was added, the more adenosine triphosphate was required. These observations suggest a quantitative relationship between the substrate for the polyphenol oxidase reaction and the adenosine triphosphate effect. By measuring redness in potato slices, it was possible to demonstrate inhibition of color with as little as $10^{-5}M$ adenosine triphosphate. Higher concentrations of adenosine triphosphate reduced redness with no apparent change in initial rates.

Addition of dihydroxyphenylalanine increased darkening in all slices. At the same time, increasing the amount of dihydroxyphenylalanine counteracted the color-preventing action of adenosine triphosphate. This could be expected if addition of adenosine triphosphate resulted in an increase in quinone-reducing material. If enough substrate were added to build up a reserve of quinone, then the rate of forming reducing substance by adenosine triphosphate, at its maximum, might be insufficient to reduce all the quinone formed. Therefore, darkening would proceed. This probably occurred in the case of eggplant and, to some extent, of mushroom. A similar situation was created in potato slices to which large amounts of substrate were added, showing that the effectiveness of adenosine triphosphate in preventing formation of color depends also on the amount of substrate present.

Nearly all experimental data indicate that the inhibiting effect of adenosine triphosphate upon color development paralleled that of ascorbate, which reduces the quinones formed in the early stages of oxidation (*7*). Neither ascorbic acid nor adenosine triphosphate is a true inhibitor, as both permit enzymatic oxidation to go on (*2*, *4*).

Color differences between adenosine triphosphate-treated and untreated potato slices were apparent in the first hour and often persisted after holding overnight. The early stage (reddish brown) of enzymatic color formation in potato slices was reversed by addition of adenosine triphosphate in the presence of air, but not under vacuum nor under nitrogen. Ascorbate, however, decolorized initial reddish brown color in the absence of air. This was true when any one or a combination of the following was also added to the slices at pH 6.2: phosphate buffer, succinate, and dihydroxyphenylalanine. Oxygen, therefore, appears necessary for the production of quinone-reducing substances by adenosine triphosphate in living tissue. Moreover, active cell particles also seem necessary for the adenosine triphosphate effect (*4*, *5*).

The prevention of browning by adenosine triphosphate was maintained much longer in slices kept cold than in slices left at room temperatures. A possible explanation is that in the cold, adenosine triphosphate is decomposed more slowly by hydrolyzing enzymes present in potato tissue (*10*). The low temperature also prolongs the metabolic activity of the cytoplasmic particles, mitochondria (*6*). Adenosine triphosphate acts indirectly via small and very labile potato cell particles (*5*), which are laborious to isolate and harder to keep in an active state. In slices, some of the same enzymatic phenomena found in carefully prepared particles could be demonstrated with far less time and effort. Direct measurement of color changes in enzymatic and other reactions upon slices is rapid and convenient.

In all plant materials studied, there was a positive correlation between the anatomical location of vascular tissue and of seeds and the location of the darkest areas. Such localization of polyphenol oxidase activity was most pronounced in sweet potato, avocado, and eggplant. To establish whether or not adenosine triphosphate interferes with color formation in slices of sweet potato and eggplant, considerable modifications in method and extensive studies would have been required.


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**BEET SUGAR IMPURITIES**

# Colorimetric Determination of Saponin as Found in Beet Sugars


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**The unsightly flocculent precipitate that sometimes forms when impure beet sugars are placed in acid solution has recently been studied. A saponin in the beet root, if not removed during the refining process, is responsible in part for this apparently harmless, but unsightly floc. An accurate, rapid colorimetric method has been developed for evaluation of finished sugars and for refinery-control measures. Visual examination of numerous sugars for floc gave results that compared well with the quantities of saponin found.**


THE COMPOSITION of acid-insoluble substances in beet sugars has been studied recently (*1*, *7*) and earlier references to the subject were included. There remained the problem of sugar refining-control measures, on which work is continuing. However, to have control, it is necessary to have available rapid and accurate analytical methods. Walker (*6*) developed a colorimetric method based upon the reaction of sugar beet saponins with antimony pentachloride. West and Gaddie (*8*) tested this method extensively to correlate results with the observed floc precipitate.

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Johnson and Diehl (*2*) improved a method based upon the turbidity produced by the reaction of certain substances present with quaternary ammonium compounds. Some saponins yield a green color with ferric chloride in the presence of alcoholic sulfuric acid (*5*). Reddy and coworkers (*4*) made use of the phenylhydrazine, sulfuric acid–ethyl alcohol reagent and the sulfuric acid–ethyl alcohol reagent for the colorimetric determinations of cortisone and a urinary conjugated 17-hydroxycorticoid.

The present study has confirmed, in part, the findings of other workers (*1*, *7*) that the acid-insoluble material in beet sugars is composed of a mixture of saponins, saponin metal salts, fats, and fatty acids. This work indicates that the method of Walker (*6*) reports a part of the fats and traces of sucrose in addition to the saponin; it also has the disadvantage of requiring hot glacial acetic acid—an acid irritating to the analyst. The method of Johnson and Diehl (*2*) presumably reports all negatively charged particles in terms of a floc number. Walker's method (*6*) and gravimetric methods require that all the sucrose be separated from the precipitated saponin—a difficult separation.

This paper reports the work done to overcome some of the limitations of previous methods and, at the same time, to provide a method which is rapid, accurate, and within the capabilities of a routine technician.

## Chromatographic Procedure

**Chromatogram Characterization of an Acid-Insoluble Fraction.** Several samples of beet sugars were acidified with hydrochloric acid to pH 1.0. The solution was filtered through a fine-porosity, fritted-glass filter and was washed with 2.0 pH hydrochloric acid water until the washings were free of sugar, as determined by 1-naphthol test. The filter was extracted with 5.0 ml. of hot methanol and the extract was chromatographed on paper.

**Chromatographic Materials and Methods.** Whatman No. 4 paper was in 4 different solvents:

| | | |
|---|---|---|
| 1. | Benzene | |
| 2. | Isopropanol | 70 vol./vol. |
| | 1-Butanol | 10 vol./vol. |
| | Benzene | 25 vol./vol. |
| | Water | 25 vol./vol. |
| 3. | Methanol | 90 vol./vol. |
| | Water | 10 vol./vol. |
| 4. | Distilled water | |

The chromatograms were run upflow about 4 inches. The strips were then dipped in a reagent solution of antimony pentachloride, 10% in chloroform, to bring out the location of saponins and fats.

In addition to fats, two forms of saponin were found to be present—the normal acid saponin and a metal salt of saponin having no mobility in the various solvents. This appeared to be calcium saponin, as it has the same chromatographic properties. Similar chromatograms using only the isopropanol solvent were tested for the presence of sugars. One gram of 1-naphthol and 15 ml. of phosphoric acid in 100 ml. of ethyl alcohol was used as a spray reagent to test for certain sugars; an ammoniacal, 10% silver-nitrate-spray reagent was used as a test for reducing sugars. Sucrose was found to be present.

### Apparatus

A spectrophotometer or photoelectric colorimeter which determines transmittance at 575 mμ. The light path through the solution may be from 1.0 to 2.5 cm.; the longer light path gives greater precision.

Glass volumetric ware.

Büchner filter funnels with fritted-glass disks (Corning No. 36060, 60 ml., fine).

Water baths.

Fisher filtrator, vacuum bell jar with base.

### Reagents

Sulfuric acid, concentrated.
1-Naphthol, 10%, in methanol.
Hydrochloric acid, concentrated.
Methanol, absolute, redistilled.
Benzene, anhydrous, redistilled.
Saponin, purified.

**Preparation of Purified Saponin.** Several workers (*1*, *3*) describe the extraction and purification of saponin in detail. An outlined method follows:

Treat beet root skins with 11.5 pH sodium hydroxide solution. Heat the slurry to 90° C. for 1 hour. Allow the mixture to cool, then filter. Adjust the filtrate to pH 1.5 and heat to 90° C.; allow the solution to cool and the floc to form. Add filter aid to the solution and filter through a Büchner funnel using Whatman No. 4 paper. Redissolve the residue with hot sodium hydroxide solution, pH 11.5. Reprecipitate the saponin with sulfuric acid; floc forms immediately. Filter the solution by gravity through paper; allow the residue to air dry. Extract the residue in a Soxhlet apparatus with benzene and with redistilled methanol. Concentrate the methanol extract by warming; treat with carbon and filter (*3*). Pour the carbon-treated extract into acidified water, pH 2. Filter by gravity through paper. Redissolve the residue in hot methanol and concentrate; add to acidified water and filter. Repeat the above purification process or the individual steps, several times. Wash the residue with distilled water until the washings show nearly neutral pH. The air dried precipitated saponin has a light tan color and an uncorrected melting point of 209–211° C.

### Analytical Procedure

**Extraction of Saponin.** Dissolve 100.0 grams of sugar to be tested in a 300-ml. Erlenmeyer flask, using 200 ml. of hydrochloric acid water, pH 1.0. Tests indicate that best results are obtained by dissolving and filtering at room temperature. Invert the flask into a fine (F-fritted) glass filter funnel. Provide suction through a suitable filtering flask. Allow approximately 1 hour for complete filtration. Wash the outside lip and interior of the Erlenmeyer flask into the funnel using portions of hot hydrochloric acid water, pH 2.0, (hereafter called acidified water). Apply gentle suction during the washing procedure. After about 40 ml. of acidified water have passed through the funnel, wash the inside part of the stem of the funnel with acidified water. The final washings can be performed with a Fisher filtrator unit.

Again wash the precipitate and test by mixing 2 ml. of the last washings with about 0.25 ml. of 1-naphthol solution and 3 ml. of concentrated sulfuric acid. Continue the washing with acidified water until the 1-naphthol test shows no sugar. Use care to wash all droplets of sugar solution out of the stem of the funnel. Dry the funnel in the oven for 1 hour at 105° C., allow to cool to room temperature, then place in a Fisher filtrator unit. Treat the precipitate with about 5 ml. of hot benzene, applied in several portions, to dissolve the fats. Make this extraction with gentle suction, followed with strong suction. The benzene extract may be discarded.

Place a glass stoppered 5-ml. volumetric flask, with a small glass funnel in its neck, in the Fisher filtrator unit. Apply approximately 5 ml. of hot methanol, in several portions, to the precipitate, and collect the extracted saponin in the 5-ml. volumetric flask. Use gentle suction for the methanol extraction. Adjust the volume of the extract to 5 ml. using more methanol and mix.

**Color Development.** Pour the contents of the 5-ml. flask, without rinsing, into a glass stoppered 10-ml. volumetric flask. With this flask held at about a 45 degree angle, add concentrated sulfuric acid, about 0.5 ml. at a time, while cooling the flask with running tap water. After adding sulfuric acid to the 10-ml. mark, stopper and place the flask in an ice water bath for about 5 minutes. Quickly mix the contents by inverting back and forth under cold running tap water. Several samples may be prepared and held in ice water for a while at this point. Heat the stoppered flask in boiling water for 20 minutes, cool to room temperature, then add concentrated sulfuric acid to the mark. Mix the contents by inverting and pour without rinsing into a colorimeter cuvette. Measure the transmittance at 575 mμ in a suitable colorimeter. Now add 0.25 ml. of the 1-naphthol solution to the solution in the cuvette, mix, and again

**Table I. Comparison of Saponin Recovery by 1-Butanol Extraction and Direct Filtration**

| Sample No. | 1-Butanol Extraction, Mg. Saponin/ 100 G. Sugar | Direct Sugar Solution Filtration, Mg. Saponin/ 100 G. Sugar |
|---|---|---|
| 1 | 0.21 | 0.24 |
| | 0.18 | 0.23 |
| | 0.24 | 0.18 |
| 2 | 0.09 | 0.12 |
| 3 | 0.58 | 0.57 |

**Table II. Determination of Absorbance Due to Various Materials and Treatments**

| | Saponin, Mg. | Sucrose, Mg. | Transmittance, % | Absorbance |
|---|---|---|---|---|
| Wave Length 575 Mμ without 1-Naphthol | | | | |
| | 0.05 | ... | 94.9 | 0.02273 |
| | 0.10 | ... | 91.3 | 0.03953 |
| | 0.15 | ... | 87.2 | 0.05948 |
| | 0.20 | ... | 83.4 | 0.07883 |
| (1) | 0.05 | 0.20 | 91.4 | 0.03905 |
| (2) | 0.10 | 0.20 | 88.6 | 0.05257 |
| (3) | 0.15 | 0.20 | 83.0 | 0.08092 |
| (4) | 0.20 | 0.20 | 78.4 | 0.10568 |
| Wave Length 575 Mμ with 1-Naphthol | | | | |
| | 0.05 | ... | 94.9 | 0.02273 |
| | 0.10 | ... | 91.3 | 0.03953 |
| | 0.15 | ... | 87.2 | 0.05948 |
| | 0.20 | ... | 83.4 | 0.07883 |
| (1) | 0.05 | 0.20 | 84.1 | 0.07520 |
| (2) | 0.10 | 0.20 | 81.6 | 0.08831 |
| (3) | 0.15 | 0.20 | 76.3 | 0.11748 |
| (4) | 0.20 | 0.20 | 72.7 | 0.13847 |

**Table III. Comparison of Calculated and Actual Quantities of Saponin in Known Mixtures**

| | Calculated Saponin, Mg. | Actual Saponin, Mg. | % |
|---|---|---|---|
| (1) | 0.057 | 0.05 | 114 |
| (2) | 0.093 | 0.10 | 93 |
| (3) | 0.163 | 0.15 | 109 |
| (4) | 0.230 | 0.20 | 115 |

measure the transmittance at 575 mμ after 5 minutes.

**Standard Curves.** Prepare two series of standards in methanol made to the mark in 5-ml. volumetric flasks, one series containing 0.00, 0.05, 0.10, and 0.20 mg. of saponin only. The other series should contain the same increments of saponin and, in addition, 0.20 mg. of sucrose in each. After development of the colors as described above, determine the transmittance and absorbance as compared to the blank, which will appear to be colorless after the heat treatment.

Using the results obtained from the above standard solutions, plot three curves of the absorbance against quantity of saponin. The three curves will be for solutions of saponin only, saponin plus sucrose, and saponin plus sucrose plus 1-naphthol. The extent to which the curves approach a straight line determines the suitability of the technique and analytical accuracy.

**Routine Standards.** After it has been established that the technique yields nearly straight lines, the procedure may be shortened for routine determinations by preparing fewer standards: blank, 0.20 mg. of saponin, 0.20 mg. of saponin plus 0.20 mg. of sucrose, and 0.20 mg. of saponin plus 0.20 mg. of sucrose plus 1-naphthol. After the absorbances, $A$, have been determined, the following formula provides the factor, $K$, for reducing the absorbance due to the sugar with 1-naphthol to the absorbance due to sugar without 1-naphthol.

$K$ having been found experimentally by using known amounts of materials, the formula may be rearranged to solve for the absorbance due to saponin only, $A_{(saponin)}$.

$$A_{(saponin)} = \frac{A_{(saponin + sugar)} - A_{(saponin + sugar + 1\text{-naphthol})} - A_{(saponin + sugar)}}{K}$$

## Experimental

A Beckman Model DU spectrophotometer with 1.0-cm. cells was used to obtain the absorption spectra of the colored solutions. For the analyses a Lumetron Model 402 E colorimeter, adapted to accept Coleman 25 × 105 mm. cuvettes, was employed to measure the transmittance of the solutions. A narrow band pass optical filter (Photovolt No. 4167) is suitable to obtain light of 575 mμ wave length. The saponin absorbance spectrum follows Beer's Law at 420 mμ and at 575 mμ. The 575 mμ wave length was chosen because the absorbances of the saponin standards, relative to the blank, are not altered when the 1-naphthol is added to a sugar-free solution.

Single acid precipitation of the saponin from the sugar solution always results in saponin contaminated with sugar. For that reason, this method which corrects for the presence of the sugar, was developed. Two alternative methods were developed to extract the saponin.

**Carbon Extraction of Saponin.** Carbon, Darco G-60, may be used for the removal of saponin. Add about 7.0 mg. of carbon to the pH 2.0 sugar solution; then filter and refilter through a medium-porosity fritted-glass funnel. Difficulty may be encountered in removing the sucrose and color bodies from the carbon. Accomplish this by washing with a hydrochloric acid–water solution of pH 2.0, containing 10% methanol. Dry the funnel, extract with methanol, and determine the saponin colorimetrically. The method does not always remove saponin as completely as with F-fritted funnels alone. Use Clorox (sodium hypochlorite solution) to remove the carbon from the pores of the fritted glass.

**Butanol-Water Solvent Extraction of Saponin.** Extract fats and saponin from pH 2.0 sugar solution with 1-butanol. Add an aliquot of the butanol phase to acidified water; bring to a boil until the butanol layer is gone. Allow the solution to come to room temperature; at the end of which time, a flocculent precipitate will appear. Filter the solution through an F-fritted funnel. Wash the residue, dry, extract, and determine colorimetrically, as above. Comparative results are shown in Table I.

## Results and Discussion

The butanol extraction method was devised in an attempt to develop a more rapid method. The method seems to have promise, but as it proved to be no faster than direct filtration of the acidified sugar solution, it was not pursued further.

The colorimetric procedure was applied to various known mixtures of saponin and sugar as shown in Table II.

The average $K$ factor from the above series was found to be 2.31, which values were placed in the formula and the quantities calculated (Table III).

To test the efficiency of recovery by this method, a sample of sugar was analyzed. In 100 grams of sugar, 0.12 mg. of saponin were found to be present. To other equal portions of the same sugar, known amounts of purified saponin were added. Table IV shows the efficiency of recovery.

The method was also tested by using 100-gram portions of sugar which were dissolved, acidified, and filtered. The filtrates, which should then be free of acid insoluble material, were retained and known amounts of saponin added to them. Table V shows the quantity of saponin originally present in the sugars and the quantities recovered.

Saponin in sugar is not objectionable

**Table IV. Efficiency of Recovery of Added Saponin**

| Sample No. | Originally Present, Mg. | Amount Added, Mg. | Total Present, Mg. | Amount Found, Mg. | Saponin Recovery, % |
|---|---|---|---|---|---|
| 1 | 0.12 | 0.10 | 0.22 | 0.19 | 86 |
| 2 | 0.12 | 0.20 | 0.32 | 0.32 | 100 |
| 3 | 0.12 | 0.40 | 0.52 | 0.46 | 88 |
| 4 | 0.12 | 0.51 | 0.63 | 0.57 | 90 |

**Table V. Quantity of Saponin Found in Sugar and Efficiency of Recovery of Saponin from Saponin-Free Filtrates**

| Sample No. | Mg. Saponin Originally Present/100 G. Sugar | | Saponin Added to Filtrate, Mg. | Saponin Recovered from Filtrate, Mg. | | Saponin Recovered from Filtrate, % | |
|---|---|---|---|---|---|---|---|
| 1 | 0.56 | 0.56 | 0.25 | 0.26 | 0.23 | 104 | 92 |
| 2 | 0.39 | 0.46 | 0.25 | 0.23 | 0.26 | 92 | 104 |
| 3 | 0.72 | 0.74 | 0.25 | 0.26 | 0.26 | 104 | 104 |

**Table VI. Suitability of Sugars Graded by Pepsi-Cola Co. Visual Estimation as Compared to Quantity of Saponin Found by Colorimetric Method**

| Sample No. | Mg. Saponin/100 G. Sugar | Visual Floc Evaluation |
|---|---|---|
| 1 | 0.02 | Negative floc |
| 2 | 0.03 | Border line pin point |
| 3 | 0.14 | Border line pin point |
| 4 | 0.50 | Heavy positive floc |
| 5 | 0.32 | Heavy positive floc |

except in acid solution where it produces an unsightly flocculent precipitate. Fats are also considered to be a component of this floc. However, the contribution of fats to the formation of floc was not pursued here. The problem is to provide a quantitative relationship of saponin content in sugar to the visually observed floc. Table VI shows the saponin found to be present in various sugar samples which had been evaluated visually by analysts of the Pepsi-Cola Co.

As the visual evaluation is entirely subjective, the correlation might be considered very good.

The method described also provides a suitable and fairly rapid method which can be used for the study of saponin removed in the beet sugar refinery.


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**MOISTURE MEASUREMENT**

# Determination of Moisture in Hops


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Moisture determination in hops by titration with Karl Fischer reagent was compared with oven drying and solvent distillation. Lower moisture values were obtained by Karl Fischer titration and by solvent distillation with iso-octane than by oven drying or solvent distillation with toluene or benzene. Higher results obtained by the latter three methods were due in large part to the liberation of water from hop components owing to chemical decomposition and concurrent decrease in the number of hydroxyl groups present. The Karl Fischer method is convenient and valid for the determination of moisture in hops.


OFFICIAL METHODS suggested for the determination of moisture in hops by the American Society of Brewing Chemists (*1*) and the Association of Official Agricultural Chemists (*2*) include oven drying and solvent distillation. Both methods suffer from serious disadvantages.

While these methods are good for the determination of moisture in many materials, special difficulties arise in hops, owing to the very heat-sensitive nature of several hop constituents and to the presence of up to 1% volatile oils.

During an evaluation of the Karl Fischer reagent for moisture determination on raw materials used in the brewery, this reagent was also studied for hops.

Comparative data on the various methods for the determination of hop moisture, with special emphasis on the use of the Karl Fischer reagent, are presented.

### Methods for Moisture Determination

The amount of moisture in a material is often determined by heating in an oven and measuring the weight loss; however, this is unsuitable for materials which are heat sensitive.

Solvent distillation, employing a water-immiscible solvent, is another commonly used method. This test is based on the boiling point of water-solvent mixtures which is lower than that of either the water or solvent alone. For this distillation, the method of Dean and Stark (*8, 16*) was employed.

Drying in vacuo over a desiccant is one of the most accurate methods, but it takes a very long time for many materials to reach constant weight.

A rapid method, specific for water,