A STUDY OF EFFECTS OF SAMPLE PREPARATION AND METHODS OF EXTRACTION ON DETERMINATION OF STARCH IN PLANT MATERIALS

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A STUDY OF EFFECTS OF SAMPLE PREPARATION AND METHODS OF EXTRACTION ON DETERMINATION OF STARCH IN PLANT MATERIALS

By

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INTRODUCTION

Many papers on the chemistry of starch have been written over the years and are still being published on various phases of the subject. However, one aspect which has been comparatively neglected is that of analytical methods for the quantitative determination of small amounts of starch in plant materials.

Because of the importance of starch data to studies of plant metabolism, it seemed desirable to determine the effectiveness of some of the analytical procedures which have appeared in the literature. The problem of such analyses is threefold: 1. the preliminary treatment of samples prior to analysis; 2. the complete extraction of starch; 3. the accurate evaluation of starch with the exclusion of any other carbohydrates which may be present. The work to be discussed deals with these three phases of starch analyses.

HISTORICAL

Some study has been devoted to the effect on starch content of preliminary treatment of plant materials prior to analysis. In 1893, Brown and Morris (1) reported observations on the effects of temperature, loss of water, and anesthetics on starch dissolution in tree leaves. Freezing of leaf samples was found by Spoehr and Wilner (2) to prevent depletion of starch. Treatment of fresh plant material with toluene, chloroform, and other anesthetics was reported by Spoehr and Wilner (3) to preserve starch.

Of the many agents which have been used to dissolve starch, concentrated calcium chloride solution is one of the oldest and is still used for that purpose. As early as 1860, Flueckiger (4) reported that starch could be dissolved in calcium chloride solution. Fellenberg (5, 6, 7) devised procedures for the determination of pure starch and starch in plant samples using calcium chloride solution as the dispersing agent. Several studies were made by Lenny (8, 9, 10) comparing calcium chloride extraction of starch from plant tissues with direct acid extraction method of Nask (11) and direct malt diastase hydrolysis procedure used by Walton and Coe (12). Sullivan (13) reported optimum conditions

for extraction of starch from plant matter with calcium chloride. More recently, calcium chloride extraction has been applied to plant materials containing less than 10 per cent starch by Hoffpauir (14, 15). A new method for starch determination in leaf samples was described by Chinoy (16) using dilute potassium hydroxide to extract starch. Niemann, Roberts, and Link (17) chose an ethanol-nitric acid mixture to solubilize starch in woody tissue prior to extraction of starch with 20 per cent aqueous ethanol. The procedure described by Hanes (18) for extracting starch from plant material such as apple fruit involved the conversion of starch to a highly soluble form by heating the tissues in alcoholic hydrochloric acid. Boiling water was then used to extract the solubilized starch from the tissues. Hassid, McCready, and Rosenfels (19) adapted the method outlined by Hanes (18) to the determination of starch in leaves and various other types of plant materials for which the original procedure was unsatisfactory.

After its isolation, starch has been evaluated in a number of ways. Fellenberg (5, 6, 7) precipitated starch from solution with iodine and collected the starch-iodine complex on a Gooch crucible. After thoroughly washing the precipitate, the crucible was dried to constant weight, ignited, and reweighed. The loss

in weight was considered to be amount of starch present. Gravimetric evaluation was used also by Rask (11) who precipitated starch from solution by addition of ethanol and collected the starch by filtration for direct weighing. Chinoy (16) precipitated starch with iodine and weighed the starch-iodine complex. Iodine solution was used also by Sullivan (13) to precipitate starch from calcium chloride solution. However, in this case the starch was subsequently hydrolyzed with hydrochloric acid and reducing power was taken as a measure of glucose, which was used as the basis for calculation of the amount of starch originally present. Hoffpauir (14) published a modification of the Sullivan (13) procedure. Walton and Coe (12) incubated plant material malt B-amylase to hydrolyze starch without preliminary extraction. Pectin was removed by precipitation with alcohol, and the resulting solution was treated with acid to completely hydrolyze any dextrins and maltose before determining reducing sugars. Malt B-amylase was used by Hanes (18) to hydrolyze starch, whereas Hassid, McCready, and Rosenfels (19) used salivary amylase in their procedure to convert starch to maltose and dextrins.

EXPERIMENTAL

Plant samples analyzed in the present study were obtained from the experimental plots of the Department of Soil Science. The plants selected were alfalfa, Medicago sativa, just before blooming, and second growths of brome grass, Bromus inermis, reed canary grass, Phalaris arundinacea, and orchard grass, Dactylis glomerata, hereinafter to be referred by the common names. None of the plots sampled had been fertilized or irrigated.

Samples were taken at about two o'clock on afternoons of clear days with temperatures of 85° F. or above. All plants in an area of approximately four square feet were cut about two inches above the ground with shears. As soon as possible after cutting, the samples were taken to the laboratory where all dead plant leaves and any extraneous matter were removed.

Each plant material was divided into approximately four equal parts for curing by four different methods. The first portion was spread out on a table and allowed to dry at room temperature until brittle. A second portion was placed in a drying room at 50° C. for twenty-four hours. Another portion was dried for eighteen hours in an electric oven at 85-90° C. The fourth portion was frozen and then placed in the electric oven

and dried for eighteen hours at 85-90° C. A total of sixteen samples resulted from four methods of handling each of the four plant varieties.

After drying, the samples were put through a large Wiley mill equipped with a coarse sieve prior to final grinding to 60-mesh with a semi-micro Wiley mill.

Defatted corn starch was prepared by the method of Schoch (20) for use as a standard in recovery tests. Lipids were removed from 5 g. of corn starch by Soxhlet extraction with 95 per cent ethanol for 48 hours. Excess alcohol was removed from the starch by allowing the paper thimble holding the starch to stand at room temperature overnight. Final drying was accomplished by spreading the starch out on a large watch glass and and heating for 24 hours in an electric oven at 105° C. The dried starch was placed in a small weighing bottle and stored over calcium chloride in a desiccator.

A portion of each plant sample was taken for moisture determination at the same time that samples were weighed for starch analysis. Moisture content of the dried plant material was measured by placing 5 g. of sample in a special aluminum pan and drying at 100° C. for 24 hours in the Brabender moisture tester. The per cent moisture was obtained by multiplying the Brabender scale reading by two since the moisture tester is calibrated for 10 g. sample weights.

Duplicate amounts of 0.5-1.0 g., depending on starch content, of each sample of a specific plant variety were weighed on the same day for extraction by the three different procedures studied as described in succeeding paragraphs. For example, six 0.5 g. portions of each of the four alfalfa samples, cured by different methods, were weighed into appropriate vessels, 200 ml. Pyrex centrifuge bottles for ethanol-hydrochloric acid and potassium hydroxide extractions and 150x25 mm. Pyrex test tubes for calcium chloride extraction. Thus, all analytical results for a given plant sample could be calculated to a common dry weight basis requiring only one moisture determination.

For the solubilization and extraction of the starch, three procedures were used. The first one studied was that of Hassid, McCready, and Rosenfels (19). The second procedure used was based on the method described by Chinoy (16). The third method followed the suggestions given by Hoffpauir (15).

In the first procedure the samples were refluxed in a boiling water bath for twenty minutes with 100 ml. of 95 per cent ethanol. The starch was solubilized by adding 1 ml. of concentrated hydrochloric acid through the top of the reflux condenser and boiling for fifteen minutes longer. After cooling, the sample was

centrifuged to pack the plant residue. The ethanol-hydrochloric acid was drawn off, and the residue was washed twice with 25 ml. portions of hot 95 per cent ethanol. Next, the residue was boiled with 100 ml. of distilled water for five minutes and then placed in a boiling water bath for thirty minutes to dissolve the solubilized starch. The mixture was cooled, centrifuged, and the supernatant liquid decanted into a 250 ml. volumetric flask. Two 25 ml. portions of hot distilled water were used to wash the residue, and the washings were added to the solution in the volumetric flask. Solubilization was repeated with 50 ml. of 95 per cent ethanol and 0.5 ml. of distilled water to dissolve any remaining starch. The water extract and plant residue were transferred quantitatively to the volumetric flask. A rubber policeman was used to scrub the last traces of starch from the centrifuge bottle. After cooling, the contents of the flask were diluted to volume and filtered.

Hydrolysis of the starch in the aqueous extract was carried out in the following manner. A 25 ml. aliquot was pipetted into a 50 ml. volumetric flask, 2 ml. of 0.2 N sodium acetate buffer of pH 5.6, 4 ml. of 0.25 N sodium chloride, and 4 ml. of saliva which had been diluted 1:1 with water and filtered were added, and the mixture was kept at 37-40° C. for four hours. After

the hydrolysis period, 1 ml. of saturated neutral lead acetate was added to the digest to precipitate water-soluble proteins and non-carbohydrate reducing substances. The flask was shaken and allowed to stand about five minutes. Excess lead was removed by adding 4 ml. of saturated disodium phosphate. The solution was diluted to volume, mixed, and filtered. A second 25 ml. aliquot taken for a blank was treated in the same way except that the diluted saliva added had been inactivated by boiling for five minutes.

Reducing values of the hydrolyzed sample and blank filtrates were determined as follows: Five ml. of the solution, containing not more than 4 mg. of maltose, and 5 ml. of alkaline potassium ferricyanide, containing 8.25 g. of potassium ferricyanide and 10.6 g. of sodium carbonate per liter of aqueous solution, were mixed in a 150x25 mm. Pyrex test tube and heated in a boiling water bath for exactly fifteen minutes. After cooling the mixture by immersing the tube in cold running water for three minutes, 5 ml. of 5N sulfuric acid were added and thoroughly mixed with the contents of the tube. Ten drops of Setopaline C indicator were added, and the solution was titrated to a golden brown color with 0.0093 N ceric sulfate using a micro burette.

The ceric sulfate solution used to titrate the starch extracts was standardized with glucose solution.

Standard glucose solution containing 1 mg. of glucose per ml. was prepared by diluting 10 ml. of 1 per cent glucose solution to 100 ml. From 0.5 to 2.5 ml. of the diluted glucose solution were pipetted into large test tubes and diluted to 5 ml. with water. Five ml. of alkaline ferricyanide solution was added to each tube followed by heating for fifteen minutes. After cooling, 5 ml. of 5 N sulfuric acid and 10 drops of Setopaline C indicator were added. The samples were titrated with ceric sulfate in the same manner as the plant sample extracts. It was found as shown in Table I that 3.220 ml. of the ceric sulfate was equivalent to 1 mg. of glucose. In terms of maltose, 2.578 ml. of the ceric sulfate was equivalent to 1 mg. Hassid (21) reported that 3.0 ml. of exactly 0.01 N ceric sulfate was equivalent to 1 mg. of glucose. From this relationship, the normality of the ceric sulfate used in the present study was calculated to be 0.0093.

Table I

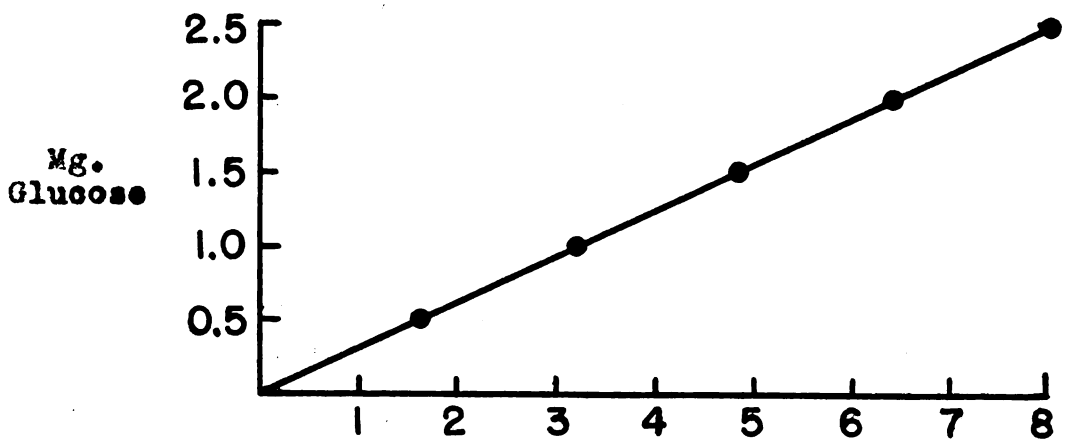
Standardization of Ceric Sulphate

Ml. standard glucose solution	Mg. of glucose	Ml. of ceric sulfate	Ml. of ceric sulfate per mg. glucose
0.5	0.5	1.600	3.200
1.0	1.0	3.225	3.225
1.5	1.5	4.845	3.230
2.0	2.0	6.427	3.214
2.5	2.5	8.058	3.223
			Av. 3.220

A linear relationship was found to exist between glucose oxidised and ferricyanide reduced under the titration conditions used in the present study. By plotting the number of mg. of glucose used to standardize ceric sulfate against the number of ml. of 0.0093 N ceric sulfate required to reach titration end-point, a straight line was obtained as shown in Figure I. This result agreed with the findings of Heinze and Murneek (22).

Figure I

Glucose-Ceric Sulfate Relationship



To calculate the reducing power in terms of maltose, the net titration value of the plant extract, obtained by subtracting the ml. of 0.0093 N ceric sulfate used to titrate the blank from that required for the

hydrolyzed aliquot, was divided by the number of ml. of 0.0093 N ceric sulfate equivalent to 1 mg. of maltose; i. e., 2.576. Starch equivalent was found by dividing the amount of maltose by the hydrolysis limit 0.890, as established by Nassid, McCready, and Rosenfels (19). The above calculations may be combined in one formula as follows:

$$\frac{(\text{ml. 0.0093 N ceric sulfate for sample titration} - \text{ml. 0.0093 N ceric sulfate for blank titration}) \times 100}{\text{mg. of plant sample} \times 0.890 \times 2.576} \times 100 = \% \text{ Starch}$$

For the second extraction procedure, that based on the method of Chinoy (16), an appropriate weight of plant sample in a 200 ml. Pyrex centrifuge bottle fitted with a reflux condenser was heated with 70 ml. of 0.7 per cent potassium hydroxide for forty minutes in a boiling water bath. After the preliminary heating period, the sample was boiled five minutes, cooled, centrifuged, and the solution was decanted into a 250 ml. volumetric flask. The extraction was then repeated with 50 ml. of the 0.7 per cent potassium hydroxide. The entire contents of the centrifuge bottle were transferred to the volumetric flask, diluted to volume, and filtered.

Two 25 ml. aliquots of the filtrate were taken for hydrolysis and for blank determination as outlined above

except that 4 N acetic acid was added to adjust the solution to a pH of 5.6 before addition of the diluted saliva.

The calcium chloride solution used for the third extraction procedure was prepared by adding 6 ml. of 0.8 per cent acetic acid for each 100 ml. of slightly alkaline calcium chloride of 1.3 density as described by Hoffpauir (15). Five ml. of calcium chloride was added to the sample in a Pyrex test tube measuring 150x25 mm. The tube was heated for fifteen minutes in an oil bath at 120° C. During the heating period, the suspension was stirred thoroughly with a glass rod. The sample was cooled, 20 ml. of water added, and the tube contents were thoroughly mixed. After centrifuging, the supernatant liquid was decanted into a 250 ml. volumetric flask. A second extraction was carried out, and the sample was transferred quantitatively to the volumetric flask and diluted to volume with water.

The solution was filtered and 25 ml. of aliquots were taken for analysis. Dilute sodium hydroxide solution was added to adjust the solutions to pH 5.6. The solutions were treated as before except that no sodium chloride was added prior to hydrolysis. In the evaluation of reducing power, the heating period and manner of titration were the same as described before.

However, the amounts of alkaline potassium ferricyanide and of 5 N sulfuric acid were increased from 5 to 10 ml.

Recovery of added starch was determined on duplicate samples of alfalfa for each of the three extraction methods studied. Exactly 50 mg. of dry defatted starch was mixed with 500 mg. of alfalfa which had been dried at 50° C. Starch extractions, hydrolysis, and determination of reducing power were carried out as previously described. The results are given in Table II.

Table II

Recovery of Starch from Alfalfa Dried at 50° C.

Sample	Ethanol-HCl treatment followed by hot water extraction of starch	0.7 per cent potassium hydroxide extraction of starch	Calcium chloride extraction of starch
500 mg. of alfalfa	35.39 mg.	28.79 mg.	38.39 mg.
	34.96 mg.	27.16 mg.	38.63 mg.
Average	35.18 mg.	27.97 mg.	38.51 mg.
500 mg. of alfalfa plus 50 mg. starch	87.24 mg.	71.63 mg.	92.90 mg.
	86.80 mg.	72.56 mg.	92.70 mg.
Average	87.02 mg.	72.10 mg.	92.80 mg.
Starch recovery	51.84 mg.	45.13 mg.	54.28 mg.
	103.68 %	90.26 %	108.56 %

To test the solubility of pure starch in the extraction reagents studied, 100 mg. of starch was weighed into appropriate vessels and treated separately with ethanol-hydrochloric acid followed by hot water extraction, with 0.7 per cent potassium hydroxide; and with calcium chloride solution. The starch appeared to dissolve completely in the hot water and in the potassium hydroxide. However, samples treated with calcium chloride formed a gel at the bottom of the tubes which was difficult to remove completely. On hydrolysis for two hours with 2 ml. of acetate buffer, 2 ml. of 0.25 N sodium chloride solution, and 2 ml. of diluted saliva as outlined by Hassid, McCready, and Rosenfels (19) and determination of reducing values, erratic results were obtained. The results in terms of per cent starch for duplicate samples, except in the case of calcium chloride treatment, are shown in Table III.

Table III

Per Cent Starch in Pure Defatted Samples

Ethanol-HCl treatment followed by hot water extraction	0.7 per cent potassium hydroxide extraction	Calcium chloride extraction
102.76	45.18	84.22
93.40	77.34	

The solubilization by ethanol-hydrochloric acid followed with hot water extraction and by 0.7 per cent potassium hydroxide were repeated on 50 mg. of defatted corn starch. Duplicate samples were analyzed in both cases. In the hydrolysis procedure, aliquots of the starch solutions were hydrolyzed for two hours with 2 ml. of acetate buffer, 2 ml. of 0.25 N sodium chloride solution, and 2 ml. of diluted saliva as described by Hassid, McCready, and Rosenfels (19). Second aliquots were hydrolyzed for four hours with 2 ml. of buffer, 4 ml. of sodium chloride, and 4 ml. of diluted saliva. Better agreement of values was obtained with the longer hydrolysis period and increased amounts of reagents as shown in Table IV. On the basis of these experimental results, the extracts from the plant samples studied were hydrolyzed for four hours with the larger volumes of reagents except for slight variations in the procedure for potassium hydroxide and for calcium chloride extracts as mentioned previously.

Table IV

Effect of Hydrolysis Conditions on Results of Analysis of Pure Starch

Hydrolysis Conditions	Ethanol-HCl treatment followed by hot water extraction	0.7 per cent potassium hydroxide extraction
2 hours with 2 ml. buffer, 2 ml. sodium chloride, and 2 ml. dilute saliva	95.10 % 91.50 %	88.70 % 96.31 %
4 hours with 2 ml. buffer, 4 ml. sodium chloride, and 4 ml. dilute saliva	97.75 % 97.03 %	95.51 % 95.66 %

RESULTS AND DISCUSSION

The percentages of starch found by each of the three solubilizing procedures described above and obtained for each of the four plant substances used are given in Tables V, VI, VII, and VIII.

Methods for preparing samples for analysis were selected from the literature and represented the procedures commonly used to dry and grind plant materials. One portion of each plant material studied was air-dried at room temperature to simulate as nearly as possible the drying of hay crops in the field. Drying at 50° C. in a warm room equipped with a ventilating fan and at 85-90° C. in an oven were used to determine the effect of temperature on starch content. A part of the fresh plant materials was frozen before final drying at 85-90° C. to determine the effect of low temperature on starch dissolution in the plant materials and also in an effort to rupture the cell walls and thus make starch extraction more complete. Samples dried at room temperature and at 50° C. had a green or yellow-green color whereas samples dried at 85-90° C. showed little or no browning with the exception of brome grass which turned dark brown. Samples which were frozen turned dark brown on drying in the oven.

The dried samples were ground to 60-mesh as

Table V

Per Cent Starch in Alfalfa
Calculated to Dry Weight Basis

(Each value given represents a separate sample)

Sample	Ethanol-HCl treatment followed by hot water extraction	0.7 per cent potassium hydroxide extraction	Calcium chloride extraction
Air-dried at room temperature	1.121 1.074 1.110 1.115	1.167 1.074 1.125 1.000	1.027 0.933 1.192 1.117
Warm room at 50° C.	6.941 7.127 7.611 7.424	6.613 6.988 6.071 6.118	7.786 8.053 7.781 7.963
Oven-dried at 85°-90° C.	6.706 6.567 6.723 6.589	5.429 5.159 4.454 4.222	6.569 7.384 6.727 7.000
Frozen and oven-dried at 85°-90° C.	7.623 7.675 7.543 7.454	5.709 5.143 4.709 4.445	8.097 7.424 7.770 7.963

Table VI

Per Cent Starch in Reed Canary Grass
Calculated to Dry Weight Basis

(Each value given represents a separate sample.)

Sample	Ethanol-HCl treatment followed by hot water extraction	0.7 per cent potassium hydroxide extraction	Calcium chloride extraction
Air-dried at room temperature	0.208 0.230 0.255 0.730	0.463 0.371 0.343 0.255	0.347 0.440 0.395 0.278
Warm room at 50° C.	0.322 0.370 0.394 0.300	0.324 0.231 0.322 0.227	0.324 0.370 0.302 0.370
Oven-dried at 85°-90° C.	0.885 0.836 0.885 0.898	0.682 0.573 0.526 0.478	1.745 0.885 0.956 0.862
Frozen and oven-dried at 85°-90° C.	0.468 0.398 0.537 0.444	0.608 0.506 0.515 0.561	0.374 0.258 0.422 0.305

Table VII

Per Cent Starch in Orchard Grass
Calculated to Dry Weight Basis

(Each value given represents a separate sample.)

Sample	Ethanol-HCl treatment followed by hot water extraction	0.7 per cent potassium hydroxide extraction	Calcium chloride extraction
Air-dried at room temperature	0.303 0.326 0.371 0.324	0.232 0.326 0.324 0.276	0.373 0.257 0.348 0.422
Warm room at 50° C.	0.564 0.467 0.537 0.586	0.560 0.560 0.492 0.562	0.467 0.584 0.561 0.656
Oven-dried at 85°-90° C.	0.328 0.233 0.305 0.348	0.352 0.258 0.210 0.280	0.447 0.517 0.374 0.468
Frozen and oven-dried at 85°-90° C.	0.329 0.393 0.395 0.346	0.195 0.301 0.302 0.196	0.307 0.460 0.533 0.488

Table VIII

Per Cent Starch in Brome Grass
Calculated on Dry Weight Basis

(Each value given represents a separate sample.)

Sample	Ethanol-HCl treatment followed by hot water extraction	0.7 per cent potassium hydroxide extraction	Calcium chloride extraction
Air-dried at room temperature	0.227 0.227 0.253 0.230	0.318 0.227 0.206 0.193	0.250 0.272 0.275 0.299
Warm room at 50° C.	0.365 0.319 0.323 0.370	0.205 0.238 0.254 0.208	0.434 0.338 0.346 0.416
Oven-dried at 85°-90° C.	0.301 0.277 0.255 0.299	0.162 0.182 0.253 0.206	0.346 0.324 0.299 0.368
Frozen and oven-dried at 85°-90° C.	0.375 0.325 0.348 0.372	0.141 0.164 0.140 0.253	0.445 0.468 0.418 0.372

recommended by Hassid, McCready, and Rosenfels (19). Chinoy (16) ground samples to 100-mesh whereas Hoffpauir (15) suggested grinding to finer than 80-mesh. Malhotra (23) determined the effect of fineness of material on chemical analyses and found that the point of diminishing return occurred at 60-mesh. In grinding the samples for the present study, some of the woody structural portions of the plant materials remained on the screen of the mill, but in general, the plant samples were fairly fine and powder-like.

In the evaluation of reducing power of solutions from calcium chloride extraction, a white precipitate formed on addition of alkaline potassium ferricyanide which dissolved readily in 5 N sulfuric acid. The effect of this precipitation on the reaction of the reducing components with the potassium ferricyanide was not investigated. However, to insure an excess of alkali and potassium ferricyanide, the amount of alkaline potassium ferricyanide added was increased from 5 to 10 ml. Subsequently, 10 ml. of 5 N sulfuric acid was added to maintain the same relative acid concentration in the final titration.

In the present study, salivary amylase was used as the hydrolytic agent for starch in the extracts obtained with three different reagents. Salivary amylase is active in the presence of fairly high salt concentrations. Sodium, potassium, and chloride ions activate

the hydrolytic action whereas Kneen, Sandstedt, and Hollenbeck (24) report that calcium ions exert a stabilizing effect on ∝-amylases.

There is some disagreement among various authors concerning the final products of salivary amylase hydrolysis of starch. Swanson (25) reported slight maltase activity after incubating corn starch with salivary amylase for more than twenty-four hours. Bourne, Hayworth, Macey, and Peat (26) found no increase in reducing power when salivary amylase was incubated with maltose, indicating the absence of maltase. In studying the end products resulting from the action of salivary ∝-amylase on potato amylose, Roberts and Whelan (27) found a mixture of maltose and maltotriose in the molecular proportion of 2.39:1. Hassid, McCready, and Rosenfels (19) calculated the reducing values found in terms of maltose assuming that no glucose was formed. Also, the last named authors found that less than 1 per cent of dextrins remained after hydrolysis of starch with salivary amylase.

The limiting hydrolysis value of 0.89 for salivary amylase was found by Hassid, McCready, and Rosenfels (19) to be constant for starches from different sources and of varying concentrations. Also, the hydrolysis limit appeared to be constant for salivary amylase from different individuals. Therefore, it was reasonably safe to assume

that the limit held for the present analyses.

Results from samples dried in different ways, Tables V through VIII, show some variations which seem to be dependent on the type of plant material studied. Alfalfa which was frozen and then dried contained more starch than samples prepared by the other curing methods. The oven-dried sample of reed canary grass had a higher content of starch whereas the sample of orchard grass cured at 50° C. contained a greater amount of starch. In the case of brome grass, the different drying procedures had little effect on the starch present. All of the plant varieties had the least amount of starch in the air-dried samples. The analytical results for the frozen samples might be explained on the basis of findings of Spoehr and Milner (3) that some plant materials show an increase in rate of starch dissolution with a decrease in temperature. Treatments which destroy protoplasm, such as freezing, do not necessarily destroy enzyme activity. However, in general, no starch dissolution occurs perhaps because of formation or deposition of some sort of protective material around the starch granules on destruction of protoplasm. Spoehr and Milner (3) also found that loss of water from leaves, as in wilting, causes an increase of starch dissolution which may account for the low starch content of the air-dried samples. Since amylase activity seems to follow the reaction rate-temperature rule, i. e.,

a 10 degree increase doubles the rate of reaction, at temperatures which do not destroy the enzyme, the rate of starch dissolution should be greater in plant samples dried at 50° C. and at 85-90° C. than at room temperature. However, if amylase were capable of hydrolyzing starch for a much shorter period of time at elevated temperatures than at room temperature as a result of enzyme destruction or formation of a protective material around the starch granules, then one might expect a higher starch content in samples dried in the warm room and drying oven than in those which were air-dried.

In general, starch values of samples extracted with calcium chloride were higher and those from 0.7 per cent potassium hydroxide were lower than those from ethanol-hydrochloric acid treatment followed by hot water extraction. The blank titrations from ethanol-hydrochloric acid treatment followed by hot water extraction were less than 1 ml. of 0.0093 N ceric sulfate indicating the presence of a small amount of non-starch reducing substance. Blank titrations from 0.7 per cent potassium hydroxide and from calcium chloride extractions were rather high, ranging from 1 ml. to 6 ml. of 0.0093 N ceric sulfate. Hemicellulose and pectic substances were probably extracted with dilute potassium hydroxide. Also, any sugars present would be extracted and might react with the potassium hydroxide. The calcium chloride

solution used was acidic and may have reacted with hemicellulose and pectic substances to release reducing materials. Also, sugars in the plant materials would be extracted. In both potassium hydroxide and calcium chloride extraction, a considerable amount of the chlorophyll in the plant samples was extracted as evidenced by green solutions. Addition of lead acetate after enzymatic hydrolysis would remove much of the reducing substances present. However, any sugars and other substances not precipitated by lead would be determined in the blank titrations. The magnitude of the various blank titrations from potassium hydroxide and calcium chloride extractions may reflect the amount of sugars present in the plant samples.

Starch values from ethanol-hydrochloric acid treatment followed by extraction with hot water are more reproducible than those from the potassium hydroxide and calcium chloride extractions. One reason may be the relatively high blank titrations in the latter two methods, particularly in the case of reed canary grass, orchard grass, and brome grass which contained less than 1 per cent of starch and required less than 0.2 ml. of 0.0093 N ceric sulfate net titration.

In view of findings presented, the procedure, other than air-drying, used in curing plant samples for starch analysis would depend upon the type of plants studied.

The extraction procedure of Hassid, McCready, and Rosenfels (19) is more satisfactory than extraction with either potassium hydroxide or calcium chloride because of the small amount of reducing substances present in the extract. Also, more reproducible starch values and better recovery of added starch were obtained by ethanol-hydrochloric acid treatment and water extraction than by other extraction procedures.

SUMMARY

1. Samples of alfalfa, reed canary grass, orchard grass, and brome grass were taken on warm, clear afternoons from unfertilized and unirrigated experimental plots of the Department of Soil Science.
2. The plant samples were each divided into four portions for drying at room temperature, at 50° C., at 85-90° C., and freezing followed by drying at 85-90° C.
3. After grinding the dried samples to 60-mesh, starch was extracted by three different procedures involving: (1) solubilization with ethanol-hydrochloric acid followed by hot water extraction; (2) 0.7 per cent potassium hydroxide extraction; and (3) calcium chloride extraction.
4. Starch in the extracts was hydrolyzed with salivary amylase and reducing power was determined by treating the hydrolyzed extract with alkaline potassium ferricyanide followed by titration of ferrocyanide formed with standard ceric sulfate solution.
5. Analytical results indicate that the choice of curing procedure to preserve the greatest amount of starch is dependent upon the particular plant studied.

6. Best reproducibility and agreement of starch values for a specific plant sample were obtained by the treatment of plant material with ethanol-hydrochloric acid followed by extraction of solubilized starch with hot water.

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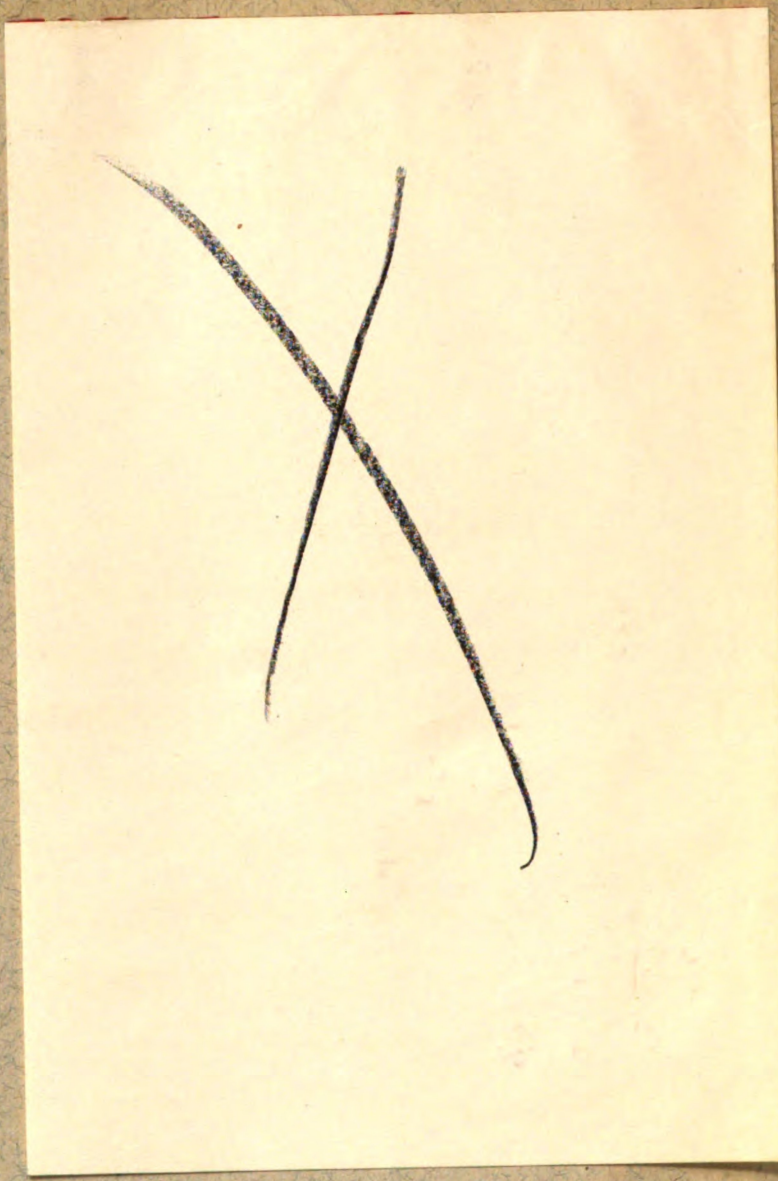

ABSTRACT

A STUDY OF EFFECTS OF SAMPLE PREPARATION AND METHODS OF EXTRACTION ON DETERMINATION OF STARCH IN PLANT MATERIALS

By

Betty Ruth Johnston

The present study was made to determine the effectiveness of some of the analytical procedures for the determination of small amounts of starch in plant materials with special attention given to preliminary sample preparation, completeness of starch extraction, and evaluation of starch with the exclusion of other carbohydrates.

Samples of alfalfa, Medicago sativa, just before blooming and second growths of brome grass, Bromus inermis, reed canary grass, Phalaris arundinacea, and orchard grass, Dactylis glomerata, were taken on warm, clear afternoons from unfertilized and unirrigated experimental plots of the Department of Soil Science. The fresh plant samples were each divided into four portions for drying at room temperature, at 50° C., at 85-90° C., and freezing followed by drying at 85-90° C. After grinding the dried samples to 60-mesh, starch was extracted by three different procedures involving: (1) solubilization with ethanol-hydrochloric acid followed by hot water extraction as described by Hassid, McCready, and Rosenfels; (2) 0.7 per cent potassium hydroxide extraction as used

by Chinoy; and (3) calcium chloride extraction as outlined by Hoffpauir. Starch in the extracts was hydrolyzed with salivary amylase. Reducing power was determined by treating the hydrolyzed extracts with alkaline potassium ferricyanide followed by titration of ferrocyanide formed with standard ceric sulfate solution.

Analytical results indicate that the choice of curing procedure to preserve the greatest amount of starch is dependent upon the particular plant studied. Best reproducibility and agreement of starch values for a specific plant sample were obtained by the treatment with ethanol-hydrochloric acid followed by extraction of solubilized starch with hot water as described by Hassid, McCready, and Rosenfels (1).

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