Vol. 18 No. 2

PLANT PHYSIOLOGY

APRIL, 1943

A SYSTEM OF ANALYSIS FOR PLANT TISSUE BY USE OF PLANT JUICE[1]

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The utilization of expressed plant juice for determining the chemical constituents of a plant is becoming increasingly important. It has the advantage of presenting the products of metabolism in a state more similar to that found in living tissue than any of the more commonly used extraction methods. Conditions of state and equilibrium, however, can probably never be identical for juice within and without the cell, for at the moment of cell rupture, the conditions that control living cellular activity are changed. To illustrate, if living leaf tissue is placed in an atmosphere of carbon dioxide, the juice becomes more basic (**8**), but if the cell liquid is expressed, and carbon dioxide bubbled through it, the juice becomes more acid.

The first important studies of expressed juice were for the investigation of physical constants such as density, refractive index, freezing point, etc. This work has been summarized by SASTRI and SREENIVASAYA (**21**). Recently, attention has been paid to the inorganic and more particularly to the organic fractions, of plant juice including the sugars and nitrogen fractions. This paper is presented to describe methods for the determinations of a portion of the inorganic and organic constituents in plant juice extracts, and calculation of the results on a fresh weight basis.

General methods

Before a representative sample of juice can be obtained from the entire tissue studied, the plant material must be treated in some manner so that the contents of all cells are represented. For example, unrepresentative juice samples are obtained by pressure alone, if the freezing point lowering is used as a criterion. This uncertainty may be obviated, to a large extent, by treating the sample in various ways: by grinding, freezing with solid carbon dioxide, liquid air, or eutectic mixtures; by vapors of volatile liquids such as ether or chloroform; or by autoclaving (**1, 6, 7, 11, 12, 21**). The work of SAYRE and MORRIS (**22**) with corn indicates that grinding is as effective in breaking cell walls as freezing. This is advantageous for there

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is some indication that during the freezing of tomato leaves the sucrose-reducing sugar equilibrium is shifted toward an increase in reducing sugars. Substances such as nitrates, inorganic phosphates, and total sugars are expressed in like amounts in successive fractions, after a preliminary grinding of corn tissue (**23**). Treatment of plant material by autoclaving at five pounds per square inch for 15 minutes gives results comparable to the freezing technique (**7**) and similarly causes the precipitation of proteins.

For the data presented in this paper, the ordinary Nixtamal mill was used for grinding leaf samples. When samples were frozen, they were first cut and wrapped tightly in a cheese-cloth bag, exposed to carbon dioxide snow as it was formed at the end of a copper tube attached to the tank of liquid carbon dioxide. This gave rapid and complete freezing of a 100-gram sample in less than a minute. Unless otherwise stated, all frozen samples used in this study were so treated.

It is evident that the preliminary treatment of plant tissue depends upon the substances to be determined. Grinding is apparently adequate for all circumstances and its only evident disadvantage, in comparison with freezing or autoclaving, is that the resulting juice contains large quantities of debris and protein. Where proteins must be removed, their partial elimination by freezing or autoclaving is helpful.

Determination of physical constants

Sayre and Morris (**23**) have proposed a formula[2] whereby the various plant constituents can be determined by analysis of expressed juice and the results calculated to the fresh-weight basis. The values obtained were comparable to those secured by an alcohol extraction. Using this equation as a basis, it was advisable to examine the methods available for the determination of specific gravity, total solids of plant juice, and the total moisture of the leaf material since these values are necessary for the calculation.

For the determination of specific gravity three methods are available: the Westphal balance, hydrometer, and pycnometer. In a series of 20 juice samples of beet and tomato leaves using the methods mentioned, the average values obtained were 1.036, 1.025, 1.022, respectively. In all instances the agreement between these methods was sufficiently accurate so that any one could be used. The hydrometer method, however, is by far the most rapid and convenient.

Gortner (**13**) has demonstrated the possibility of determining total solids and subsequently the moisture content of plant juice with the refractometer. This method has been shown (**17**) to give high results in comparison with oven drying for juice samples of soybean, corn, wheat, and oats. Sayre and Morris (**23**) found that for corn it was necessary to correct the total solids determined by refractometer when leaf juice was used, but no correction was needed for juice from stalk tissue.

[2] $$\text{Percentage of sugar in tissue} = \frac{\dfrac{\text{Gm. of sugar in 100 ml. sap}}{\text{Gm. of moisture in 100 ml. sap}} \times \text{Total moisture of tissue in gm.}}{}$$

In the present study a comparison has been made between the toluene distillation method (**3**) for moisture in plant juice and the refractometer method. The refractometer was adjusted to give the theoretical value for water at 20° C. The scale reading gives the refractive index of the juice directly, and SCHÖNROCK'S (**2**) table was used to translate refractive index values to total solids. The percentage of juice moisture is the difference between the percentage of total solids and 100. In table I, the results by the above mentioned methods are expressed in terms of moisture content rather than total solids.

The estimation of total moisture in leaf tissue was also made by oven drying at 100° C. for 24 hours. In order to be assured of the accuracy of this method it was examined in two ways. First, samples were prepared by cutting the leaves into small sections and weighing aliquots into aluminum dishes. One series was frozen with carbon dioxide snow, the other was untreated. The freezing technique was introduced to make certain that the cell walls were broken so that all leaf moisture could be removed. Using juice from tomato leaves and beet leaves and beet petioles the agreement between frozen and non-frozen samples was within 0.5 per cent. Similarly, with the same tissues the average of six determinations gave the following results: oven drying, 86.88 per cent.; toluene distillation, 86.75 per cent. moisture.

CHEMICAL METHODS

In a study of the nitrogen metabolism of plants certain fractions are usually determined; nitrate, ammonia, alpha-amino, amide, and total nitrogen. Carbohydrate analyses usually include sucrose and reducing sugars, which in turn may be divided into glucose, fructose, and non-sugar reducing substances.

NITRATE NITROGEN.—BURRELL and PHILLIPS (**4**) for alcohol extracts and FREAR (**10**) for plant juice have shown that clarification of plant juice is possible with silver sulphate, copper sulphate, and a mixture of calcium

TABLE I

PERCENTAGE OF MOISTURE IN JUICE FROM BEET LEAVES AS DETERMINED BY REFRACTOMETER AND TOLUENE DISTILLATION METHODS

METHOD	MOISTURE, PERCENTAGE								
	1	2	3	4	5	6	7	8	AVERAGE
	%	%	%	%	%	%	%	%	%
Refractometer	91.93	89.93	93.20	93.90	95.20	93.90	94.50	95.20	93.46
Toluene distillation	92.00	91.40	92.90	94.50	95.00	93.00	93.40	93.90	93.26

hydroxide and magnesium carbonate or calcium hydroxide alone. BURRELL states that it was necessary to make a preliminary treatment with sodium peroxide for alcoholic extracts. FREAR did not try this for plant juice but secured satisfactory results. Nitrate is then determined in the clarified juice by the phenol-disulphonic acid method (**15**).

AMMONIA NITROGEN.—While studying the various methods for ammonia, SCHLENKER (**24**) found that when either 52 per cent. potassium carbonate or heavy magnesium oxide was added to plant juice and the sample aspirated, there was a rapid evolution of ammonia during the first two hours but that with continued aeration ammonia continued to be given off in small amounts over a long period of time. A more satisfactory procedure for the determination of ammonia was devised by shaking juice with 2.0 ml. of sodium permutit (FOLIN) for 5–7 minutes to adsorb the ammonia, then removing the juice by washing, thus eliminating the glutamine and asparagine. Ammonia is freed from the permutit by sodium hydroxide and aspirated as usual the Van Slyke-Cullen aspiration block.

PUCHER (**19**) later reported that the amide nitrogen of glutamine is hydrolyzed quite readily, more easily than that of asparagine. Therefore, it is to be expected that when glutamine is present in a plant juice which is treated with an alkali such as potassium carbonate and aspirated, some of the glutamine amide nitrogen will be included with that of the preformed ammonia. It can be shown that asparagine is only slightly hydrolyzed by potassium carbonate (**27**).

GERDEL (**11**) modified the SESSION and SHIVE method for the determination of ammonia and nitrate nitrogen; but, as this method is based on the release of ammonia directly from plant material in the presence of alkali, high results are obtained.

ALPHA-AMINO NITROGEN.—The VAN SLYKE (**30**) method for the estimation of amino nitrogen has been examined by STUART (**29**) who found that high results are obtained when using water or alcoholic plant extracts. A number of substances such as ammonia, dihydroxy phenols, and tannic acid were found to yield nitrogen with nitrous acid. Several means were used to eliminate these error-producing substances. The lowest amino nitrogen values were obtained after adding calcium oxide and using vacuum aspiration at 40–45° C. for one hour with subsequent removal of excess lime. It was further demonstrated that amino acid and asparagine nitrogen could be recovered quantitatively after this treatment. It is probable, however, that a portion, if not all, of the glutamine present was destroyed during the vacuum distillation.

Ninhydrin, a specific reagent for alpha-amino acids, has been used to determine the amino nitrogen in plant extracts (**28**). If the results using this reagent are taken as true values, the figures obtained by the nitrous acid method are too high even after treatment with lime. The reliability of the nitrous acid method depends on the extent to which interfering substances can be removed and apparently lime treatment is insufficient. In the use of ninhydrin it is necessary to precipitate proteins and desirable to remove preformed ammonia. Because of specificity, methods using ninhydrin will undoubtedly give results that are close to the true alpha-amino nitrogen content of plant extracts.

GLUTAMINE AND ASPARAGINE AMIDE NITROGEN.—These amides are usually determined together by hydrolysis with normal sulphuric acid but CHIBNALL

and WESTALL (**5**) and VICKERY *et al.* (**31**), by adjusting the hydrogen-ion concentration of the solution were able to hydrolyze glutamine in the presence of asparagine, with the inclusion of only a small amount of the latter. At pH 6.0–6.5 between 98.5 and 99.0 per cent. of glutamine amide nitrogen is hydrolyzed after two hours at 100° C. In the presence of normal sulphuric acid for three hours both amides are completely hydrolyzed. Asparagine amide nitrogen is obtained by difference on the assumption that only these two amides are present. This procedure has been applied to plant juice (**27**).

PUCHER (**20**) developed a method for the estimation of glutamine which supplements the method just described. This was based on the ethyl-acetate extraction of the pyrrolidone-carboxylic acid formed during the nearly neutral hydrolysis of glutamine. After extraction the carboxylic acid was hydrolyzed to glutamic acid. By determining amino nitrogen before and after the second hydrolysis the amount of glutamine present was calculated.

ORCUTT and WILSON (**18**) advocate the use of 20 per cent. sodium bisulphite to avoid humin formation which occurs during the determination of total amide by 10 per cent. sulphuric acid. It is probable that bisulphite may be used on juice samples to avoid the necessity of precipitating the protein fraction. Determinations of total amides were made on protein-free and protein-containing juice treated with 10 per cent. sulphuric acid and 20 per cent. sodium bisulphite (table II).

It is evident that bisulphite is sufficiently active to hydrolyze plant juice proteins so that it is necessary to remove these proteins before using this reagent; the same holds true for sulphuric acid.

DETERMINATION OF SUGARS.—The colorimetric method of FOLIN (**9**) using an alkaline copper tartrate oxidizing solution gives satisfactory results with plant juice (**25, 26**). In the preparation of the tissue extract, freezing is not always a satisfactory method, for in some instances during this process there is a shift in the sucrose-reducing sugar equilibrium with an increase in the reducing sugars. In tomato leaf reducing sugar increased 0.13–1.70 mg. per ml. of juice. This difference is not due to sampling errors for, when a sample of plant juice expressed from ground beet leaf was divided into two portions and only one portion frozen, its reducing sugar content was increased by 0.39 mg. per ml. as compared with the unfrozen juice sample.

However, storage of corn leaves or juice from the same samples of corn leaves in a cold storage room at approximately 0° C. for a period of over a month had no effect on the sucrose-reducing sugar equilibrium (unpublished data).

Procedure for analysis

STANDARDS

The proposed methods for nitrogen compounds, with the exception of nitrate, are all based on the colorimetric determination of ammonia by Nesslerization after alkaline aspiration. The sugar methods depend on the reduction of phospho-molybdate reagent by cuprous oxide and comparison

of the resulting blue solution with a standard in a colorimeter. No one set of working standards can be recommended because of the great variations in concentrations of plant constituents, depending on a number of factors. The dilute stock standards generally used were as follows: 40 mg. ammonia nitrogen per liter from ammonium sulphate, 200 mg. glucose (Bureau of Standards) per liter, and 10 mg. nitrate nitrogen per liter from potassium nitrate.

TABLE II

TOTAL AMIDE-NITROGEN IN CLARIFIED AND UNCLARIFIED BEET JUICE

TREATMENT	AMIDE-NITROGEN PER MILLILITER OF PLANT JUICE	
	10% H_2SO_4	20% $NaHSO_3$
	mg.	*mg.*
Unclarified	0.0167	0.0117
Clarified*	0.0020	0.0050
Unclarified	0.0696	0.0486
Clarified	0.0150	0.0147

* Clarified by 20 per cent. tannic acid solution. Preformed ammonia removed from all samples.

Ammonia is aspirated into approximately 0.01 normal sulphuric acid and determined in all cases by Nesslerization. After aeration the samples are transferred to 50-ml. volumetric flasks and made up to within 3 ml. of the mark, mixed, and Nessler's reagent added. Aliquots are so chosen that the ammonia nitrogen concentration in the final dilution is never greater than 0.0016–0.004 mg. per ml.

If a photoelectric colorimeter is available the use of transmission-concentration curves dismisses the need of working standards.

PREPARATION OF SAMPLE

Grind the sample in a Nixtamal mill or freeze with solid carbon dioxide and allow to thaw. Express the juice by means of a hydraulic press and centrifuge the resulting juice. Determine total solids by the Abbé refractometer and specific gravity with a hydrometer. Dry a fresh leaf sample in an oven at 100° C. overnight for total moisture.

TOTAL SOLUBLE NITROGEN

Filter plant juice through asbestos to remove all suspended material. Pipette a suitable aliquot and 1 ml. of an acid mixture[3] into a large Pyrex test tube and add a glass bead. To insure the reduction of nitrate add a small amount of zinc, and heat if necessary. Heat over a small flame to complete digestion. Place the tubes in an aspiration block, make the usual connections, add 1 ml. of toluene and sufficient sodium hydroxide to make the mixture alkaline, and aspirate.

For soluble inorganic nitrogen, an alternative procedure is available (**11**).

[3] 5 ml. 5 per cent. $CuSO_4$, 30 ml. 85 per cent. H_3PO_4, 10 ml. concentrated H_2SO_4. This combination is mixed 1: 1 with water and used as suggested in the text.

Place an aliquot in the tubes in the aspiration block, add Devarda's alloy, and then alkali, causing reduction of nitrates during the alkaline rather than the acid stage. This requires a long aspiration period.

NITRATE NITROGEN (10)

Pipette a juice aliquot, 1 or 2 ml., in a 100-ml. volumetric flask containing 50 ml. water and 1 or 2 ml. of 5 per cent. $CuSO_4 \cdot 7\ H_2O$, mix and add a sufficient volume of saturated Ag_2SO_4 solution to precipitate chlorides. Finally add enough solid $Ca(OH)_2$ $MgCO_3$, 4 + 10 mixture, to precipitate excess copper. Make to volume, mix, and filter through a dry paper. Evaporate a 25-ml. aliquot of the sample to dryness. Prepare a standard by evaporating 10 ml. of a solution containing 10 p.p.m. of N from KNO_3 to dryness. Add 1 ml. of phenoldisulphonic acid solution and allow to react with the nitrate. Then add water followed by sodium hydroxide until the characteristic yellow color appears. Dilute the standard and sample to 100 ml. and compare in a colorimeter.

AMMONIA NITROGEN (24)

Add 1 to 5 ml. of juice to 2.5 ml. of permutit (FOLIN) in an ammonia aspiration tube and shake at least three minutes. Remove extraneous juice by washing with water and decanting a sufficient number of times to remove all color. To the permutit add approximately 5 ml. of water and 1 ml. of toluene. Place the tubes in an aspiration block and add 4 ml. of 10 per cent. NaOH; connect the apparatus and aspirate 2.5 hours into approximately 0.01 N H_2SO_4. To reduce the time, the reaction tubes may be immersed in a water bath at 55–60° C. and aspirated 30 minutes. If ammonia is determined by Nessler's reagent the normality of the acid need only be approximate.

CLARIFICATION OF JUICE SAMPLE FOR THE DETERMINATION OF AMIDE AND ALPHA-AMINO NITROGEN

Treat 10 ml. of juice with 5 ml. of 5 per cent. sodium tungstate in 0.33 N H_2SO_4. After proteins and colored substances have been precipitated, centrifuge to remove suspended material. Pour a sample of the supernatant liquid into a test tube and shake with a sufficient amount of permutit to remove preformed ammonia.

AMIDE NITROGEN (27)

Pipette 5 ml. or less of the protein- and ammonia-free juice into an ammonia aspiration tube; add 1 ml. 6 N H_2SO_4 and sufficient water, if necessary, to make the total volume 6 ml. At the same time place 10 ml. of phosphate buffer pH 6.0–6.5 in a second ammonia tube and add a suitable aliquot of the protein and ammonia-free juice. Place both tubes, fitted with stoppers containing capillary tubes, in a boiling water bath. Hydrolyze the first tube three hours for total amide nitrogen, and the second tube two hours for glutamine amide nitrogen. At the end of the period, cool the tubes, place in an aspiration block, and add 1 ml. of toluene. For total

amides add 3–4 ml. of a 10 per cent. NaOH solution. To make the glutamine tube alkaline add 2 ml. of 52 per cent. K_2CO_3. Aspirate and determine ammonium nitrogen as above. The difference between total amide nitrogen and glutamine amide nitrogen is considered to be asparagine amide nitrogen.

Amino nitrogen (28)

Transfer a protein- and ammonia-free sample containing not more than 0.20 mg. alpha-amino nitrogen into an ammonia tube, add 1 ml. phosphoric acid (2 ml. 85 per cent. H_3PO_4 + 1 ml. H_2O) and 0.5 ml. ninhydrin solution (15 mg.). Close the tube with a stopper containing a capillary glass tube 5 cm. long. Place in a boiling water bath for 45 minutes. Remove to an aspiration block, add 4 ml. saturated NaOH solution and aspirate 30 minutes at 50–60° C., or two hours at room temperature. Determine ammonia by titration or Nesslerization. In samples where glutamine and asparagine are extremely high due to abnormal environmental conditions, it is advisable to utilize the sample used for the determination of total amides. After the removal of total amide nitrogen as ammonia, adjust the contents of the tube containing the alkaline mixture to pH 1.0 with concentrated phosphoric acid, add 15–30 mg. of ninhydrin, and place the tube in a boiling water bath for 45 minutes. After cooling determine the ammonia by alkaline aspiration as previously described.

Clarification of juice for the sugar determination

Pipette an aliquot of juice, 1 or 2 ml., into a centrifuge tube (15 or 50 ml.) and dilute to 10 ml.; add 1 or 2 ml. saturated neutral lead acetate solution and centrifuge. Add 10 per cent. Na_2HPO_4 solution until the sugar solution is blue to bromthymol blue, dilute to volume, and again centrifuge.

Determination of total sugar (25)

Transfer an aliquot of the clarified plant juice to a Folin sugar tube, add 2 drops of a 0.5 per cent. Wallenstein's invertase scale preparation. After letting stand for two hours at room temperature, make the solution alkaline and add 2 ml. of Folin alkaline copper tartrate (9). Place the tubes in a boiling water bath for 15 minutes, remove and cool, and add the acid molybdate solution for color development; make the solution to volume.

Reducing sugar

Determine this fraction in the lead-free, clarified juice, using a 1- or 2-ml. aliquot, by the Folin alkaline tartrate method mentioned above.

Non-sugar reducing substance (26)

Place 2 ml. of a thoroughly washed 10 per cent. yeast suspension in a 15-ml. centrifuge tube and centrifuge to destroy the suspension. Pour off the supernatant liquid and dry the sides of the tube thoroughly with strips of filter paper. Add an aliquot of clarified juice or fresh juice, about 4 ml., and stir. The tube is placed in a water bath at 37–39° C. and agitated frequently. Centrifuge after 20 minutes and determine residual reducing

power of the sample. This value is used to correct the results for total and free reducing sugars.

Summary

This paper summarizes efforts during a considerable period to select and adapt chemical methods for the analysis of plant juice. Most of the procedures are applicable to other types of plant extracts. The nitrate and sugar methods, however, cannot be used with alcohol or water extracts if a visual colorimeter is used because the precipitating reagents involved do not give the necessary colorless filtrates.

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