresis of the sapogenin portion is successful only if an organic solvent is added to the buffer to increase the solubility of the triterpene organic acids. The sapogenin from the hydrolysis of saponin in fraction A and pure oleanolic acid migrate at the same rate in a buffer composed of 60 volumes of ethyl alcohol and 40 volumes of pH 10 buffer. However, as a mixture of ursolic and oleanolic acids, which are isomeric triterpene acids, was not resolved, further work along this line was abandoned.

The use of these techniques in this investigation has been extremely helpful, because valuable information can be gained from only a few milligrams of material in a relatively short time. The application of some of these methods to the rapid quantitative determination of floc in refined sugar has been considered, but has not yet been investigated thoroughly.

Discussion

The work on floc at this laboratory has been concerned with the material actually formed when a refined sugar is dissolved in water and acidified. The authors have, therefore, chosen to consider that all materials isolated by filtration (other than obvious gross foreign particles) are components of the floc. It is probably true that the acid-insoluble saponin material is responsible for the formation of the floc on acidification. Because of its colloidal nature, however, it tends to act as a scavenging agent as it slowly forms aggregates, picking up impurities present in the original sugar or in the water used to prepare the sirup. For example, one sample of isolated floc contained colloidal decolorizing carbon particles passed by the filters in the refining process. The silica content and the brownish coloring matter of the floc are probably picked up in the same manner. The fat component of the floc may come from the oils added to the processing liquors to prevent foaming during the boiling operations. The saponin, acting as a soap in neutral solution, may carry along some of the oil which ordinarily remains in the crystallizing liquors.

Investigation of fraction A will be continued to try to complete the knowledge of its constitution and properties. The results obtained thus far are similar to those of previous workers (*5*) on sugar beet saponin, who also failed to account quantitatively for all the hydrolysis products. It is hoped that more adequate information about the nature of fraction A may suggest improvements in the methods for its elimination from processing juices.

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BROMIDE RESIDUES

Determination in Fresh Fruits after Fumigation with Ethylene Dibromide

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A method is described for the determination of ethylene dibromide and bromide residues in fresh fruits and vegetables which were fumigated with ethylene dibromide. Papaya and pineapple flesh showed no increase in ethylene dibromide or bromide content 6 days after fumigation. Avocado flesh retained 7.3 p.p.m. of ethylene dibromide and 5.5 p.p.m. of bromide. Banana flesh retained no ethylene dibromide, but an excess of 28 p.p.m. of bromide.

ETHYLENE DIBROMIDE (1,2-dibromoethane) is an excellent fumigant for destroying infestations of the oriental fruit fly (*Dacus dorsalis* Hendel) and the melon fly (*Dacus cucurbitae* Coq.) in fresh fruits and vegetables. The potential usefulness of this material was first demonstrated in laboratory screening tests made by Balock and Lindgren (*3*), and its adoption as an approved treatment was the result of subsequent developmental research conducted by Balock (*2*). The use of ethylene dibromide permits the treatment of most fruits without injuring them, as was often the case with earlier methods, and it has already increased the movement of Hawaiian crops to mainland markets.