# Acid Beverage Floc from Sugar Beets

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## ABSTRACT

**Acid beverage floc (ABF), a flocculated turbid material that can form in sugar-sweetened, acidified, carbonated beverages after several days standing, is a customer problem to beverage bottlers and their suppliers of sugar. ABF from beet sugar has been reported to be caused by a saponin from the beet plant, and recent work has shown the presence of several saponins in sugarbeet. ABF from cane sugar is caused when a negatively charged cane polysaccharide forms a colloidal network with protein under acid conditions. Our investigations show that isolation and test procedures for saponins, as reported in the literature, are actually for oleanolic acid. ABF from beet sugar is proposed to have a two factor basis: a negatively charged component and a positively charged component interact at acid beverage pH, forming a coacervate and subsequently coagulating into a floc. The negatively charged factor can be oleanolic acid, any of the saponins that contain a glucuronic acid moiety, or beet cell wall polysaccharide containing uronic acids. The positively charged component can be protein or peptide, with isolectric point above the beverage pH of 2.5 to 3.0. ABF can be made by adding these components to non-floccing sugars.**

**Additional Key Words:** *Beta vulgaris* L., saponin, acid beverage floc, oleanolic acid, beet sugar, carbonated beverage

Acid beverage floc (ABF), which can form in sugar-sweetened carbonated soft drinks after several days standing, has been ascribed to both beet and cane sugars. In general, any haze or turbidity in a soft drink is referred to as "floc," but specific characteristics define acid beverage floc, most notably that shaking will make it disappear. Beet and cane flocs can appear as turbidity or as "cotton ball floc." Beet sugar floc is more granular in appearance and less fluffy than cane sugar floc. Beet sugar floc long has been ascribed to saponins (Eis et al. 1952; van der Poel et al. 1966; Carruthers et al. 1967), but in our tests, authentic saponin added to non-floccing sugars in amounts approximating those reported in floccing sugar did not necessarily produce floc. The literature supports this: Eis (1952) says that "separated floc can produce effervescence and flocculation when sufficient neutral solution of the floc is added to carbonated beverages." By separated floc, the author meant all material that was precipitable at pH 2. In the authors' experience, "sufficient" is far above the <1 to 30 ppm levels of saponin reported in white sugars. Sufficient levels are above several hundred ppm. The objective of this study was the isolation of sugarbeet saponins for further study of their effect on acid beverage floc formation. The evidence for sugarbeet saponins being the cause of floc may be circumstantial.

Acid beverage floc from cane sugars has two causative factors: a polysaccharide containing glucuronic acid and a protein. At least one specific regional acid beverage floc is caused by a specific microbial infection. In the general case, the polysaccharide is derived from plant cell wall material. The protein may be of cane origin or the residual from enzyme addition. The glucuronic acid and the primary amine residues are oppositely charged at beverage pH, and, through charge attraction, combine to form a coacervate as the basis for a floc network. Suspended solids, colloidal material, and high molecular weight soluble polymers such as dextran and starch can come out of solution and enhance the appearance of a floc that has already formed.

Many tests for ABF are available, but none is good and all take several days to show results.

**Saponins**

Saponins are a class of compounds widely distributed in the plant kingdom in legumes, roots, shrubs and bushes, in varying degrees of concentration. Various saponins have been used as soaps because of their surface active properties. Saponin-containing plants, or their extracts, have been used in herbal medicine, in treatment of various complaints including liver and cholesterol related diseases (Ireland et al. 1986), and as anti-fungal

agents (Hallanoro et al. 1990).

Saponins fall into two classes: triterpene-based and steroid-based. The attachment of sugar group(s) and glucuronic acid to the base aglycone defines the molecule as a saponin. Sugarbeet (*Beta vulgaris* L.) is known to contain at least three triterpene-based saponins, all of which are glucuronic acid glycosides of oleanolic acid (Ridout et al. 1994), as shown in Figure 1. Two additional compounds, referred to as seco-glycosides of saponins, recently have been isolated from beet leaves and roots (Massiot et al. 1994). Up to six beet saponins have been postulated.

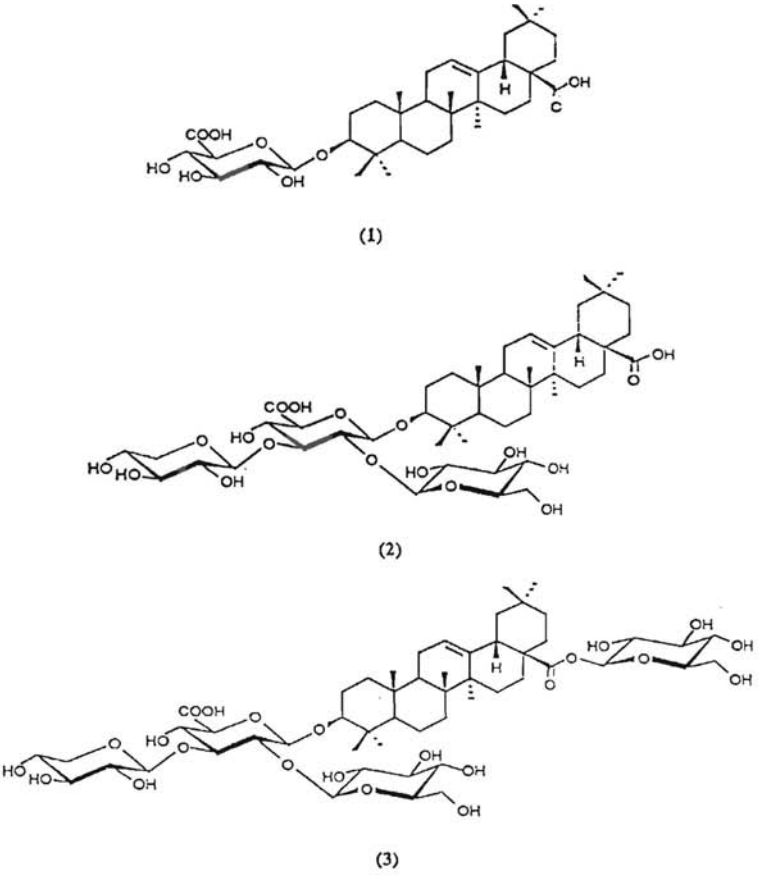



**Figure 1**. Three Saponins of Sugarbeet.

Saponins are reported in sugarbeet at levels of 0.01% to 0.2% of beet (Carruthers et al. 1961; van der Poel et al. 1966; Hallanoro et al. 1990; Schiweck et al. 1991), and at less than 100 ppm, generally less then 20 ppm, in white sugar. Saponins are most densely concentrated just under the sugarbeet skin, where they function as plant defense compounds against disease and frost damage. They are also located in cell membranes (Hallanoro et al. 1990). They are most highly concentrated in small beets grown in warm climates.

In recent work comparing isolation systems and their products we suggested that material reported as "saponin" in sugars and process streams may in fact be oleanolic acid (Roberts et al. 1996). Oleanolic acid is derived from saponins by hydrolysis. The purpose of the work reported here was to determine whether oleanolic acid, derived from saponin by hydrolysis under processing conditions, is the actual flocculating agent in ABF, rather than saponin, as previously reported.

## MATERIALS AND METHODS

### Extraction of Saponins

Beet peelings were obtained from fresh sugarbeet in the S.P.R.I. labs and subjected to several methods of extraction. The resulting extracts were evaluated by thin layer chromatography and by GC-MS.

**1. Method of Rother (1962): aqueous extraction.** Fresh beet peelings weighing 5.5 kg were covered with water in a blender and divided into small pieces. The slurry was heated to 90EC and filtered on fabric. The residue was suspended in water, heated, and filtered again on fabric. The filtrate was adjusted to pH 1.5 with HC1, heated to 90EC for one hour, and allowed to settle overnight. After settling, the supernatant liquid was decanted. The residue was mixed with filter aid and filtered; the filtered residue was washed with water, adjusted to pH 1.5 with HCl, and allowed to air dry. The dried residue was extracted in a Soxhlet extractor with ethanol, the ethanol solution was concentrated and poured into water at pH 1.5. The precipitate was dissolved in hot ethanol and again precipitated by pouring into pH 1.5 water. The precipitate was filtered off on hardened paper, dissolved in water, and evaporated to dryness at low temperature. The yield from the 5.5 kg of fresh beet peeling was 3.0 g of brown material. Analysis of this material by TLC (as described below) showed oleanolic acid (the aglycone, or sapogenin) and nothing corresponding to saponins. Mass spectroscopy analysis confirmed the presence of oleanolic acid. Apparently the harsh acidic treatment hydrolyzed the saponins, leaving only oleanolic acid in the isolation.

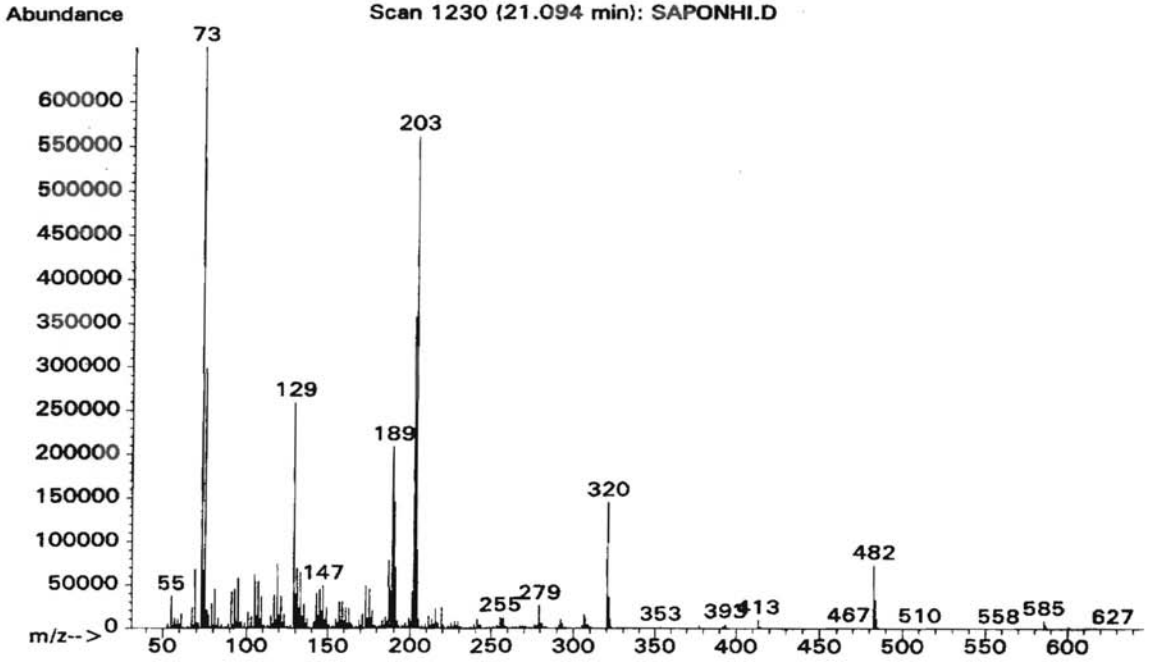
**2. Method of Ridout et al. (1994): aqueous extraction.** In another experiment, 1734 g of beet peel was ground in a blender. The slurry was filtered on fabric and the residue mashed with water. The pH of the filtrate was adjusted to 1.5 with HCl, heated to 85EC for 15 minutes, cooled overnight, and filtered on fabric coated with filter aid. The filtrate was returned to the filter twice more and the residue was washed with warm 1N HCl. All filtrates were discarded. The filter was then washed with warm 2N NaOH solution until the filtrate was clear. The filtrate was placed in a large beaker and HCl was added to reduce the pH to 1.5. The precipitate was collected on fabric coated with filter aid as before, washed with 1N HCl, and the filtrate discarded. The filter was then washed with warm 2N NaOH. The filtrate was acidified to pH 1.5 with HCl, filtered through Whatman 542 paper, washed with water, and extracted with 500 ml of warm ethanol. The filtrate was evaporated to dryness, then taken up in water and freeze dried, yielding 2.0 g of brown material. TLC analysis showed oleanolic acid but no saponin.

**3. Method of Ridout et al (1994): methanol extraction.** Freeze dried beet peel (650 g) was crumbled into small pieces and extracted in a Soxhlet extractor with methanol. The methanol was evaporated under reduced pressure; the residue was dissolved in water and extracted several times with 1-butanol. The butanol was evaporated and the residue was dissolved in water and dialyzed against flowing tap water in a 12,000 MW cut-off bag for 24 hours. The material remaining in the bag was filtered, concentrated, and freeze dried, yielding 5.6 g of cream colored material. Thin-layer chromatography and mass spectrometry showed that the material contained saponins.

**Thin layer chromatography of isolates from aqueous extraction**

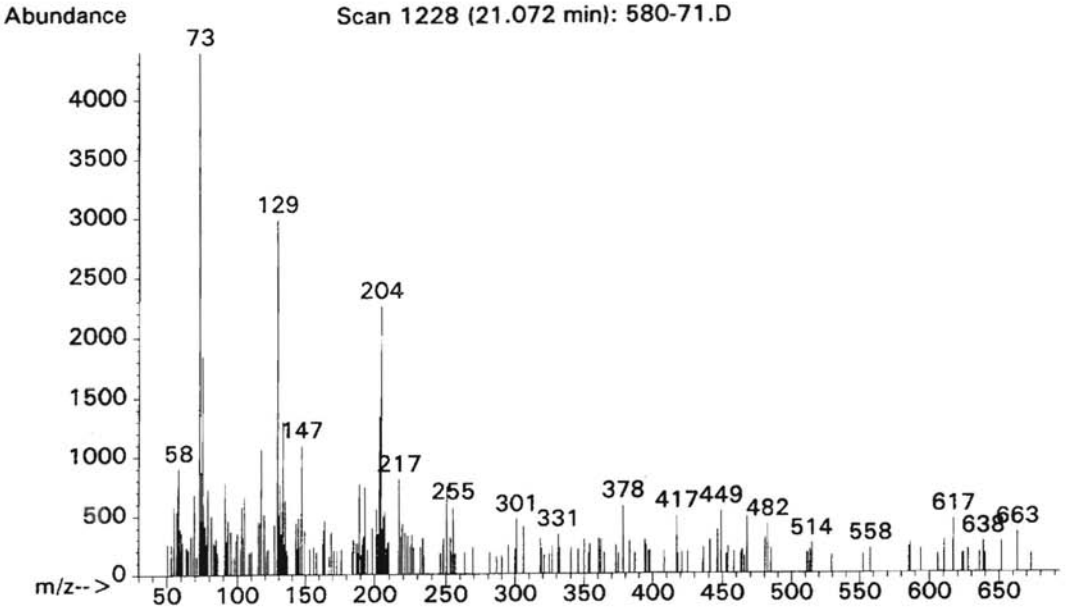
Isolates prepared by the traditional aqueous extraction methods (1 and 2), with repeated extractions at pH 1.5 and washing with base showed only oleanolic acid in the final dried extract and no saponins. Oleanolic acid identification on thin layer chromatography (solvent system: chloroform; methanol; water 65:35:10), made visible by 2N $H_2SO_4$ or anisaldehyde spray, was confirmed by gas chromatography-mass spectrometry identification, as shown in Figures 2 through 4.

Method 1 (Rother et al. 1962), aqueous extraction at low pH, yielded 3g (0.05% on beet peel) brown solids; Method 2 (Ridout et al 1994), yielded 2 g (0.12% on beet peel) of brown material. Method 3 (Ridout et al. 1994), similar to that of Ireland (1986) using methanol extraction and not including low pH treatment, yielded 5.6 g (0.8% on beet peel) of cream colored material. Thin layer chromatography of the methanol extracted material showed five major components, two of which traveled with an authentic



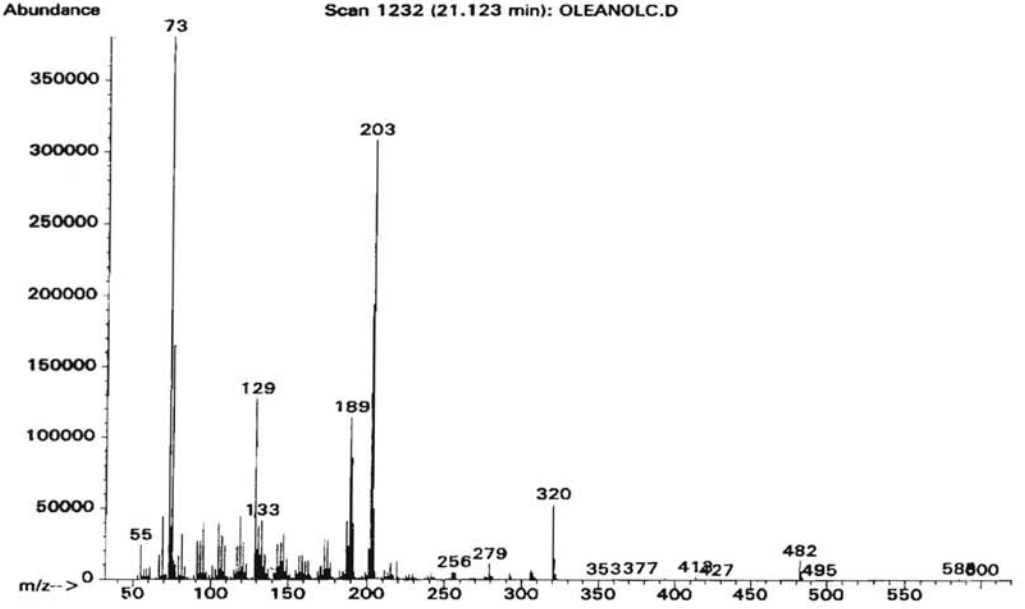

**Mass spectum showing oleanolic acid in aqueous extract of beet peel.**

**Figure 2**. Mass Spectrometric Analysis of Sugarbeet Extracts.




**Mass spectrum of methanolic extract of beet peel, showing absence of oleanolic acid.**

**Figure 2.** (Continued)




**Mass spectrum of authentic oleanolic acid TMS. Match quality with beet peel extract is 99%.**

**Figure 2.** (Continued)

saponin (probably soybean) obtained from Sigma Chemical Co. It should be noted that "saponin extracts" supplied to S.P.R.I., Inc., by several sugar companies (sponsoring companies of S.P.R.I., Inc.) appeared to consist mainly of oleanolic acid.

**Gas chromatography-mass spectrometry (GC-MS)**

Samples containing oleanolic acid were converted to the trimethylsilyl (TMS) derivative by use of Pierce Tri-Sil" in pyridine solution. Gas chromatography (GC) was performed on a Hewlett Packard 5890. GC conditions were: 250EC for 10 min; increase temperature 5EC per min to 310EC for 10 min. The column was a 30 m x 0.25 mm fused silica with 0.25F film thickness of 5% phenyl methyl silicone. Oleanolic acid eluted at 21.11 minutes under these conditions. Mass spectrometry (MS) was conducted with a Hewlett Packard 5972 mass selective detector.

**Charged species at beverage pH**

Moving boundary electrophoresis on oleanolic acid was conducted in sucrose solution at pH 3, adjusted with phosphoric acid. Oleanolic acid moved towards the anode. The oleanolic acid was thereby shown to have a negative charge at pH 3 in sucrose solution (simulating acid carbonated beverage conditions).

## RESULTS AND DISCUSSION

**Composition and Structure of Beet Saponin Isolates**

Saponins are known to exist in variety in any one plant - a single structure is not common. Variations in the sugar moiety structure and linkage position are observed. Sugarbeet saponins are no exception. The three forms shown in Figure 1 all have oleanolic acid, a carboxylic acid triterpene, as their base unit.

Comparison of the aqueous methods of extraction with the methanolic method indicated that the sugarbeet saponins are indeed present in the peel. Chromatographic and mass spectroscopic data demonstrate that the saponins extracted by acidic aqueous methods have been hydrolyzed by the strong acid treatment so that only the aglycone (or sapogenin), oleanolic acid, remains. This observation throws some doubt on earlier work, all of which isolated saponins by aqueous extraction with strong acid treatment. These earlier results may have been due to the presence of oleanolic acid only and not to saponin, as ascribed. Earlier workers did not have the benefits of GC-MS but had to rely on colorimetric tests, which may give a false positive for saponins when oleanolic acid is present.

In recent work, Ridout et al. (1994) found saponins by aqueous extraction, not in extracts of beet roots, but only in extracts from beet molasses, where the compounds may be expected to concentrate. Subsequent investigations by the same workers (Massiot et al. 1994) found saponins in methanol extracts of sugarbeet roots and leaves.

**Beverage Floc**

Floc tests (50 Brix, phosphoric acid to pH 2) were run on non-floccing sugars with the addition of various amounts of beet extract, or commercial saponin not from beet, or oleanolic acid. Saponin and oleanolic acid were also tested in combination with gelatin or α-amylase protein. The methanolic extract of beet root formed a floc, as did the combination of oleanolic acid and protein.

The observation that saponins are apparently hydrolyzed during the acid extraction raises a basic question about causes of floc formation. The original assumption was that floc material was acid insoluble, and therefore the aqueous extraction method at low pH was developed. Saponins, if they pass through processing into the white sugar, might be hydrolyzed in carbonated beverages, where pH is about 2 to 2.5. In that case oleanolic acid and not saponin would be the immediate cause of floc formation.

In our studies, only the material isolated from sugarbeet by methanolic extraction was able to form floc when added to non-floccing sugar. This material contained whole saponin, indicating that saponin alone does form beverage floc. This methanolic extract contained many other compounds from beet in addition to saponins. The material isolated by acid extraction was able to form floc only when a protein was also added.

The observations on hydrolysis explain why Eis (1952) observed flocculation only after adding back a relatively large quantity of isolated floc material. The amount added probably was sufficient to form a haze rather than a true floc.

In past work (Roberts 1996; Clarke, 1992) we have observed that isolated beet sugar floc from beverages contained beet cell wall polysaccharide with galacturonic acid residues and protein. The polysaccharide, given the trivial name Indigenous Beet Polysaccharide (IBP), is comparable to the sugarcane cell wall polysaccharide, which contains glucuronic acid groups, and can cause acid beverage floc when in combination with a protein. At beverage pH the acid groups become negatively charged, the protein groups become positively charged, and charge attraction brings the molecules together to form a coacervate and then a flocculating network that entraps colloidal and suspended material to form a visible floc. We propose that a similar mechanism can be responsible for beet sugar floc. A carboxylic-acid containing molecule, such as saponin in a form that con-

tains glucuronic acid, oleanolic acid, or cell wall polysaccharide, becomes negatively charged at low pH. A molecule containing an amino-group, such as protein, peptide, or other, becomes positively charged. The two come together from charge attraction to initiate a floc network. This explains the observation of floc without saponin present since another negatively charged molecule, possibly oleanolic acid, can participate. Both saponin and oleanolic acid contain a glucuronic acid group. Beet cell wall polysaccharides contain galacturonic acid groups. This mechanism accounts for the presence of saponin without floc, if insufficient protein or positively charged amino group is present. It also accounts for the presence of floc without saponin, if oleanolic acid or IBP is forming a coacervate with protein.

Floc can be made in non-flocculating beet or cane sugars by the addition of oleanolic acid and protein, as shown in Table 1.

**Table 1.** Determination of floc formation. Preparation: 240 g sugar, 500 ml water, 60 ml formaldehyde, pH 2-3, 10 days. If added: oleanolic acid, 5 mg; protein, 5 mg.

| Sugar | Addition | Results |
|---|---|---|
| Beet, non-flocculating | | no floc |
| Beet, non-flocculating | methanol extract | floc |
| Beet, non-flocculating | oleanolic acid | floc |
| Beet, non-flocculating | protein | no floc |
| Beet, non-flocculating | oleanolic acid + protein | heavy floc |
| Beet, floc positive | | fine floc |
| Beet, floc positive | saponin | fine floc |
| Beet, floc positive | oleanolic acid | heavy floc |
| Cane, non-flocculating | | no floc |
| Cane, non-flocculating | oleanolic acid + amylase | fine floc |
| Cane, non-flocculating | oleanolic acid + gelatin | coarse turbidity |
| Cane, non-flocculating | oleanolic acid + beef serum | coarse turbidity |
| Cane, non-flocculating | oleanolic acid + dextranase | fine floc |
| Cane, non-flocculating | oleanolic acid + invertase | fine floc |

**Saponin Tests**

All the postulates and observations in the literature depend on the validity of saponin tests The traditional tests must be re-evaluated using instrumental analysis to distinguish between saponin and oleanolic acid. Literature discussion on the most frequently used test, the antimony pentachloride test, points out that its color reaction is not specific for triterpenes (van der Poel et al. 1966). Therefore, the test cannot distinguish between saponin and oleanolic acid.

## CONCLUSIONS

Comparison of traditional aqueous acid extracts of "saponin" from sugarbeet substrates with methanol extraction has shown that aqueous extraction yields only the aglycone of beet saponins, oleanolic acid. Chromatographic and mass spectrometric evidence support this.

A re-examination of the role of saponin in acid beverage floc and in foaming is recommended since oleanolic acid may be responsible for some of the problems ascribed to saponin.

It is proposed that two factors are required to form floc, a molecule that is negatively charged at beverage pH, such as saponin, oleanolic acid, or cell wall polysaccharide, and a molecule that is positively charged at low pH, such as protein or peptide The two molecules combine in solution through charge attraction to form a coacervate that develops into a floc network.

A quick test is needed to identify the presence of floc-causing factors in sugars used in acid carbonated beverages.

## ACKNOWLEDGEMENTS

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