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A method for routine measurements of total sugar and starch content in woody plant tissues

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Summary Several extraction and measurement methods currently employed in the determination of total sugar and starch contents in plant tissues were investigated with the view to streamline the process of total sugar and starch determination. Depending on the type and source of tissue, total sugar and starch contents estimated from samples extracted with 80% hot ethanol were significantly greater than from samples extracted with a methanol:chloroform:water solution. The residual ethanol did not interfere with the sugar and starch determination, rendering the removal of ethanol from samples unnecessary. The use of phenol–sulfuric acid with a phenol concentration of 2% provided a relatively simple and reliable colorimetric method to quantify the total soluble-sugar concentration. Performing parallel sugar assays with and without phenol was more useful for accounting for the interfering effects of other substances present in plant tissue than using chloroform. For starch determination, an enzyme mixture of 1000 U α-amylase and 5 U amyloglucosidase digested starch in plant tissue samples more rapidly and completely than previously recommended enzyme doses. Dilute sulfuric acid (0.005 N) was less suitable for starch digestion than enzymatic hydrolysis because the acid also broke down structural carbohydrates, resulting in overestimates of starch content. After the enzymatic digestion of starch, the glucose hydrolyzate obtained was measured with a peroxidase–glucose oxidase/*o*-dianisidine reagent; absorbance being read at 525 nm after the addition of sulfuric acid. With the help of this series of studies, we developed a refined and shortened method suitable for the rapid measurement of total sugar and starch contents in woody plant tissues.

Keywords: acid hydrolysis, analysis, colorimetric, enzymatic digestion, ethanol extraction, MCW extraction, phenol–sulfuric acid.

Introduction

Many physiological studies of growth and reserve allocation in plants require the separate measurements of sugars and starch in tissues. Generally, water-soluble sugars are extracted from the tissue sample and starch content is determined in the residue. The process is time-consuming and costly. Two methods are frequently used for the extraction of soluble sugars: the first, a hot ethanol solution (Ebell 1969, MacRae et al. 1974, Rose et al. 1991); and the second, a methanol:chloroform:water solution (Dickson 1979, Rose et al. 1991). Both methods are thought to be effective in removing soluble sugars from plant tissues, but their efficacies on sugar and subsequent starch determinations have not been compared.

Plant extracts contain diverse mixtures of sugars. The presence of glucose, fructose, galactose, sucrose, maltose, melibiose, raffinose and stachyose have been reported from tissues of beech (Dietrichs and Schaich 1964), aspen (Wildman and Parkinson 1979), poplar (Fege and Brown 1984, Sauter 1988) and birch (Sauter and Ambrosius 1986). Although these sugars can be separately determined by high-performance liquid chromatography (HPLC) (Casterline et al. 1999) and gas chromatography (GC) (Carlsson et al. 1992), the process is expensive especially when only the total amount of glucose equivalents is of interest (e.g., reserve and carbon allocation studies). The enzymatic method based on NADPH absorption (Blunden and Wilson 1985, Hendrix 1993) requires a specific enzyme for each of the sugars, making it a relatively expensive and lengthy process. Methods that measure reducing sugars (Ashwell 1957, Miller 1959) exclude many of the oligosaccharides (e.g., sucrose), unless they are first hydrolyzed. Colorimetically, the anthrone method (Scott and Melvin 1953) can detect all types of sugars; however, because of the large difference in absorption coefficients among the different types of sugars, it is known to produce errors in the analysis of sugar mixtures (Ashwell 1957). A simpler colorimetric method for sugar determination uses phenol–sulfuric acid (Dubois et al. 1956). Although Buysse and Merckx (1993) optimized the phenol–sulfuric acid method for a sugar mixture of glucose, fructose and sucrose by adjusting the amount of phenol, an optimization of this method for a mixture that includes all dominant sugars present in plant extracts has not been reported.

Alcohol-soluble substances present in plant extracts, including chlorophylls, lipids and proteins, react with the concentrated sulfuric acid in the sugar assay and therefore significantly interfere with the absorbance reading (Ashwell 1957). To remove these substances, the use of chloroform is considered easier and more effective than hexane, activated charcoal or ion-exchange columns (Haslemore and Roughan 1976, Dickson 1979, Fege and Brown 1984). As an alternative to the removal of these interfering substances, Ashwell (1957) sug-

gested that running parallel assays for each sample with and without a color developer could also provide a correction of the absorbance readings. This approach has yet to be tested in sugar assays using phenol–sulfuric acid.

To estimate starch content in tissue samples, most methods hydrolyze the starch to glucose, which is subsequently assayed. Among these methods are perchloric acid hydrolysis (MacRae et al. 1974, Rose et al. 1991), which is considered dangerous because of the instability of perchloric acid; sulfuric acid hydrolysis (Smith et al. 1964), which is considered the simplest and most rapid; and enzyme digestion with amylase and amyloglucosidase (Smith 1969, Haissig and Dickson 1979, Rose et al. 1991, Hendrix 1993), which is considered the most accurate but the most labor intensive. Grotelueschen and Smith (1967), Greub and Wedin (1969) and Kozloski et al. (1999) compared the sulfuric acid and enzymatic methods on legume roots, grasses and cereals; however, they determined the total nonstructural carbohydrate content without separating the soluble sugars from starch. In addition, the authors used sulfuric acid at concentrations of 0.2 N or higher, which could break down structural carbohydrates. So far, few data are available on the use of more dilute acid for the exclusive estimation of starch in woody plant tissues. Rose et al. (1991) described methods for digesting starch with enzyme mixtures of α-amylase and amyloglucosidase, but the completeness of digestion was not tested in their studies.

Currently, the removal of the ethanol after extraction is among the most time-consuming steps in the determination of sugar and starch in tissue samples. Buysse and Merckx (1993) showed that, in pure sugar solutions, absorbance readings increased for glucose but decreased for fructose and sucrose, with increasing ethanol content when analyzed by the phenol–sulfuric acid method. However, the effects of residual ethanol on the analysis of total sugar in plant extracts and on the subsequent analysis of starch have not been investigated.

The overall objective of this paper was to review selected methodologies for total sugar and starch analyses in plant tissues, and to shorten the existing procedures to establish a protocol suitable for rapid routine measurements of total sugar and starch reserves in plant tissues.

Materials and methods

Plant materials and preparation

Leaf, stem and root samples of aspen (*Populus tremuloides* Michx.), black spruce (*Picea mariana* (Mill.) BSP.) and lodgepole pine (*Pinus contorta* Loudon) seedlings and saplings were oven-dried at 68 °C for 2–3 days, ground in a Wiley mill to pass 40-mesh and stored in airtight containers at room temperature, in the dark, until analysis. Tissue samples analyzed in the different studies varied by type and collection.

Comparison of sugar extraction methods

Two methods of soluble sugar extraction, hot ethanol and methanol:chloroform:water (MCW, 12:5:3, v/v/v), were compared on dried samples of five different woody plant tissues (leaves of aspen, spruce and pine, aspen roots and pine shoots). For each tissue sample, five subsamples (50 mg) were extracted three times with 5 ml of 80% ethanol, by boiling the samples in glass tubes capped with glass marbles in a 95 °C water bath for 10 min each. After each extraction, the tubes were centrifuged at 2500 rpm for 5 min, and the supernatants of the three extractions combined for sugar analysis. A fourth ethanol extraction yielded less than 0.5% of the total sugar in the first three extracts. The residues remaining in the tubes were stored wet at –20 °C for starch analysis. Whether residues were oven-dried or wet had no effect on the subsequent starch analysis (see below).

A second set of five subsamples was extracted three times with 5 ml of MCW solution. Sample tubes were loosely capped, placed in a sonic bath for 5 s, and left at room temperature for 10 min. Samples were then centrifuged at 2500 rpm for 10 min and the supernatants of the three extractions were combined (Rose et al. 1991). An earlier study had shown that three extractions with MCW removed about 98% of the water-alcohol-soluble compounds from leaf material (Dickson 1979). A 5-ml sample was taken from each combined extract, mixed with 3 ml of deionized water (dH_2O) and separated into two phases by centrifuging at 2500 rpm for 5 min. The chloroform phase was discarded and the methanol:water phase was analyzed for sugar. The residues were oven-dried at 50 °C overnight to remove the residual solvent, and stored at –20 °C for starch analysis.

For both extraction methods, sugar concentrations in extracts were determined by the phenol–sulfuric acid method without removing the aqueous ethanol or methanol solvent.

Optimization of phenol dosage and absorbance wavelength for sugar mixtures

Oligosaccharides are hydrolyzed by concentrated sulfuric acid during the phenol–sulfuric assay and form monomers, namely glucose, fructose and galactose (Sturgeon 1990). Therefore, in the analysis of mono- and oligosaccharides in a plant extract, the intermediate product after acid hydrolysis is mostly a mixture of glucose, fructose and galactose, which are thus the principal compounds measured in the sugar assay. Thus optimizing the phenol concentration and absorbance wavelength for these three monomers should increase the accuracy of total sugar estimation in plant extracts based on a single measurement.

For the optimization, five 0.5 ml solutions of glucose, fructose, or galactose (50 μg ml^{-1}) were mixed with 1 ml of a phenol solution at one of five concentrations (1, 1.5, 2, 3 and 4% phenol) followed by the rapid addition of 2.5 ml of concentrated sulfuric acid (H_2SO_4). After 10 min of color development in the dark and an additional 30 min of cooling in a water bath at 22 °C, absorbance was measured at wavelengths ranging from 465 to 505 nm in 5 nm increments. Each sugar and phenol combination was replicated five times.

After optimization of the phenol concentration and the absorption wavelength, the absorption coefficient of the sugar mixture (1:1:1, glucose, fructose, galactose; GFG) was compared with the absorption coefficients of the single sugars: glucose, fructose, galactose, sucrose, maltose, melibiose, raffinose and stachyose, to verify that the GFG mixture can be used

as the calibration standard for the colorimetric analysis of the single sugars. Because the oligosaccharides increase in molecular weight after hydrolysis to monomers, the concentrations of all sugar solutions were standardized based on their monomer equivalent.

The sugar assay was also verified on prepared pure sugar mixtures (Table 1) with known concentrations, from 50 to 200 μg ml^{-1}. The composition of the pure sugar mixtures was derived from information on plant extracts found in the published literature (Wildman and Parkinson 1979, Sauter and Ambrosius 1986, Sauter 1988). Sugar concentrations were determined against the GFG mixture standard.

Interfering substances in the ethanol extract

Two methods that account for the interfering effects on sugar determination of compounds (primarily pigments and proteins) found in plant extracts were compared. One method physically removes the interfering substances with chloroform, whereas the other method corrects the absorbance readings based on parallel assays.

Raw ethanol extracts were made from three samples each of four plant tissue types (leaves of aspen, pine and spruce, and pine roots). To clean the plant extracts with chloroform by fractionation, 1 ml of chloroform was mixed with 3 ml of extract to form a monophasic liquid. After shaking with 5 ml of dH_2O and centrifuging at 2500 rpm for 5 min, two phases separated. The chloroform phase was discarded and the aqueous phase was assayed for sugar. The experiment was repeated by increasing the chloroform to extract ratio from 1:3 to 1:2.5 and 1:2. For comparison, sugar assays were carried out on the raw extracts before chloroform treatment.

In the second method, the raw plant extracts were subjected to parallel sugar assays: with and without phenol (the phenol solution was replaced in the control by an equivalent volume of dH_2O). In the assay with phenol, the absorbance (A) measured is given by:

$$A = a_s[\text{sugar}]_u \quad (1)$$

where a_s is the absorption coefficient of the GFG standard and $[\text{sugar}]_u$ is the sugar concentration uncorrected for interference. However, when taking the interference into account:

$$A = a_s[\text{sugar}]_c + A_I \quad (2)$$

where $[\text{sugar}]_c$ is the corrected sugar concentration and A_I is the absorbance of interfering substances. In the parallel assay without phenol, the absorbance (A') measured is given by:

$$A' = a'_s[\text{sugar}]_c + A'_I \quad (3)$$

where a'_s is the absorption coefficient of the GFG standard without phenol and A'_I is the absorbance of interfering substances without phenol. Assuming that the interfering substances react with the concentrated H_2SO_4 similarly in both assays and thus have the same interfering absorbance, i.e., $A_I = A'_I$, then, subtracting Equation 3 from Equation 2 gives:

$$A - A' = (a_s - a'_s)[\text{sugar}]_c \quad (4)$$

or

$$[\text{sugar}]_c = \frac{A - A'}{a_s - a'_s} \quad (5)$$

The corrected sugar concentration of each extract solution was calculated according to Equation 5 after the absorption coefficients of the GFG standard were determined from standard curves.

The parallel assay method was verified on mixtures containing a GFG standard mixed with a pine leaf extract that contains significant amounts of interfering substances. Four mixtures were made, with 0.5 ml of a leaf extract combined with 0, 2.5, 5.0 or 7.5 ml of 100 μg ml^{-1} GFG sugar solution producing samples with decreasing interfering substances relative to sugar content.

Acid hydrolysis versus enzymatic digestion for starch estimation

To explore the possibility of using a dilute acid for hydrolyzing the starch content of woody plant tissues instead of digestive enzymes, eight pure polysaccharides (cotton cellulose, citrus pectin, starch from arrowroot, corn and potato, and hemicellulose including arabic, damar and guaiac gum) and four de-sugared tissue samples (shoots and roots of aspen and spruce) were tested.

The method of acid hydrolysis was adapted from Grotelueschen and Smith (1967); however, our sulfuric acid concentration was 40 times lower (0.005 N) than that used by the previous authors. Plant tissue (20 mg), starch (5 mg), and cellulose, hemicellulose and pectin (50 mg) samples were refluxed for 0.5 and 1 h with 5 ml of 0.005 N H_2SO_4 in a 95 °C water bath. After hydrolysis, the contents were vacuum filtered through Whatman No. 40 filter paper. Filtrates were analyzed for glucose, by the phenol–sulfuric acid method and concentrations determined against a glucose standard. Starch content of the samples was estimated by multiplying the glucose content by the glucose equivalent of 0.9.

A replicate set of samples was digested with an enzyme mixture containing α-amylase and amyloglucosidase (Rose et al. 1991). Samples were weighted as described above. Each

Table 1. Sugar composition (SC) as a percentage of total sugar. Sugar compositions found in plant extracts are based on published values for various tissues (Wildman and Parkinson 1979, Sauter and Ambrosius 1986, Sauter 1988).

Sugar	% SC found in plant extracts	Pure sugar mixtures (%)		
		Mix 1	Mix 2	Mix 3
Glucose	7.2–36.7	30	15	5
Fructose	6.3–52.8	50	25	5
Galactose	0–23.7	20	10	0
Sucrose	0–40.2	0	25	35
Maltose	0–36.6	0	10	25
Raffinose	0–10.3	0	5	10
Stachyose	1.6–20.7	0	10	20

sample was heated with 2 ml of 0.1 N sodium hydroxide (NaOH) in a 50 °C water bath for 30 min with intermittent mixing. After neutralizing with 2.5 ml of 0.1 N acetic acid, 0.5 ml of a digestive enzyme mixture containing 400 U ml^{-1} of α-amylase (from *Bacillus licheniformis*, ICN-190151, ICN Biomedicals, Aurora, OH) and 2 U ml^{-1} of amyloglucosidase (from *Aspergillus niger*, Sigma A-1602, Sigma Chemicals, St Louis, MO) in 0.05M sodium acetate buffer (pH 5.1) was added. The combined solution was incubated for 48 h in a 50 °C water bath. To determine the amount of glucose hydrolysate, the digest was centrifuged at 2500 rpm for 10 min and the supernatant diluted with 0.05 M sodium acetate buffer, as required. To 0.2 ml of the diluted sample solution, 2 ml of a peroxidase–glucose oxidase/*o*-dianisidine reagent (PGO-color solution, prepared by dissolving one capsule of PGO enzymes (Sigma P-7119) in 100 ml of dH_2O and mixed with 1.6 ml of *o*-dianisidine solution (50 mg of *o*-dianisidine dihydrochloride (Sigma D-3252) in 20 ml of dH_2O) was added and mixed. The absorbance was read at 450 nm, after leaving the mixture in darkness at room temperature for 45 min. The amount of glucose was calculated against a glucose standard prepared in the sodium acetate buffer solution. A preliminary test showed that using phenol–sulfuric acid for the glucose assay after enzyme digestion of starch was not possible, because the concentrated H_2SO_4 hydrolyzed the enzyme and therefore gave unreliable results.

Efficiency of enzymatic digestion of starch

To examine the amount of enzyme and time required for complete hydrolysis of starch in plant tissues, tissue samples from aspen and spruce roots, aspen shoots, spruce and pine leaves, as well as purified potato starch (Sigma S-2630) were digested with either 400 U ml^{-1} of α-amylase + 2 U ml^{-1} of amyloglucosidase (Rose et al. 1991) or 2000 U ml^{-1} of α-amylase + 10 U ml^{-1} of amyloglucosidase. The high dosage was calculated from the guideline set out by Haissig and Dickson (1979), based on a 50 mg sample weight with a starch content of 30%. Tissue samples (50 mg) and potato starch samples (5 mg) were digested for either 24 or 48 h.

Measurement of glucose hydrolysate from enzymatic digestion of starch

During starch solubilization, colored impurities are readily noticeable when sample material is heated with sodium hydroxide, resulting in a brown solution. The brown color is maintained throughout the enzyme digestion process and interferes with the colorimetric measurement of glucose hydrolysates obtained after the digestion (Haissig and Dickson 1979). To overcome this interference, parallel assays were performed to test at which wavelength an absorbance correction can be made.

After ethanol extraction, the residues of five different tissues (leaves of aspen, spruce and pine, aspen roots and pine shoots) were digested with 0.5 ml of an enzyme mixture containing 2000 U ml^{-1} of α-amylase and 10 U ml^{-1} of amyloglucosidase for 20 h at 50 °C. Samples of potato starch and an enzyme blank were also included. Because the enzyme mixture contained a small amount of glucose, the total amount of glucose hydrolysate was corrected for all samples. After digestion, the resulting solution of each sample was split into four subsamples for glucose measurement. The first subsample was analyzed with the addition of 2 ml of PGO-color solution alone (as described above) (Haissig and Dickson 1979, Rose et al. 1991), whereas the second subsample was analyzed with 2 ml of PGO-color solution followed by the addition of 0.4 ml of 75% H_2SO_4 (Ebell 1969, Rose et al. 1991). To determine the amount of interference from sample impurities, the third subsample was analyzed with 2 ml of dH_2O alone and the fourth subsample with 2 ml of dH_2O plus 0.4 ml of 75% H_2SO_4. Absorbances of all four subsamples were read at wavelengths from 400 to 575nm in 25nm increments. The experiment was repeated five times.

The effects of residual ethanol on sugar and starch analyses

To determine the effect of residual ethanol on the sugar assay, a sample of spruce leaf tissue was extracted with ethanol, and equal aliquots of the extract were transferred to 25 test tubes. After drying in a 50 °C oven overnight, ethanol of five concentrations (0, 20, 40, 60 and 80%) was added to redissolve the residue in replicates of five. A GFG standard (100 μg ml^{-1}) was also prepared in ethanol of the same five concentrations. All samples were assayed for sugar, and absorbances were read and corrected for interference.

The effect of the residual ethanol on the starch analysis was studied on six tissue samples (leaves and roots of aspen and spruce, and leaves and shoots of pine). From each sample, 10 subsamples were extracted with ethanol and residues were randomly divided into two sets of five. One set was oven-dried at 50 °C overnight to remove the residual ethanol and water, and the second set was kept frozen at –20 °C overnight without drying. Both sets of residue were analyzed enzymatically for starch as described above.

Statistical analysis

In all studies, sugar and starch contents were evaluated by analysis of variance of paired comparisons (SAS Institute, Cary, NC).

Results and discussion

Comparison of sugar extraction methods

Overall, samples extracted with methanol:chloroform:water (MCW) solution gave total nonstructural carbohydrate (TNC) estimates 6 to 29% lower than samples extracted with ethanol. Estimates of sugar content in the five different plant tissue samples were 7 to 16% lower when extracted with MCW than when extracted with 80% hot ethanol ($P < 0.001$). Similarly, starch content estimated in MCW-extracted samples was 2 to 96% lower than in samples extracted with ethanol ($P = 0.019$) (Table 2). Some of the differences could be explained by the extraction process. During the MCW extraction, sugars are removed from the tissues by brief sonication without any further agitation, whereas samples extracted with ethanol are agitated constantly by boiling and thus sugars might be removed more efficiently. Further, starch grains are gelatinized by boiling in

ethanol, so they dissolve better in the NaOH during starch digestion (Rose et al. 1991). Although not tested, the large range of differences between the two extraction methods, especially in starch content, might indicate that the success of the extraction technique can vary with tissue type (e.g., leaf versus root) (Table 2).

Optimization of phenol concentration and absorbance wavelength for sugar mixtures

Absorption peaks of glucose, fructose and galactose were observed at 490 nm, which is similar to the findings reported by Dubois et al. (1956). Our study indicates that a phenol concentration of 2% produced the smallest difference in the absorbance readings among the three sugars (Figure 1). As a result, measuring absorbance at 490 nm with the use of 2% phenol in the sugar assay gave the closest match to the absorption coefficients of the three basic monomers without compromising the sensitivity of the assay (Figure 1C).

Based on a phenol concentration of 2% and an absorption wavelength of 490 nm, the standard curves of the GFG standard and the sugars (glucose, fructose, galactose, sucrose, maltose, melibiose, raffinose and stachyose) were linear up to 200 μg ml^{-1}. Although there were differences in absorption coefficients among the sugars, which ranged from 0.00628 cm^2 μg^{-1} for fructose to 0.00716 cm^2 μg^{-1} for glucose, the absorption coefficient of the GFG standard (0.00679 cm^2 μg^{-1}) was close to the mean absorption of the eight single sugars (0.00668 cm^2 μg^{-1}). Considering that fructose and galactose are equivalent to glucose in molecular weight, this sugar assay using GFG as a reference standard can approximate the glucose equivalent of the tested sugars.

The sugar assay quantified 96 to 100% of the total sugar contained in the pure sugar mixtures, indicating that it can satisfactorily estimate the glucose equivalent of sugar mixtures. However, when working with plant tissues that might contain sugars other than those tested in this study, absorbances of those particular sugars need to be tested against the GFG standard.

Interfering substances in the ethanol extract

The sugar concentrations were 2 to 7% lower in extracts treated with chloroform than in extracts without the chloroform treatment ($P < 0.001$); however, increasing the amount of chloroform resulted in no further reduction in the sugar estimates ($P = 0.968$). By running parallel assays with and without phenol, the sugar concentrations in the extracts estimated after the interference correction were 11 to 36% lower ($P < 0.001$) than those without correction, suggesting that the chloroform fractionation removed only a portion of the interfering substances from the plant tissue extracts. In addition, another study indicated that the compounds removed by chloroform (primarily chlorophylls and carotenoids) absorbed mainly at wavelengths below 420 nm (data not shown). These substances would interfere only slightly with sugar absorption at 490 nm. As increasing the amount of chloroform did not reduce the estimated quantity of sugar, interfering substances that remained were likely water-soluble compounds such as proteins and other non-carbohydrate reducing agents (Sturgeon 1990).

When extracts treated with and without chloroform were analyzed in parallel assays, the sugar estimates after interference corrections were not significantly different ($P = 0.448$). Using the parallel-assay method on the four GFG/pine leaf extract mixtures gave sugar recoveries between 98 and 99%. This indicates that the correction of interference by means of parallel sugar assays can give accurate estimates of sugar in plant extracts.

The amount of interfering substances was found to be closely related to the sugar content of the plant tissue. The ratio of the corrected to the uncorrected sugar content measured in leaf, shoot and root samples of aspen, spruce and pine were found to be constant within each plant-tissue type (Table 3). Consequently, we believe that for each type of plant tissue the sugar content can be calculated from Equation 1 and corrected using the ratio obtained from a group of representative samples. This saves time by eliminating the need for parallel sugar assays of all samples.

Comparison of acid hydrolysis and enzymatic digestion methods for starch estimation

After half an hour, 0.005 N sulfuric acid hydrolyzed the three sources of starch (arrowroot, corn and potato) by 67 to 86%,

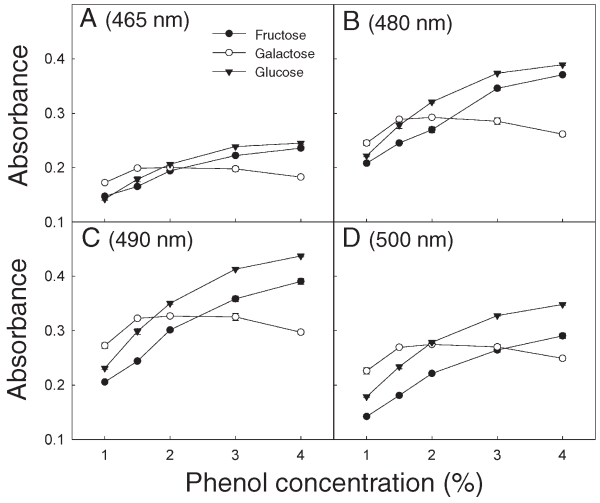

Figure 1. Absorbance of glucose, fructose and galactose (50 μg ml^{-1}) at various wavelengths and phenol concentrations in the phenol–sulfuric acid sugar assay. Data represent means and SE of 5 replicates.

Table 2. Sugar and starch contents (% dry mass) of plant tissue samples extracted with either 80% hot ethanol (ETOH) or methanol:chloroform:water (MCW) solution. Values are means of 5 subsamples.

Sample	Sugar (%)		Starch (%)	
	ETOH	MCW	ETOH	MCW
Aspen leaf	24.7	22.9	0.28	0.01
Aspen root	10.4	9.3	11.82	11.57
Pine leaf	7.3	6.1	3.17	1.38
Pine shoot	7.5	6.8	0.72	0.26
Spruce leaf	11.8	10.6	4.15	2.13

Table 3. Mean ratio ± SD of corrected to uncorrected sugar contents measured in ethanol extracts of various plant tissues from *n* different trees.

Species	Tissue	Ratio	*n*
Aspen	Leaf	0.88 ± 0.04	29
	Shoot	0.91 ± 0.02	39
	Root	0.89 ± 0.03	28
Pine	Leaf	0.74 ± 0.03	39
	Shoot	0.82 ± 0.04	39
	Root	0.81 ± 0.03	19
Spruce	Leaf	0.75 ± 0.04	38
	Shoot	0.83 ± 0.01	18
	Root	0.80 ± 0.04	20

arabic gum and citrus pectin by 74 and 53%, respectively, and cotton cellulose and guaiac gum both by 1%. Damar gum was not hydrolyzed. Thus, even at low concentrations, sulfuric acid can partially hydrolyze structural carbohydrates in plant tissues, giving rise to an overestimate of starch content. However, the enzyme mixture digested the three sources of starch by 84 to 89% without digesting cellulose or hemicellulose, although it broke down 19% of the citrus pectin. As woody tissues contain little pectin (Kramer and Kozlowski 1979), starch estimation by enzyme digestion can be considered acceptable.

Sulfuric acid hydrolysis gave generally higher estimates of starch content than the enzyme method for all plant tissue samples (P = 0.004), suggesting that sulfuric acid hydrolyzed some structural carbohydrates to glucose (Table 4).

Efficiency of enzymatic digestion of starch

Independent of enzyme dosage (P = 0.942) and digestion time (P = 0.285), there was, overall, no significant difference in the amount of purified starch digested. However, in plant tissues incubated with the low enzyme dosage, starch estimates after 48 h of digestion were 3 to 16% higher (P = 0.013) than those digested for only 24 h (Table 5), suggesting that the low dosage may have been insufficient for complete hydrolysis. Haissig and Dickson (1979) reported that without enough enzyme, the amount of glucose yielded by starch digestion increases with digestion time, but full digestion is not achieved. In our tests, samples digested for 24 h with the high enzyme dose yielded starch estimates that were 5 to 28% higher than samples that had been digested for 48 h with the low enzyme dose (P < 0.001). However, extending the digestion time to 48 h at the high dose did not result in higher glucose yields (P = 0.060). This indicates that the high enzyme dose is preferable, particularly for rapid assays. A separate test showed that, at the high enzyme dose, the digestion period could be further shortened to 20 h without compromising the starch estimation (P = 0.765).

Table 4. Starch content (% dry mass) of plant tissue samples estimated by enzyme digestion (Enzyme) and dilute sulfuric acid (H_2SO_4) hydrolysis (n = 2). Abbreviations: OS = old shoot; and NS = new shoot.

Sample	Starch estimated (%)		
	Enzyme (48 h)	H_2SO_4 hydrolysis (0.005 N)	
		0.5 h	1 h
Aspen OS	0.40	3.08	4.47
Aspen root	2.78	4.46	6.55
Spruce NS	0.62	8.10	10.25
Spruce root	0.94	3.68	4.31

Measurement of glucose hydrolysate from enzymatic digestion of starch

Colored impurities formed during the enzymatic digestion of starch had a very broad absorption spectrum with an absorption maximum at or below 400 nm (Figure 2A). Absorption curves of sample solutions analyzed with the addition of dH_2O showed that the degree of interference at 525 nm was less than half that at 450 nm (Figure 2A). The addition of sulfuric acid to the sample solution lowered the absorption at 525 nm by between 0 to 50%, depending on the type of plant tissue (Figure 2B). Samples analyzed with PGO-color solution alone had an absorption maximum at 450 nm where interference is still high (Figure 2C). In contrast, samples analyzed with PGO-color solution and sulfuric acid had absorption peaks at 525 nm where interference was significantly less (Figure 2D). Combining these effects, the interfering substances will have a smaller impact on the measurement of glucose hydrolyzate when absorbance is measured at 525 nm after the addition of sulfuric acid.

After making corrections for interference in the plant samples by subtracting the absorbance readings obtained without

Table 5. Mean starch content ± SD (% dry mass) of plant tissue samples estimated following digestion with low (400 U ml^{-1} α-amylase + 2 U ml^{-1} amyloglucosidase) and high (2000 U ml^{-1} α-amylase + 10 U ml^{-1} amyloglucosidase) enzyme dose for 24 or 48 h (n = 5).

Sample	Low enzyme dose		High enzyme dose	
	24 h	48 h	24 h	48 h
Aspen shoot	2.96 ± 0.05	3.05 ± 0.09	3.90 ± 0.06	3.87 ± 0.07
Aspen root 1	5.30 ± 0.23	5.49 ± 0.12	6.20 ± 0.27	6.33 ± 0.12
Aspen root 2	7.40 ± 0.05	8.58 ± 0.27	9.05 ± 0.17	9.79 ± 0.37
Pine leaf	19.9 ± 0.2	20.8 ± 0.1	22.2 ± 0.2	22.6 ± 0.5
Spruce leaf	23.4 ± 0.4	25.0 ± 0.5	26.5 ± 0.5	27.4 ± 0.5
Spruce root	7.87 ± 0.56	9.17 ± 0.59	10.1 ± 0.3	11.7 ± 0.4
Potato starch	99.4 ± 3.7	97.3 ± 3.2	99.6 ± 3.1	96.8 ± 7.9

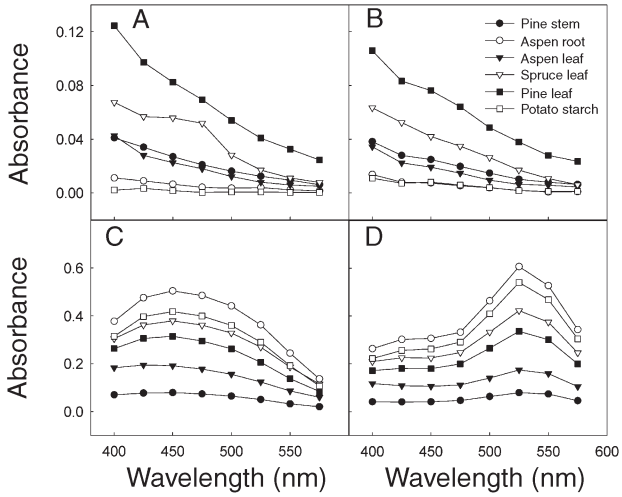

Figure 2. Absorption curves of sample solutions after digestion with α-amylase and amyloglucosidase. Tissue materials were extracted with hot ethanol before digestion. Data are means of 5 replicates. (A) Analyzed with dH_2O. (B) Analyzed with $dH_2O + H_2SO_4$. (C) Analyzed with peroxidase–glucose oxidase/*o*-dianisidine reagent (PGO-color solution). (D) Analyzed with PGO-color solution + H_2SO_4.

the PGO-color solution from those with the PGO-color solution, there was no detectable difference in starch content when estimated at 450 or 525 nm ($P = 0.267$).

The color developed with PGO-color solution darkens over time and absorbance readings increased by about 3% within the first hour. However, when sulfuric acid was added, the enzymatic action of the PGO-color solution was stopped, and the color of the solution turned from orange to pink and darkened only by about 0.1% per hour. Therefore, we believe that the addition of sulfuric acid allows for the more accurate estimation of glucose hydrolysate concentrations.

Effects of residual ethanol on sugar and starch analyses

Increasing ethanol content in plant extracts and GFG standard solution samples resulted in decreased absorbance readings and consequently underestimation of sugar concentrations. At ethanol concentrations of 20 to 80%, absorbance readings decreased from 3 to 13%. There was no difference in the effect of ethanol on absorbance readings between the GFG solution and the plant extract ($P = 0.466$).

Generally, sample extracts are diluted with water (1:10) before the sugar assay and, as a result, only 8% or less of ethanol will be present in the sample solution. By interpolating our results, this concentration will result in a 1% reduction in absorbance. However, if the sugar content of the tissue sample is expected to be less than 1% of dry mass (no dilution), residual ethanol concentrations up to 80% are possible. In that case, the expected reduction in absorbance associated with the presence of alcohol will be 13% (e.g., a tissue sugar content of 1% will measure as 0.87%).

Ethanol that remained in the residues after ethanol extraction had no significant effect on the subsequent measurement of starch content. No significant difference was found in the starch contents of oven-dried samples and undried samples containing residual ethanol ($P = 0.619$).

Reproducibility and application

Combining the above findings and amendments of existing procedures provides a method that yielded consistent results. Throughout this study, we used a benchmark tissue sample of aspen root to assess the precision and reproducibility of the method. Sugar and starch estimations of the benchmark sample remained consistent throughout the 61 tests over a period of 10 months during which different batches of chemicals were used. The sugar and starch contents in the benchmark sample were 15.09 ± 0.57% (SD) and 3.38 ± 0.17% (SD), respectively.

Overall, our method requires small samples (50 mg) and only common laboratory equipment. It is practical for all tissues of woody plants, including leaves, buds, stems, bark and roots, is sensitive enough to detect a sugar or starch concentration of 0.04%, and can handle samples with up to 40% sugar and 30% starch content. For samples with higher anticipated carbohydrate contents, sample size should be reduced to half. Starting with a ground tissue sample, both sugar and starch assays can be completed in 27 h, including incubation time. In routine measurements, one operator working with one set of equipment can finish a complete analysis of 60 ground samples in four working days, including preparation and clean-up work.

Conclusion

Based on the above studies, we have developed a rapid method for the measurement of total sugar and starch in woody plant tissues. Briefly, 50 mg of dried ground tissue sample is extracted with 80% hot ethanol. The ethanol extract is analyzed for sugar using phenol–sulfuric acid against a GFG standard, with 2% phenol and absorbance read at 490 nm. Interference is corrected by running a parallel sugar assay without phenol. The residue is analyzed for starch by enzymatic digestion with a mixture of 1000 U of α-amylase and 5 U of amyloglucosidase for 20 h, followed by the colorimetric measurement of glucose hydrolyzate with a peroxidase–glucose oxidase/*o*-dianisidine reagent. Absorbance is read at 525 nm after the addition of H_2SO_4.

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