Standard Protocols (Revised April 26, 2021)

Standardized protocols and procedures can precisely and accurately quantify non-structural carbohydrates

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Tree Physiology **38**: 1764-1778 (https://doi.org/10.1093/treephys/tpy118).

Note: *Since a certified plant based standard is currently not available for NSC analysis, we strongly suggest using lab internal standards (one synthetic (constructed, see Table 1 next page)) and one plant sample) that needs to be analyzed with each batch to confirm stability of measurements and quality assurance.*

Protocols:

Table 1: Components, their type, and suppliers to assemble two synthetic samples (s1 and s2) that can be used to test protocols. The last column indicates which of the three quantification methods used in our study can detect these components. See reference for more details.

Component	Type	Supplier and catalogue no.	Sample S1 (g)	Sample S2 (g)	Method used to detect component
Cellulose (washed)*	Cellulose	Sigma S3504	1.0215	1.0217	-
Lignin (washed)*	Hemicellulose	Sigma 370959	0.5411	0.1785	-
Pectin, from apple	Hemicellulose	Sigma 76282	0.0902	0.095	-
Xylan, from beechwood	Hemicellulose	Sigma X4252	0.4494	0.8068	-
Gum, Arabic	Polysaccharide	Acros Organics 258852500	0.0302	0.065	-
Myo-inositol	sugar alcohol (cyclitol)	PL Biochemicals 3204	0.0605	0.0597	-
Sorbitol	sugar alcohol (alditol)	Sigma S7547	0.0601	0.0638	-
Chlorophyllin sodium copper salt	Pigment	Sigma C6003	0.0601	0.0794	-
Chlorogenic acid	Phenolic	Sigma C3878	0.0318	0.0077	-
Glucose	Monosaccharide	Sigma G7528	0.0638	0.0696	**I**on **C**hromatography, **E**nzyme, **A**cid
Fructose	Monosaccharide	Sigma F0127	0.0812	0.1156	IC, E, A
Galactose	Monosaccharide	Sigma G0750	0.0162	0	IC, A
Sucrose	Disaccharide	Sigma S0389	0.1565	0.0972	IC, E, A
Maltose (monohydrate)	Disaccharide	Sigma M5885	0.0625	0	A
Melibiose	Disaccharide	Sigma M5500	0.0454	0	A
Raffinose (pentahydrate)	Oligosaccharide	Sigma R0250	0.0609	0	A
Corn starch (washed)*	Starch	Sigma S5296	0.127	0.3026	IC, Hexokinase, PGO

* Component was washed with deionized water at 4°C to remove any free glucose.

Protocol 1: Sugar Extraction

General

This method uses hot ethanol to extract sugars and other soluble compounds from plant samples. The extract is used for sugar quantification (Protocols 3 – 5), while the residue (pellet) is used for starch determination (Protocol 2).

Equipment and supplies

1. Drying oven at 60 °C
2. Desiccator with indicating desiccant
3. Analytical balance, readability 0.1 mg or lower
4. Device for heating 2 mL micro-centrifuge tubes up to 90 °C, e.g. a thermomixer or a hot water bath
5. Vortex mixer
6. Centrifuge for 2 mL micro-centrifuge tubes, with speeds up to 13,000 g
7. (Optional) A centrifugal evaporator, e.g. Speedvac
8. Pipette, 100-1000 µL
9. 2 mL plastic screw-cap micro-centrifuge tubes, with rubber O-ring cap seal (e.g. Fisher Scientific 02-682-558). Micro-centrifuge tubes with snap-caps are not suitable for this procedure, because they may pop open during heating.

Chemicals and solutions

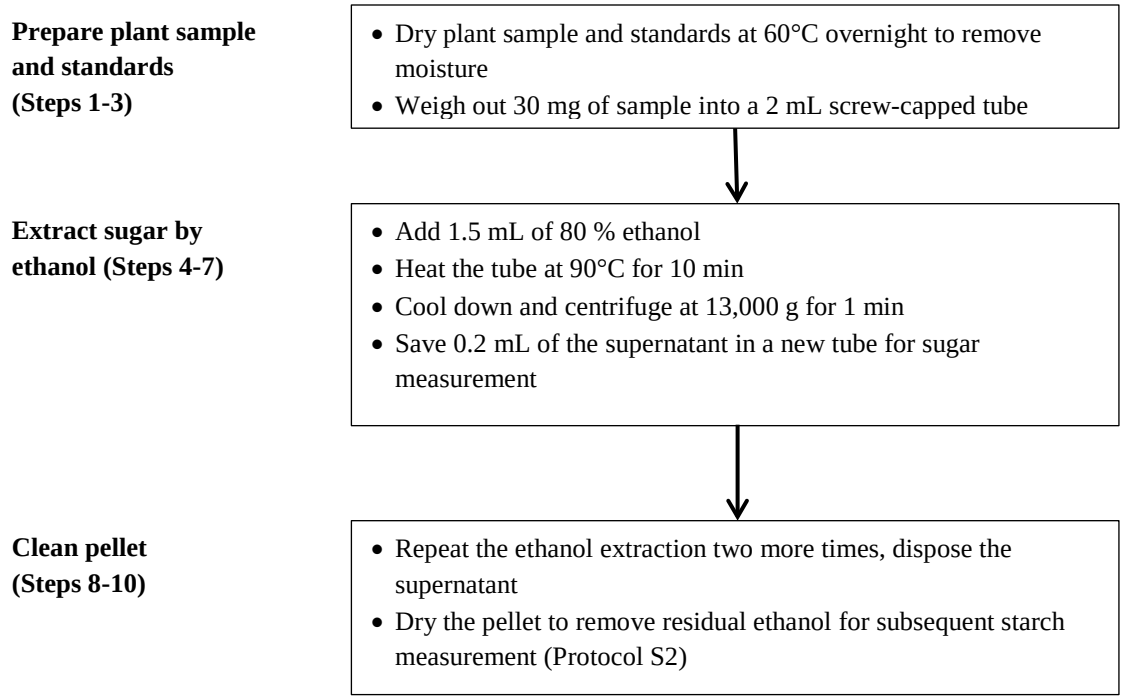
Please refer to Material Safety Data Sheet for all chemicals. All reagents should be of analytical grade.

1. Deionized water (dH_2O): Grade Type 2 or higher (please note that a higher grade is needed in IC measurements), resistivity at 25 °C > 1 MΩ-cm, filtered through 0.2 µm.
2. 80% (v/v) ethanol solution: For 1 L, mix 843 mL of 95% ethanol (absolute or 5% methylated) with 157 mL of dH_2O.
3. Two NSC control standards, one synthetic (constructed) with known sugar and starch concentrations (for examples see Table 1) and one plant sample (see note below and also main body of paper).

Note: *Since a certified plant based standard is currently not available for NSC analysis, we strongly suggest using lab internal standards (one synthetic (constructed, see Table 1 above)) and one plant sample) that needs to be analyzed with each batch to confirm stability of measurements and quality assurance.*

Procedure

Summary: Step numbers refer to those described in the Details table below



Details:

Step	Procedure	Notes
1	Dry ground plant sample and the two NSC control standards in a 60 °C drying oven overnight and keep them in a desiccator.	
2	Weight out about 30 mg of sample into a 2 mL screw-cap micro-centrifuge tube and record the actual weight (**W_{sample}**) in mg.	
3	For every batch of sample tubes, include three more tubes for the three controls. The first two contain about 30 mg of the two NSC control standards, record the actual weight (**$W_{control}$**) in mg. They serve as the quality control of the whole procedure. The third tube is an empty tube. It is an assay blank that serves both as the sugar blank in the sugar assay and the enzyme blank for the starch assay.	
4	Add 1.5 mL (**V1**) of 80 % ethanol (note (a)) and firmly cap each tube, including the assay blank. Shake the tubes thoroughly to suspend the solids.	(a) Sample intake : solvent ratio = 1 g : 50 mL.
5	Heat the tubes at 90 °C for 10 min.(note (b))	(b) In a thermomixer or hot water bath

6	After boiling, let sample cool to room temperature (note (c)). Shake to mix the content inside the tube, then centrifuge at 13,000 g for 1 min.	(c) This is necessary to ensure correct volume of the sugar extract.
7	Transfer 0.2 mL or 1.0 mL (note (d)) of the supernatant by aspiration using a pipet into a new 2 mL screw-cap micro-centrifuge tube. This is the sugar extract from which the sugar concentration will be determined (see Protocols 3 to 5). Cap the tube firmly right away to avoid evaporation which otherwise will increase the sugar concentration of the extract. (note (e))	(d) For sugar measurement using the enzyme (Protocol 4) or acid (Protocol 5) methods, transfer 0.2 mL. For sugar measurement using the IC (Protocol 3) method, transfer 1.0 mL. (e) Sugar measurement can be done later by saving the extract at 2-8 °C for up to a week, or in a -20 °C freezer for longer storage.
8	Aspirate and dispose the rest of the supernatant, make sure that all solids of the pellet remain inside the tube from which starch concentration will be determined later.	
9	Residual sugar extract trapped within the pellet is removed by repeating the extraction (Steps 4 to 8, skip Step 7) 2 more times. Dispose all supernatant resulted from these two extractions.	
10	To prepare the pellet for starch determination, leave the tube with the pellet uncovered overnight under the fume hood to evaporate the residual ethanol. Alternatively, the pellet can be dried in a centrifugal evaporator or in a drying oven at 50 °C. Cap the tube and store in a -20 °C freezer for starch analysis later (Protocol 2); also include the assay blank tube.	
11	**Values obtained:** (note (f)) Unknown sample input weight, in mg = $\mathbf{W_{sample}}$ Control standard input weight, in mg = $\mathbf{W_{control}}$ Extract volume, in mL = $V_{extract}$ = **V1** = 1.5 mL	(f) For final calculations see Protocol 6.

Protocol 2: Starch Digestion

General

This method uses α-amylase and amyloglucosidase to convert starch to glucose. The two enzymes are applied in separate steps to avoid non-specific digestion of other non-starch carbohydrates by the enzymes. After digestion of the pellet, the glucose hydrolysate produced can be measured by one of the three quantification methods described in Protocols 3, 4 and 5.

Equipment and supplies

1. Device for heating 2 mL micro-centrifuge tubes up to 85 °C, e.g. a thermomixer or a hot water bath
2. Centrifuge for 2 mL micro-centrifuge tubes, with speeds up to 13,000 g
3. 2 mL plastic screw-cap micro-centrifuge tubes, with rubber O-ring cap seal (e.g. Fisher Scientific 02-682-558). Micro-centrifuge tubes with snap-caps are not suitable for this procedure, because they may pop open during heating.
4. Vortex mixer
5. pH meter
6. Analytical balance, readability 0.1 mg or lower
7. Pipettes, 10-100 µL and 100-1000 µL

Chemicals and Solutions

Please refer to Material Safety Data Sheet for all chemicals. All reagents should be of analytical grade.

1. Pellet from the sugar extraction (including those from the standards)
2. Deionized water (dH_2O): Grade Type 2 or higher (please note that a higher grade is needed in HPAE-PAD measurements), resistivity at 25 °C > 1 MΩ-cm, filtered through 0.2 µm.
3. Sodium acetate (NaOAc) buffer solution (25 mM, pH 4.6): Dissolve 1.025 g of sodium acetate in 450 mL of deionized water. Adjust to pH 4.6 with acetic acid. Bring to a total volume of 500 mL with dH_2O. Storage: At 2-8 °C up to 6 months.
4. α-amylase solution (600 units/mL): Calculate the amount of α-amylase powder (from *Bacillus licheniformis*, Sigma A4551) required based on the tested activity values stated in the Certificate of Analysis of each lot, which can be pulled out online from Sigma webpage by entering the catalogue no. and lot no. (**CAUTION! Do not use the information on the bottle label**). Use the Certificate, it gives the tested values of "% Protein", "units/mg Protein" and "% Purity". Thus,

   Enzyme activity units/mg powder = units/mg Protein × (% Protein / 100) × (% Purity / 100)

   ***Note**: One enzyme activity unit of α-amylase used in this protocol is defined as the amount of enzyme that liberates 1 mg of maltose from starch in 3 min at pH 6.9 at 20°C.*

   Dissolve the required amount of enzyme powder in dH_2O, 1 mL per sample. Mix with a stir bar.
   ***Note:** The powder will not completely dissolve. Prepare fresh solution for same-day-use only.*

5. Amyloglucosidase solution (12 units/mL): Calculate the amount of amyloglucosidase powder (from *Aspergillus niger*, Roche #11202367001, available from Sigma under cat. no. ROAMYGLL) required based on the tested values stated in the Certificate of Analysis of each lot, which can be pulled out online from Sigma webpage by entering the catalogue no. and lot no. **(CAUTION! Do not use the information on the bottle label)**. Use the Certificate, it gives the tested values of enzyme activity in u/mgL (i.e. units/mg lyophilizate). Thus,

   Enzyme activity units/mg powder = u/mgL

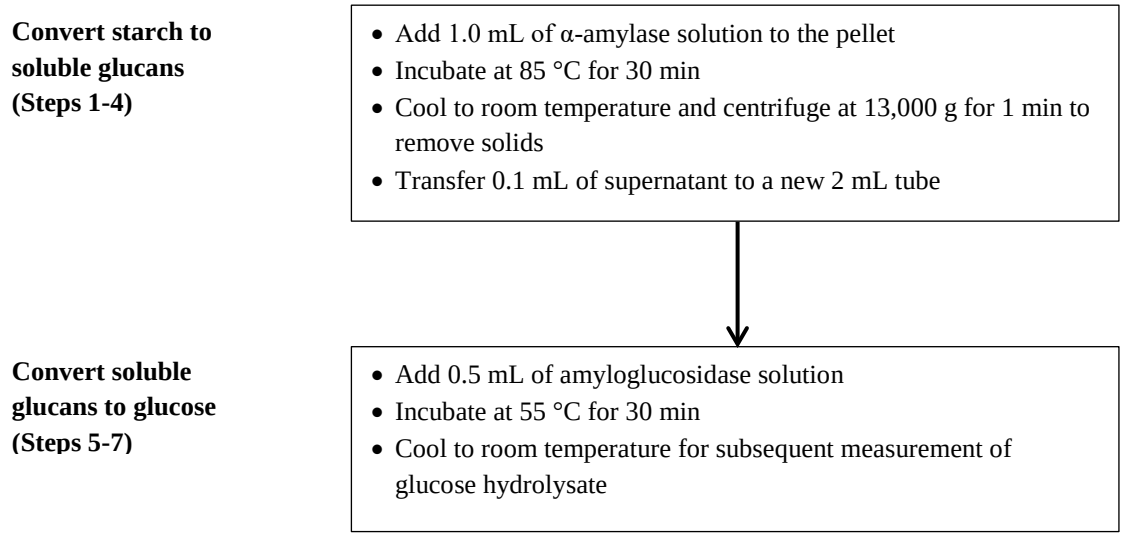
   This enzyme product contains some glucose, which is accounted for by running the assay blank in this procedure.

   ***Note**: One enzyme activity unit of amyloglucosidase used in this protocol is defined as the amount of enzyme that liberates 1 µmole of glucose from starch per minute at pH 4.8 and 60°C.*

   Dissolve the required amount of enzyme powder in 25 mM NaOAc buffer, 0.5 mL per sample. Mix with a stir bar. Prepare fresh solution for same-day-use.

Procedure

Summary: Step numbers refer to those described in the Details table below



Details:

Step	Procedure	Notes
1	Obtain the screw-cap micro-centrifuge tube containing the whole pellet from the sugar extraction (from Step 10 of Protocol 1, including the assay blank), warm up to room temperature. Add 1.0 mL (**V2**) of the α-amylase solution to the tube, including the assay blank. Firmly put on the screw cap and mix the content by vortex gently to suspend the solids. (notes (a), (b))	(a) The dried pellet is very hydrophobic, and it may not initially mix well with the α-amylase solution. (b) (Optional) Add glass beads to each sample tube, including the assay blank. This may ease the process in Step 2 below. Use equal amount of glass beads in each tube, otherwise it will upset the centrifuge in Step 4.
2	Heat the tube at 85 °C for 30 min. Shake to suspend the solids by vortex gently after the first 10 min of incubation. Make sure the pellet has completely broken up and all solids are suspended.	
3	Let sample cool to room temperature and mix the solution by vortex.	
4	Centrifuge at 13,000 g for 1 min. Transfer 0.1 mL (**V3**) of the supernatant free of any solids to a new 2 mL screw-cap micro-centrifuge tube.	
5	Add 0.5 mL (**V4**) of the amyloglucosidase solution, put on the screw cap firmly and mix by vortex.	
6	Heat the tube at 55 °C for 30 min.	
7	Let sample cool to room temperature and mix the solution by vortex. This is the digested sample. Follow the required steps (Protocols 3, 4 or 5) for measurement of the glucose hydrolysate. (note (c))	(c) The measurement of glucose hydrolysate can be done later by saving the digested sample at 2-8 °C up to 2 days.
8	**Values obtained:** (note (d)) Equivalent volume of digested sample, in mL V_{starch} = (**V3** + **V4**) / **V3** × **V2** = 6 mL Where: **V2** = volume of α-amylase solution = 1.0 mL **V3** = volume of an aliquot after α-amylase digestion put to the subsequent amyloglucosidase digestion = 0.1 mL **V4** = volume of amyloglucosidase solution = 0.5 mL	(d) For final calculations see Protocol 6.

Protocol 3: Quantification of NSCs by Ion Chromatography (IC) (HPAE-PAD)

General

This IC method employs the High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD) to quantify the concentration of glucose, fructose, sucrose and galactose in the ethanol extracted solutions and the glucose hydrolysate in the starch digested solutions. Additional sugars and carbohydrates can be determined with appropriate standards and equipment.

Equipment and supplies

1. Ion Chromatography (IC) system equipped with a gradient pump, a column oven and an electrochemical detector and autosampler (e.g. DIONEX ICS-3000). It is possible to run this system with an automated eluent generator and a self-regenerating suppression or with an eluent organizer.
2. Analytical balance, readability 0.1 mg or lower
3. Centrifuge for 2 mL micro-centrifuge tubes, with speeds up to 13,000 g
4. Vortex mixer
5. Class A volumetric flasks, of nominal capacities of 5, 10 and 100 mL
6. 2 mL micro-centrifuge tube, snap-capped
7. HPLC vials, 1.5 mL (silanized optional)
8. Lid for HPLC glass, 9 mm ultrabond, PTFE)
9. Disposable membrane filter, pore size 0.2 µm, suitable for hydrophilic liquids (e.g. Multoclear-13, PVDF)
10. Disposable 2 mL syringes with Luer-Lock tips, sterile
11. Pipette, 100-1000 µL, and filtered tips suitable for chloroform

Chemicals and Solutions

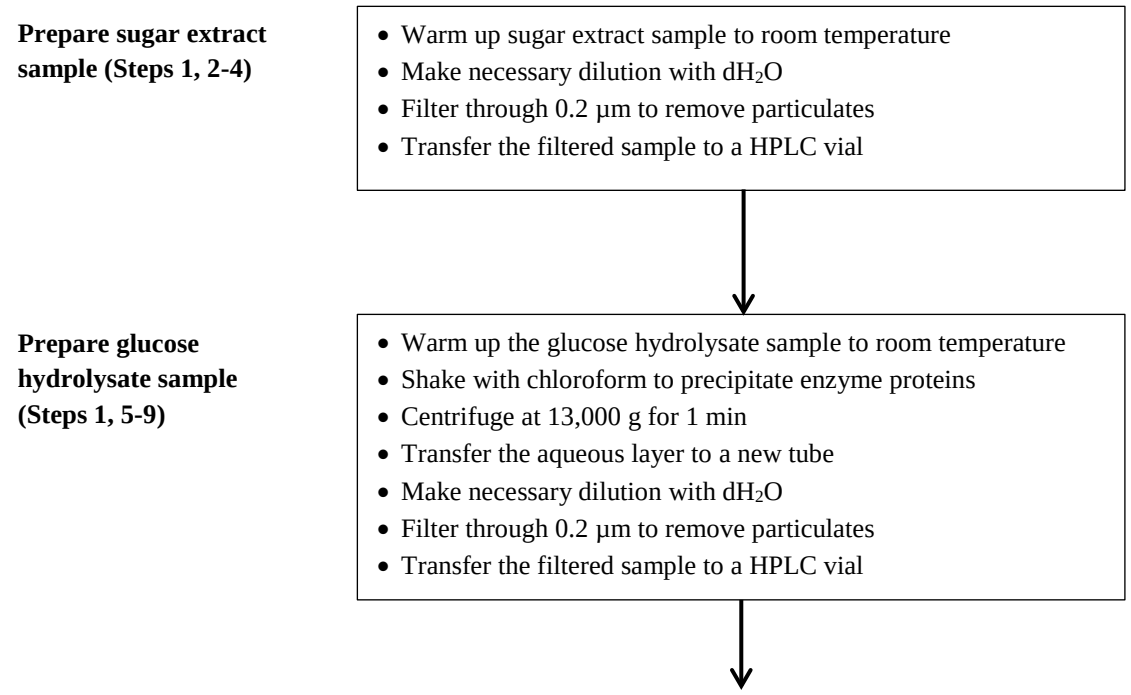
Please refer to Material Safety Data Sheet for all chemicals. All reagents should be of analytical grade. Only deionized water of Grade Type 1 should be used for this procedure.

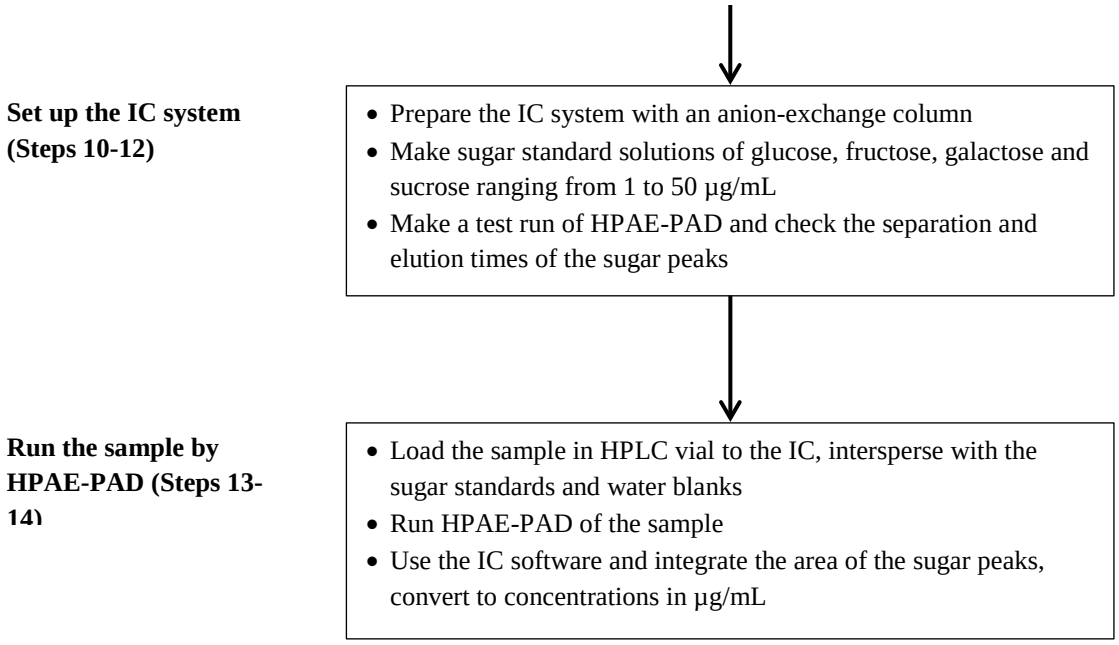
1. Deionized water (dH_2O): Grade Type 1, resistivity at 25°C > 18 MΩ-cm.
2. Chloroform: HPLC grade, stabilized with ethanol.
3. Sugar standards: Use chemicals of highest purity for preparing standards; D-glucose (≥99.5%, e.g. Sigma G7528), D-fructose (≥99%, e.g. Sigma F0127), D-galactose (≥99%, e.g. Sigma G0750) and sucrose (≥99.5%, e.g. Sigma S0389).
   CAUTION! Sugar standards have to be oven-dried at 60 °C for 2 h and cooled to room temperature in a desiccator before preparing standard solutions.
4. Sugar stock standard solutions (1000 mg/L): Separately dissolve 100 mg of each of the standard sugars (glucose, fructose, galactose and sucrose) in 100 mL of dH_2O using volumetric flasks. Mix well.
   Storage: Split the stock solutions in aliquots in separate bottles. Save them in a -20 °C freezer up to one year.

5. Working sugar standard solutions (1, 5, 10, 20, 30, 40 and 50 µg/mL): Into a series of seven 100 mL volumetric flasks, add 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mL of the sugar stock standard solution with a calibrated pipette to each flask. Fill each flask to 100 mL with dH_2O. Storage: At 2-8 °C for up to one week.
6. Sodium hydroxide (NaOH) eluent solution:
   Work from a concentrated stock solution of high quality (e.g. Sodium hydroxide solution 30%, Suprapur by EMD Millipore, #105589). The stock solution should not be older than 2 years. After opening the bottle the solution could be stored up to 6 months under dark and cool conditions. Dispose the remaining ¼ to ⅕ of your stock solution, especially if you notice a precipitation.
   Prepare the NaOH eluent solution in concentration according to the column manufacturer specification. The solution must be freshly prepared every two weeks. Use only deionized and degassed water for dilution. The prepared eluent solution has to be kept in a plastic eluent bottle under a helium atmosphere to avoid carbon dioxide contamination from the air. **CAUTION! DO NOT prepare NaOH eluent solution from pellets because they are coated with a layer of carbonate! Carbonate in the eluent can significantly reduce retention times for carbohydrates**.

Procedure

Summary: Step numbers refer to those described in the Details table below)





Details:

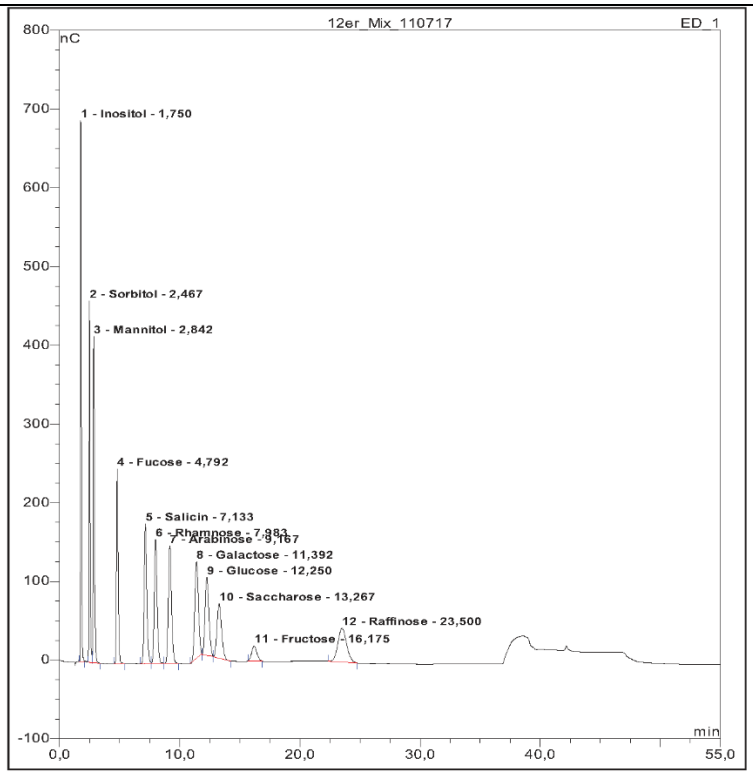
Step	Procedure	Notes
1	For ethanol extracts derived from Protocol 1, prepare samples according to Step 2 to 4 (note (a)). For enzyme-digested solutions derived from Protocol 2, prepare samples according to Step 5 to 9 (note (b)).	(a) Ethanol-extracted sample solutions have to be diluted and sterile-filtered before measured by IC. (b) Enzyme-digested sample solutions must be run through an additional cleaning step to remove the enzyme proteins before measured by IC.
2	Obtain the tube containing the 1 mL sample extract (from Step 7 of Protocol 1, including the assay blank), warm up to room temperature.	
3	If required sample should be diluted by transferring a defined volume into a volumetric flask and leveling to target volume with dH_2O (note (c)). Do not do dilution on the assay blank.	(c) The sample shall be diluted until the carbon concentration is in the range of the IC calibration.
4	Filter the diluted sample through a 0.2µm mesh filter using a syringe (note (d)), collect the filtrate in a 1.5 mL HPLC vial. Go to Step 10 for IC measurement.	(d) Pre-rinse the filter with 1 ml of the diluted sample.
5	Obtain the tube containing the 0.6 mL sample solution (from Step 7 of Protocol 2, including the assay blank), warm up to room temperature if required.	
6	Add 0.6 mL of chloroform and mix well by vortex and inversion. (note (e))	(e) **CAUTION!** Carefully read safety and operation instructions for chloroform. Work under fume hood, wear gloves and eye protection goggles.

<table>
<tr><td>7</td><td>Centrifuge at 13,000 g for 1 min. Transfer the aqueous phase (upper layer) into a 2 mL micro-centrifuge tube with a pipette. Avoid any transfer of the chloroform phase (lower layer) (note (f)), or any particles that may present at the interface between the two layers.</td><td>(f) Do not insert the pipette tip into the chloroform layer, because any chloroform residues may interfere with the IC measurements by overlaying with sugar peaks.</td></tr>
<tr><td>8</td><td>Samples can be analyzed as-is or, if required, diluted using volumetric flasks and leveling to target volume with dH_2O (note (g)). Do not do dilution on the assay blank.</td><td>(g) The sample shall be diluted until the carbon concentration is in the range of the IC calibration.</td></tr>
<tr><td>9</td><td>Filter the diluted sample solution through a 0.2µm mesh filter using a syringe (note (h)) and collect the filtrate in a 1.5 mL HPLC vial. Go to Step 10 for IC measurement.</td><td>(h) The filtration is not absolutely essential if the aqueous phase is clean and free from particles and if no dilution is required.</td></tr>
<tr><td>10</td><td>Set up the IC system (note (i)). Shown in the table below is an example using DIONEX ICS-3000 with CarboPac columns:
<table>
<tr><td>Column:</td><td>Dionex CarboPac PA10 (4 × 250mm)</td></tr>
<tr><td></td><td>Dionex CarboPac PA10 (4 × 250mm) guard column</td></tr>
<tr><td>Eluent:</td><td>A – 250 mM NaOH Flush</td></tr>
<tr><td></td><td>B – 18 mM NaOH mobile Phase</td></tr>
<tr><td>Temperature:</td><td>40 °C</td></tr>
<tr><td>Flow Rate: See chromatogram</td><td>1 mL/min</td></tr>
<tr><td>Inj. Volume:</td><td>25 µL</td></tr>
<tr><td>Detection:</td><td>Integrated Amperometry, quadruple pulse waveform (note (j))</td></tr>
<tr><td>Working Electrode:</td><td>PTFE Gold, disposable electrode</td></tr>
<tr><td>Reference Electrode:</td><td>Ag/AgCl</td></tr>
<tr><td>Standard:</td><td>Glucose, fructose, galactose and sucrose separately, 1-50 µg/mL. (note (k))</td></tr>
<tr><td>Pressure:</td><td>1800-2000 psi</td></tr>
</table>
References:

* Weiss, J., 2001, Ionenchromatographie, 3rd. Edition XII, 940 p. ISBN: 978-3-527-28702-4 ,Wiley-VCH, Weinheim Quality
* DIN 10780. Determination of free and total carbohydrates in coffee extract by high performance anion-exchange chromatography
* Assurance Report given by CarboPac manufacturer or Dionex
</td><td>(i) Anion-Exchange Chromatography provides the possibility to change the elution times by changing the molarity of the eluent, the temperature, pressure or the flow rate. For example DIONEX provides four different columns suitable to analyze carbohydrates.

(j) Use the conditions specified as optimum by the manufacturer.

(k) The limit of quantification (LOQ) or acceptance criteria has to be fulfilled for each analyte. The LOQ is true if the signal to noise ratio is at least 9:1.</td></tr>
</table>

11	Put the chromatograph into operation. Wait for the chromatograph to reach steady-state condition. Run HPAE-PAD according to the protocol detailed in Step 10 above with single sugar standards (1, 5, 10, 20, 30, 40 and 50 µg/mL) and sugar mixtures (e.g. mixture of 5 different sugars, each at 10 µg/mL) and water blanks (note (l)). The analytical run time with eluent B (including injection/loading, cleaning and equilibration) is about 27 min for each sample, depending on the sugars to be determined (note (m)). Start the clean-up only when the last monosaccharide has been eluted. Flush with eluent A for 10 min to prevent adsorption of carbonate and to remove remaining matrix.	(l) Make sure that the standard calibration curves are of good fit. In order to detect changes in retention time the DIN 10780 suggests injecting the standard solution after every fourth injection. (m) Retention times may vary from column to column.
12	 Typical HPAE-PAD chromatogram of a mixture of sugars	
13	Set up the sample vials (from Step 4 or Step 9 above) at the IC and analyze by HPAE-PAD. Measure the unknown samples and the assay blank interspersed with the standards and water blanks. Always inject the same volume as used for the calibration curve. (note (n))	(n) Make sure that samples fall within the calibration range. If samples are low in concentration, specifically verify a good fit of the calibration curve at its lower end or set up a second method with increased sample injection volume.

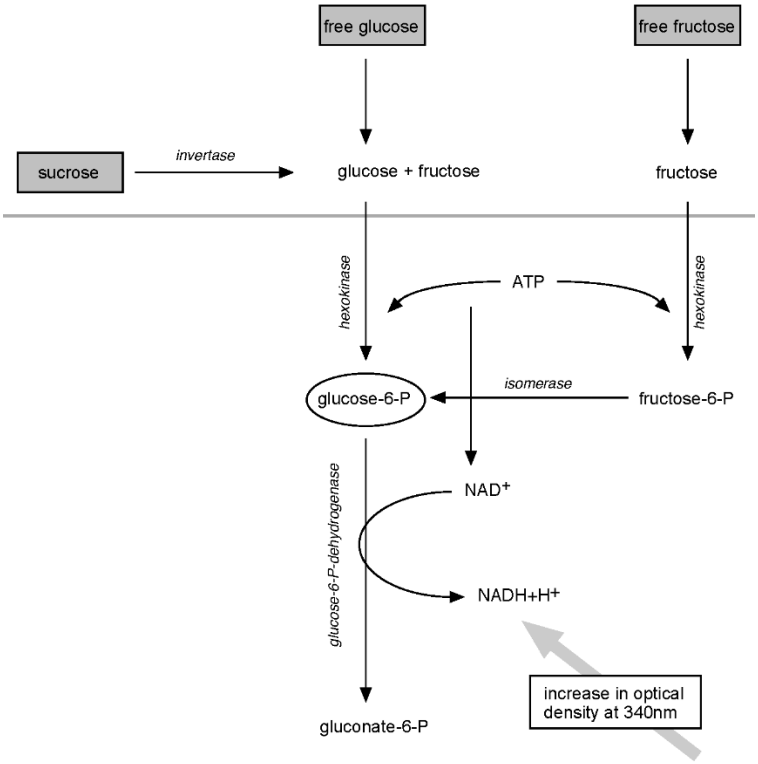
14	Use the IC software (e.g. Thermofisher scientific/DIONEX C*hromeleon)* software to integrate and calculate the concentrations of the individual sugars in mg/L (=µg/mL). **Values obtained**: (note(o)) For ethanol extracts derived from Protocol 1, multiple the sugar concentrations obtained from the software with the sample dilution factor from Step 3 above. Then subtract the corresponding sugar concentrations of the assay blank from those of the unknown sample. It gives the concentrations of the individual sugars in the sample extract, [glucose], [fructose], [galactose] and [sucrose] for glucose, fructose, galactose and sucrose, respectively in µg/mL. For enzyme-digested sample solutions from Protocol 2, multiple the glucose concentration obtained from the software with the sample dilution factor from Step 8 above. Then subtract the glucose concentration of the assay blank from that of the unknown sample. It gives the concentration of the glucose hydrolysate in the solution after starch digestion, [glucose hydrolysate], in µg/mL.	(o) For final calculations see Protocol 6.

Protocol 4: Quantification of glucose, fructose and sucrose by enzyme

General

This method measures free glucose, fructose and sucrose in an aqueous solution through NAD-linked enzymatic assays. Invertase is used to break down sucrose to glucose and fructose. Free glucose and free fructose are phosphorylated to glucose-6-P and fructose-6-P by hexokinase. After conversion of fructose-6-P to glucose-6-P by isomerase, the total amount of glucose-6-P is oxidized to gluconate-6-P in the presence of NAD+ as catalyzed by dehydrogenase, which is quantified by the reduction of NAD+ to NADH which causes an increase in absorbance at 340 nm that is directly proportional to the glucose-6-P concentration. This method is adapted for the use of 96-well microplates.

***Note**: Researchers can choose to analyze all sugars individually by sequentially following each part below, or only the specific sugar(s) of interest. If sucrose (part C) is of interest, free glucose (part A) needs to be analyzed as well.*



Equipment and supplies

1. Device for heating 2 mL micro-centrifuge tubes up to 90 °C, e.g. a thermomixer or a hot water bath
2. Drying oven at 60 °C
3. (Optional) A centrifugal evaporator, e.g. Speedvac
4. Centrifuge for 2 mL micro-centrifuge tubes, with speeds up to 13,000 g

5. Microplate shaker capable of holding four microplates (e.g. Talboys Advanced 1000MP Microplate Shaker)
6. Microplate reader with optical performance at 340 nm wavelength (e.g. Thermo Fisher Multiskan EX, or BioTek ELX800UV)
7. 96-well microplates, flat-bottom clear polystyrene, for optical absorbance measurement, >250 uL capacity (e.g. Thermo Scientific Nunc MicroWell 96-Well Microplates, product no. 260895)

Chemicals and solutions

Please refer to Material Safety Data Sheet for all chemicals. All reagents should be of analytical grade.

***Note**: The volumes shown below allow for the analysis of 96 samples, including the 3 extraction controls (certified/internal standards: a lab plant material control sample, synthetic control sample, assay blank), 6 serial dilutions of glucose, and up to 2 sugar standards (fructose, sucrose), leaving room for 85 unknown samples.*

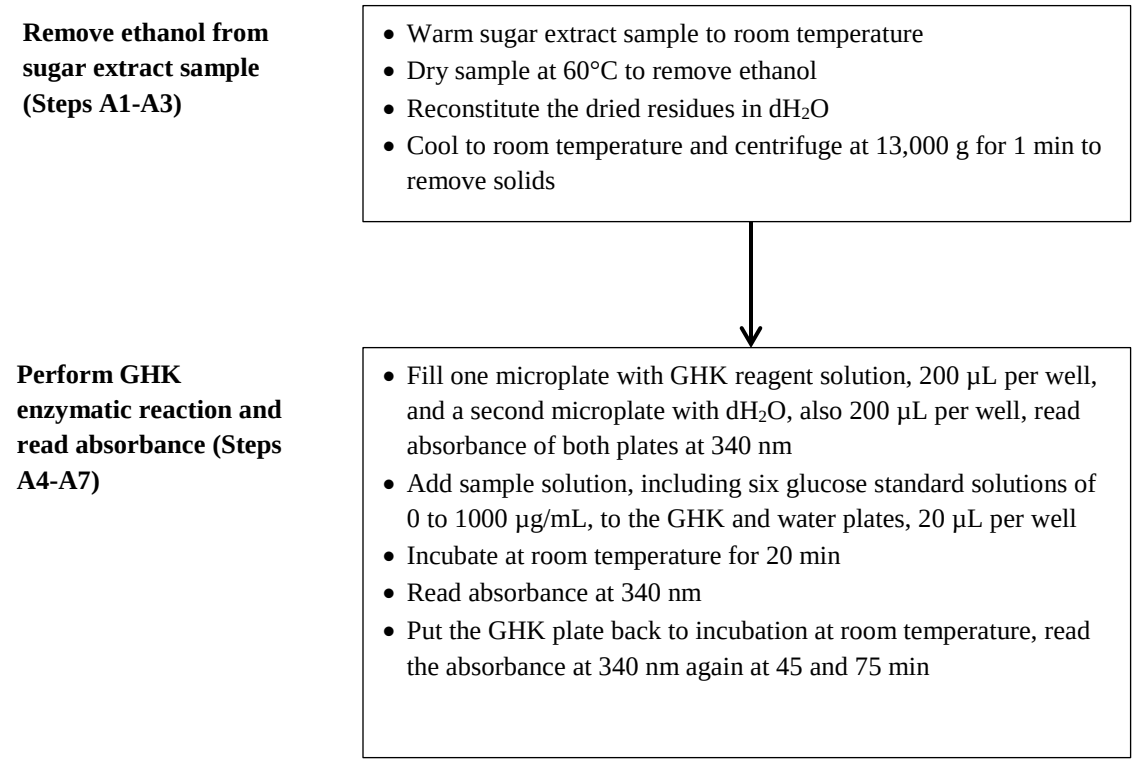
1. Deionized water (dH_2O): Grade Type 2 or higher, resistivity at 25 °C > 1 MΩ-cm, filtered through 0.2 µm.
2. Sodium acetate (NaOAc) buffer solution (25 mM, pH 4.6): Dissolve 1.025 g of sodium acetate in 450 mL of deionized water. Adjust to pH 4.6 with acetic acid. Bring to a total volume of 500 mL with dH_2O. Storage: At 2-8 °C up to 6 months.
3. Invertase solution (60 U/mL): Dissolve 600 U of invertase (Sigma I9274, from baker's yeast (*S. cerevisiae*)) in 10 mL of 25 mM NaOAc buffer. Calculate the amount of invertase powder required based on the tested values stated in the Certificate of Analysis of each lot, which can be pulled out online from Sigma webpage by entering the catalogue no. and lot no. (**CAUTION! Do not use the information on the bottle label**). Use the Certificate, it gives the tested values of enzyme activity in units/mg solid. It is important that this invertase product contains minimal amount (≤ 0.01%) of isomerase.
   Note: One enzyme activity unit (U) of invertase used in this protocol is defined as the amount of enzyme that will hydrolyze 1.0 µmole of sucrose to glucose and fructose per min at pH 4.6 at 25°C.
   Solution is good at least for four weeks at 4°C.
4. Hexokinase-glucose 6-phosphate dehydrogenase solution (GHK):
   a. <u>For use in Procedure Part A, C and D only</u>:
      Add 50 mL of dH_2O to the bottle of Glucose Assay Reagent (Sigma G3293-50ML), invert gently to dissolve. Solution is good at least for four weeks at 4°C.
   b. <u>For use in Procedure Part B only</u>:
      Prepare the GHK solution as in #4a above. Add 250 units of phosphoglucose isomerase (Sigma P5381-5KU, from baker's yeast (*S. cerevisiae*), Type III, ammonium sulfate suspension, ≥400 units/mg protein) to the GHK bottle, calculate the amount of enzyme required based on the tested values stated in the Certificate of Analysis of each lot. (**CAUTION! Do not use the information on the bottle label**). Use the Certificate, it gives the tested values of "mg protein/mL" and "units/mg Protein". Thus,
      Enzyme activity units/mL = units/mg Protein × mg protein/mL
      Note: One enzyme activity unit of phosphoglucose isomerase used in this protocol is defined as the amount of enzyme that will convert 1.0 µmole of D-fructose 6-phosphate to D-glucose 6-phosphate per min at pH 7.4 at 25 °C.
      Invert gently to mix. Solution is good at least for four weeks at 4°C.

5. Glucose standard stock solution (1 mg/mL): Sigma G6918
6. Glucose standard solutions: Make serial 1:2 dilutions of the 1 mg/mL stock solution with dH_2O to obtain glucose standard solutions of 1000 to 62.5 µg/mL. Prepare fresh solutions for same-day-use.
7. Fructose and sucrose standards (1000 µg/mL): Separately dissolve 100 mg of each sugar (D-fructose (≥99%, e.g. Sigma F0127), sucrose (≥99.5%, e.g. Sigma S0389)) in 100 mL of dH_2O, use volumetric flasks.
   CAUTION! Sugar standards have to be oven-dried at 60 °C for 2 h before preparing solutions.

Procedure

Part A - Free Glucose: Quantification of free glucose concentration in sample solutions (from Protocol 1)

Summary: Step numbers refer to those described in the Details table below



Details:

Step	Procedure	Notes
A1	Place the tube containing the 0.2 mL (**V5**) sample extract (from Step 7 of Protocol 1, including the assay blank) without cap in a 60 °C drying oven for 4 h. (note (a) and (b))	(a) Ethanol in the sample solution needs to be removed for this assay. (b) Alternatively, the sample solution can be dried in a centrifugal evaporator at 60 °C for at least 2 h.
A2	Reconstitute the dried residue in water by adding 1.0 mL (**V6**) (note (c)) of dH_2O to the tube, put on the screw cap, and shake by vortex for 5 s to mix. Heat the tube at 90 °C for 5 min, then shake again for 5 s to mix. Repeat the heating and shaking process one more time.	(c) This volume of reconstitution results in a 5× dilution of the original sample extract. It is suitable for the analysis of plant samples of free sugar concentrations from 0.5 to 25% dry weight.
A3	Let the tube cool down to room temperature. Centrifuge at 13,000 g for 1 min to remove any suspending solids. Sugar concentration is determined using only the supernatant. (note (d))	(d) Sugar measurement can be done later by saving the samples. Transfer the supernatant to a new 2 mL micro-centrifuge tube and store at 2-8 °C for up to one week or in a -20 °C freezer for longer storage. Warm up to room temperature and mix well before use.
A4	Get two new 96-well microplates, fill each well of the first plate with 200 µL of GHK and the second plate with 200 µL of dH_2O. Use the GHK solution prepared in Chemicals and Solutions #4a. Measure absorbance of both plates at 340 nm. These are the background values of the GHK absorption, Abs(GHK,self), and the water absorption, Abs(water,self).	
A5	Add 20 µL of sample solutions from Step A3 above to the GHK plate and the water plate, three wells (replicates) per sample per plate. Also include six standard glucose solutions of 0, 62.5, 125, 250, 500 and 1000 µg/mL, one well per concentration per plate (note (e)). Put on the cover lid and let them react on a plate shaker at 300 rpm for 20 min at room temperature.	(e) If running a sucrose assay, add one well with sucrose standard of 1000 µg/mL and one well with fructose standard of 1000 µg/mL. These standards serve as a quality assurance for the efficacy of the invertase enzyme used in the sucrose assay (part C)
A6	Read absorbance at 340 nm of the GHK plate, Abs(sample,GHK), and the water plate, Abs(sample,self), for the unknown samples and Abs(glucose, GHK) and Abs(glucose, self) for the glucose standard solutions.	
A7	Return the GHK plates to the shaker and re-measure absorbance after 45 and 75 min. For the calculation, only the maximum value of absorbance from the three time points (i.e. 20, 45 and 75 min) is used.	

A8	**Values obtained:** (note (f)) For glucose standard curve: [glucose] = m × Abs(glucose) + b Where: [glucose] = concentration of standard solutions, in µg/mL Abs(glucose) = (Abs(glucose,GHK) – Abs(GHK,self)) – (Abs(glucose,self) – Abs(water,self)) Absorbance of sample solution: Abs(sample) = (Abs(sample,GHK) – Abs(GHK,self)) – (Abs(sample,self) – Abs(water,self)) Absorbance of assay blank solution: Abs(blank) = (Abs(blank,GHK) – Abs(GHK,self)) – (Abs(blank,self) – Abs(water,self)) Concentration of free glucose in the sample extract, in µg/mL: (notes (g), (h)) [sugar, glu] = {(Abs(sample) - Abs(blank)) × m + b} × V6 / V5 <u>(Optional) (notes (e), (i))</u> Also calculate [sugar, glu] of the sucrose and fructose standard, $[\text{sugar, glu}]_{\text{sucrose std}}$ and $[\text{sugar, glu}]_{\text{fructose std}}$, respectively.	(f) Calculate the average value of [sugar, glu] from the three replicates of each sample in Step A5 and use the average in the final calculations in Protocol 6. (g) Use m and b from glucose standard curve (h) V5 = 0.2mL V6 = 1.0mL (i) The values of $[\text{sugar, glu}]_{\text{sucrose std}}$ and $[\text{sugar, glu}]_{\text{fructose std}}$ will be used in the calculations of Part C

Part B - Free Sugar (Glucose + Fructose): Quantification of free sugar (glucose + fructose) concentration in sample solutions (from Protocol 1)

Summary: Step numbers refer to those described in the Details table below

Remove ethanol from sugar extract sample (Step B1)

- Warm sugar extract sample to room temperature
- Dry sample at 60°C to remove ethanol
- Reconstitute the dried residues in dH_2O
- Cool down to room temperature and centrifuge at 13,000 g for 1 min to remove solids

Convert fructose to glucose, perform GHK enzymatic reaction and read absorbance (Step B2)

- Fill one microplate with GHK-isomerase reagent solution, 200 µL per well, and a second microplate with dH_2O, also 200 µL per well, read absorbance of both plates at 340 nm
- Add sample solution, including one fructose standard of 1000 µg/mL and six glucose standard solutions of 0 to 1000 µg/mL, to the GHK and water plates, 20 µL per well
- Incubate at room temperature for 20 min
- Read absorbance at 340 nm
- Put the GHK plate back to incubation at room temperature, read the absorbance at 340 nm again at 45 and 75 min

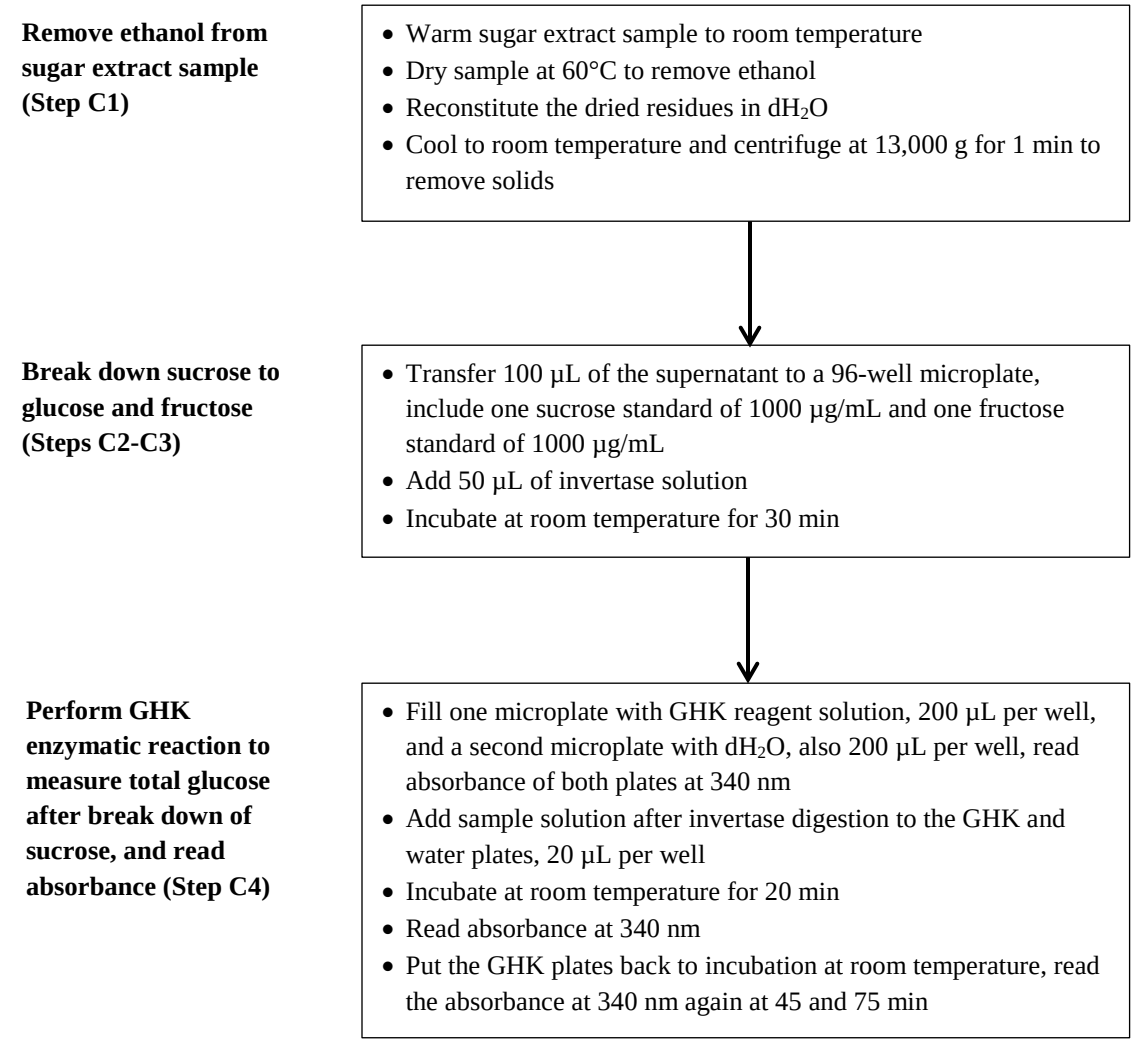
Details:

Step	Procedure	Notes
B1	Follow Step A1 to A3 of Part A above to remove ethanol from the sample solutions. (note(a))	(a) Ethanol in the sample solution needs to be removed for this assay.
B2	Run the assay as described in Part A, from Step A4 to A7. In Step A4, use the GHK solution prepared in Chemicals and Solutions #4b that contains isomerase. In Step A5, use the sample solutions from Step B1 above; also include one fructose standard of 1000 µg/mL. (note(b))	(b) The fructose standard serves as a quality assurance for the efficacy of the isomerase used. A 100% recovery of the standard is expected.
B3	**Values obtained:** (notes (c), (d), (e)) Concentration of free glucose and fructose combined in the sample extract, expressed as glucose-equivalent, in µg/mL: [sugar, glu+fru] = {(Abs(sample) – Abs(blank)) × m + b} × V6 / V5 Concentration of free fructose in the sample extract, expressed as glucose-equivalent, in µg/mL: (note (f)) [sugar, fru] = [sugar, glu+fru] - [sugar, glu] Percentage recovery of fructose standard: (note (g)) % recovery = {(Abs(sample) – Abs(blank)) × m + b} / 1000 × 100%	(c) Calculate the average value of [sugar, glu+fru] from the three replicates of each sample in Step B2 and use the average in the final calculations in Protocol 6. (d) Refer to Step A8 in Part A above for the calculation of the glucose standard curve and sample absorbance. (e) V5 = 0.2mL V6 = 1.0mL (f) Use the average value of [sugar, glu] from Part A Step A8. (g) If the % recovery of fructose standard is lower than 95%, replace the isomerase with fresh ones.

Part C - Sucrose: Quantification of sucrose concentration in sample solutions (from Protocol 1)

***Note**: In addition to cleaving fructose from sucrose, invertase cleaves fructose from raffinose family oligosaccharides. Therefore, in order to isolate sucrose, we measure sucrose as two times the concentration of glucose hydrolyzed from sucrose rather than as (fructose + glucose).*

Summary: Step numbers refer to those described in the Details table below



Details:

Step	Procedure	Notes
C1	Follow Step A1 to A3 of Part A above to remove ethanol from the sample solutions. (note(a))	(a) Ethanol in the sample solution needs to be removed for this assay.

C2	Transfer 100 µL (**V7**) of sample solution to a 96-well microplate, one well per sample. Include the sample solution from the assay blank. Also add one well with sucrose standard of 1000 µg/mL and one well with fructose standard of 1000 µg/mL. (note (b))	(b) The sucrose and fructose standards serve as a quality assurance for the efficacy of the invertase enzyme used in Step C3 below. A 100% recovery of the sucrose standard and a 0% of the fructose standard are expected.
C3	Add 50 µL (**V8**) of invertase solution to each well, put on the cover lid and let it react on a plate shaker at 300 rpm for 30 min at room temperature.	
C4	Run the assay as described in Part A, from Step A4 to A7. In Step A5, use the sample solutions from Step C3 above, and omit the six glucose standard solutions..	
C5	**Values obtained:** (note (e)) Concentration of total glucose in the sample solution after invertase digestion, in µg/mL: (notes (f), (g)) [sugar, total glucose] = {(Abs(sample) - Abs(blank)) × m + b} × V6 / V5 × (V7 + V8) / V7 Concentration of sucrose in the sample extract, expressed as glucose-equivalent, in µg/mL: (note (h)) [sugar, suc] = ([sugar, total glucose] - [sugar, glu]) × 2 Percentage recovery of fructose standard: (note (h), (i)) % recovery = ([sugar, total glucose]$_{\text{fructose std}}$ - [sugar, glu]$_{\text{fructose std}}$)/ 1000 × 100% Percentage recovery of sucrose standard: (note(h), (i), (j)) % recovery = ([sugar, total glucose]$_{\text{sucrose std}}$ - [sugar, glu]$_{\text{sucrose std}}$) × 2 × (342.3 / 360.3) / 1000 × 100%	(e) Calculate the average value of [sugar, suc] from the three replicates of each sample in Step C4 and use the average in the final calculations in Protocol 6. (f) Refer to Step A8 in Part A above for the glucose standard curve and the calculation of sample absorbance. (g) V5 = 0.2mL V6 = 1.0mL V7 = 0.1mL V8 = 0.05mL (h) Use the [sugar, glu], [sugar, glu]$_{\text{sucrose std}}$ and [sugar, glu]$_{\text{fructose std}}$ values from Part A, Step A8. (i) If the % recovery of the fructose standard is higher than 5%, or of the sucrose standard lower than 95%, replace the invertase with fresh ones. (j) Accounts for conversion of sucrose (molar mass 342.3) to glucose (molar mass 180.16) + fructose (molar mass 180.16)

Part D - Starch: Quantification of glucose hydrolysate concentration in sample solutions after starch digestion (from Protocol 2)

Summary: Step numbers refer to those described in the Details table below

Prepare glucose hydrolysate sample solution (Steps D1-D2)

- Warm the glucose hydrolysate sample to room temperature
- Make a 2× dilution with dH_2O

↓

Perform GHK enzymatic reaction and read absorbance (Step D3)

- Fill one microplate with GHK reagent solution, 200 µL per well, and a second microplate with dH_2O, also 200 µL per well, read absorbance of both plates at 340 nm
- Add sample solution, including six glucose standard solutions of 0 to 1000 µg/mL, to the GHK and water plates, 20 µL per well
- Incubate at room temperature for 20 min
- Read absorbance at 340 nm
- Put the GHK plate back to incubation at room temperature, read the absorbance at 340 nm again at 45 and 75 min

Details:

Step	Procedure	Notes
D1	Obtain the tube containing the 0.6 mL (**V9**) sample solution (from Step 7 of Protocol 2, including the assay blank), warm up to room temperature if required.	(a) Enzyme-digested sample solutions derived from Protocol 2 can be run through this assay without any treatment to remove residual enzymes.
D2	Add 0.6 mL (**V10**) of dH_2O to the tube, including the assay blank. Mix thoroughly by vortex. (note(b))	(b) This results in a 2× dilution of the original sample solution. It is suitable for the analysis of plant samples of starch concentrations from 0.5 to 35% dry weight.
D3	Run the assay as described in Part A, from Step A4 to A7 (note(c)). In Step A5, use the sample solutions from Step D2 above.	(c) Since only glucose should be present after starch hydrolysis, phosphoglucose isomerase is not needed.
D4	**Values obtained:** (notes (d), (e), (f)) Concentration of glucose hydrolysate in the solution after starch digestion, in µg/mL: [glucose hydrolysate] = {(Abs(sample) - Abs(blank)) × m + b} × (V9 + V10) / V9	(d) Calculate the average value of [glucose hydrolysate] from the three replicates of each sample in Step D3 and use the average in the final calculations in Protocol 6. (e) Refer to Step A8 in Part A above for the calculation of the glucose standard curve and sample absorbance. (f) V9 = 0.6mL V10 = 0.6mL

Protocol 5: Quantification of sugars by acid and starch by peroxidase-glucose oxidase

General

This method measures the total sugar concentration of plant extracts, in water or in ethanol, using phenol and concentrated sulfuric acid. All soluble sugars, including mono- and oligo-saccharides are hydrolyzed to the basic sugars (glucose, fructose and galactose) by the sulfuric acid during the process and are measured collectively as total sugar. Glucose hydrolysate resulting from starch digestion is measured using a peroxidase-glucose oxidase-o-dianisidine solution and a 75% sulfuric acid solution.

Equipment and supplies

1. Visible wavelength spectrophotometer (e.g. Genesys 10S UV-Vis Spectrophotometer, by Thermo Scientific, Madison, WI, USA)
2. Semi-micro disposable cuvettes, polystyrene, 1.5 mL volume (e.g. Fisherbrand GD14955127). Use only cuvettes made of polystyrene as the acid will affect reading in other types of plastic cuvettes.
3. Centrifuge for 2 mL micro-centrifuge tubes, with speeds up to 13,000 g
4. Pump dispenser for concentrated acids
5. Glass test tubes, 16 mm dia. × 100 mm long. Clean glassware is crucial to this assay. The sulfuric acid used in the assay will hydrolyze any foreign objects present in the tube and thus darken the solution, causing error in absorbance readings.

Chemicals and solutions

Please refer to Material Safety Data Sheet for all chemicals. All reagents should be of analytical grade.

1. Deionized water (dH_2O): Grade Type 2 or higher, resistivity at 25 °C > 1 MΩ-cm, filtered through 0.2 µm.
2. Glucose-fructose-galactose (GFG) stock solution (1 mg/mL):
   a. Prepare three solutions of 2 mg/mL of D-glucose (≥99.5%, e.g. Sigma G7528), D-fructose (≥99%, e.g. Sigma F0127) and D-galactose (≥99%, e.g. Sigma G0750) separately by dissolving 100 mg of each sugar in 50 mL of dH_2O, use volumetric flasks. Combining all three sugar solutions together gives 150 ml of a 2 mg/mL GFG solution. **CAUTION! Sugar standards have to be oven-dried at 60 °C for 2 h before preparing solutions.**
   b. Prepare 100 mL of 0.2 % (w/v) benzoic acid solution by dissolving 0.2 g of benzoic acid (e.g. Alfa Aesar Benzoic acid, 99%, A14062) in 100 mL of dH_2O. It takes about 3-4 h to dissolve at room temperature with a magnetic stir bar.
   c. Combine 100 mL of the 2 mg/mL GFG solution (step a) with 100 mL of the benzoic acid solution (step b) and mix well. The result is a 1 mg/mL GFG stock solution with 0.1% benzoic acid as preservative.
   Storage: In a brown bottle at 2-8 °C for up to two years.
3. Phenol solution (2 %): Dissolve 20.0 g of phenol crystals (≥ 99%, e.g. Fisher A92-500) in 1 L of dH_2O. Alternatively, dilute 22.5 mL of phenol liquid (≥ 89%, e.g. Fisher A931l-1) up to 1 L with dH_2O. Work under the fume hood.

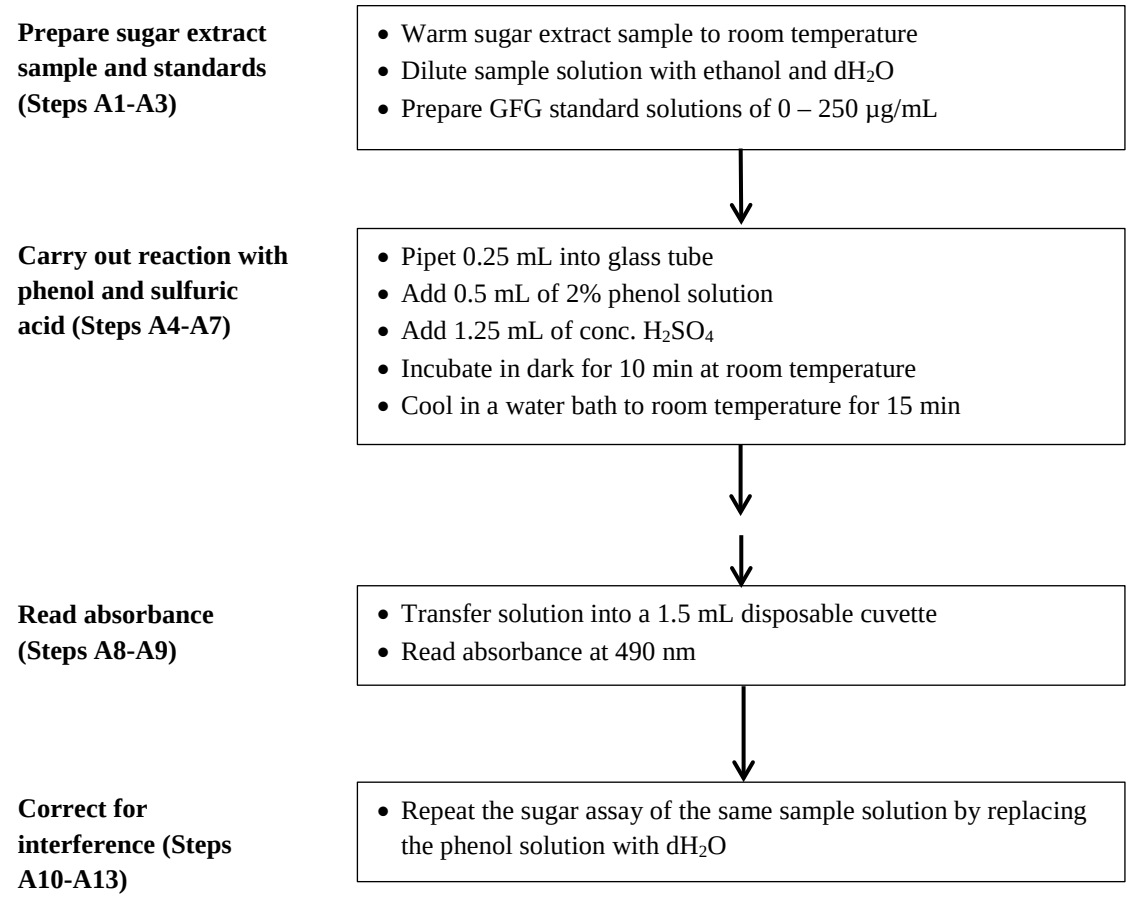
Storage: In glass bottle at room temperature under the fume hood for up to two years.

4. Concentrated sulfuric acid (H_2SO_4) **(**for **Part A)**: ≥ 95 % purity.
5. 75% sulfuric acid (H_2SO_4) solution **(**for **Part B)**: **Slowly** add 750 mL of concentrated H_2SO_4 to 250 mL of dH_2O in an 1 L Erlenmeyer flask placed in ice water under a fume hood. **CAUTION! Do not add the water to concentrated acid**.
   Stir with a magnetic stir bar to mix. To avoid overheating of the solution during mixing, spread the addition of acid over a longer time period, allowing cooling between acid additions.
6. Peroxidase-glucose oxidase (PGO)-color reagent solution: Dissolve one pill of PGO enzyme (Sigma P7119) per 100 mL of dH_2O. Also dissolve 100 mg of o-dianisidine dihydrochloride (Sigma D3252) in 40 mL of dH_2O under the fume hood.
   CAUTION! Since a solution of o-dianisidine dihydrochloride > 0.1 % is regarded a potential carcinogen, this stock solution of 0.25 % should be handled with caution.
   Add 1.6 mL of the o-dianisidine dihydrochloride solution for each 100 mL of PGO enzyme solution and gently mix. The combined reagent solution has a concentration of o-dianisidine dihydrochloride of 0.004 %. The reagent solution is light sensitive; hence minimize its exposure to light.
   Storage: Prepare the PGO-color reagent solution fresh for same-day-use; however, solution can also be stored at 2-8 °C for up to 7 days. The o-dianisidine dihydrochloride stock solution can be stored at 2-8 °C for up to a year.
7. Glucose standard stock solution (1 mg/mL): With 0.1% benzoic acid as preservative, Sigma G6918

Procedure

Part A. Measurement of total sugar concentration in sample solutions (extract from Protocol 1)

Summary: Step numbers refer to those described in the Details table below



Details:

Step	Procedure	Notes
A1	Obtain the tube containing the 0.2 mL (V5) sample extract (from Step 7 of Protocol 1, including the assay blank), warm up to room temperature.	
A2	Add 1.0 mL (V11) of 80% ethanol and mix well by vortex. Then carefully transfer 0.25 mL of the sample extract into a glass test tube and dilute it 10× (D1) by adding 2.25 mL of dH_2O. Do the same for the unknown samples and the assay blank. Mix thoroughly by vortex twice. (note (a))	(a) This dilution factor is suitable to analyze plant samples of total sugar concentration 0.5 to 50% dry weight.

<table>
<tr>
<td>A3</td>
<td>Prepare GFG standard solutions for the construction of the standard curve (note (b)). In separate glass test tubes, add the solutions as shown in the table below.
<table>
<tr><th>Concentration of GFG standard solution (µg/mL)</th><th>Volume of 1 mg/mL GFG stock solution (mL)</th><th>Volume of 80% ethanol (mL)</th><th>Volume of dH_2O (mL)</th><th>Total volume of GFG standard solution (mL)</th></tr>
<tr><td>0</td><td>0.0</td><td>0.5</td><td>4.5</td><td>5.0</td></tr>
<tr><td>50</td><td>0.25</td><td>0.5</td><td>4.25</td><td>5.0</td></tr>
<tr><td>100</td><td>0.5</td><td>0.5</td><td>4.0</td><td>5.0</td></tr>
<tr><td>150</td><td>0.75</td><td>0.5</td><td>3.75</td><td>5.0</td></tr>
<tr><td>200</td><td>1.0</td><td>0.5</td><td>3.5</td><td>5.0</td></tr>
<tr><td>250</td><td>1.25</td><td>0.5</td><td>3.25</td><td>5.0</td></tr>
</table>
Mix twice thoroughly by vortex.</td>
<td>(b) These standards solutions must bear the same ethanol concentration as in the diluted unknown sample solutions prepared in Step 2 above, which is 8% ethanol.</td>
</tr>
<tr>
<td>A4</td>
<td>Each sample is run in duplicates, pipet 0.25 mL each of the diluted sample solutions (Step 2) and the GFG standards (0 to 250 µg/mL) into two glass test tubes (A and B).</td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td>Under the fume hood, pipet 0.5 mL of 2% phenol solution into all tubes (note (c)).</td>
<td>(c) To achieve good mixing, aim the phenol solution directly into the sample and avoid running it down the tube walls.</td>
</tr>
<tr>
<td>A6</td>
<td>Keep samples under the fume hood and rapidly deliver 1.25 mL of conc. H_2SO_4 directly to the solution (note (d)). Limit the delivery time to about 1 s. The solution should turn yellow immediately. Mix lightly by vortex right after addition of the acid.</td>
<td>(d) A pump dispenser is highly recommended for this procedure. (CAUTION! Use procedures recommended for the handling of concentrated acids). Be consistent in the way of adding the acid. It greatly affects the variance of data. There will be a clear quenching sound, and it generates a lot of heat. Hold the tube only above the solution line. It is this heat that is essential for the reaction to take place.</td>
</tr>
<tr>
<td>A7</td>
<td>Cap the tubes with glass marbles and let sit in the dark for 10 min at room temperature. Place them in a water bath at room temperature for another 15 min to cool down further. (note(e))</td>
<td>(e) The yellow color developed is stable for hours.</td>
</tr>
</table>

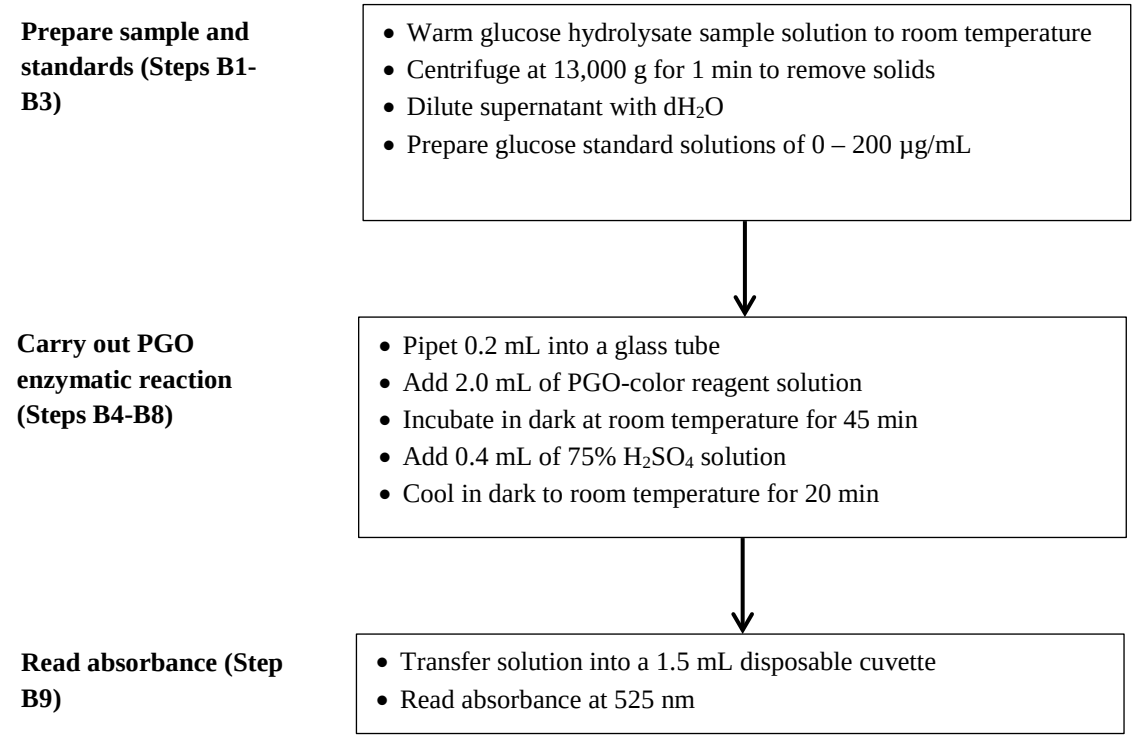
A8	Pour the whole solution into a 1.5 mL disposable cuvette and read absorbance at 490 nm (**A490**) using a spectrophotometer. Zero with the solution developed from the 0 µg/mL GFG standard. Finish the reading within 30 minutes after being transferred to the plastic cuvette to avoid measurement errors. (note (f), (g) and (h)) Average value of the absorbance readings (**$A490_{mean}$**) of the duplicates from test tubes **A** and **B** is used for the calculation of that sample solution.	(f) If the absorbance readings from test tubes **A** and **B** differ by more than 10 %, repeat the assay for that particular sample solution (steps 4 to 8). (g) Alternative to using disposable cuvettes, the absorbance can be read using microplates. Transfer the sample solution to 96-well microplates, 3 wells for test tube **A** and 3 wells for test tube **B** of each sample, 200 µL per well. Read the absorbance using a microplate reader. (h) Put waste into chemical disposal as it contains phenol and sulfuric acid.
A9	**Values obtained**: (note (i)) Build the GFG standard curve in linear regression, set the y-intercept to zero: $A490_{mean\ GFG} = a1 \times [GFG]$ Where: $A490_{mean\ GFG}$ = average of the absorbance readings, **$A490_{mean}$**, of GFG standard a1 = absorption coefficient of GFG standard [GFG] = concentration of GFG standard solutions, in µg/mL Total sugar concentration in the sample extract, uncorrected for interference, expressed as glucose-equivalent, in µg/mL: $[sugar, total]_{uncorrected} = (A490_{mean\ sample} - A490_{mean\ blank}) / a1 \times (V5 + V11) / V5 \times D1$ Where: $A490_{mean\ sample}$ = average of the absorbance readings, **$A490_{mean}$**, of unknown sample $A490_{mean\ blank}$ = average of the absorbance readings, **$A490_{mean}$**, of assay blank D1 = dilution factor on extract solution = 10	(i) For final calculations see Protocol 6.

A10	Run a parallel sugar assay of the same sample solution to correct for potentially interfering substances in the sugar assay. (note (j)) Repeat Steps 4 to 8 using the same diluted sample solution used in Step 4, including the GFG standards, except that in Step 5 the 0.5 mL of 2 % phenol is replaced by 0.5 mL of dH_2O. (note (k)) Read the absorbance at 490 nm (**A490'**) in the same way as in Step 8. Average value of the absorbance readings (**A490'$_{mean}$**) from test tubes **A** and **B** is used for the calculation of that sample solution.	(j) **CAUTION!** Concentrated H_2SO_4 hydrolyses other compounds present in the sample solution, such as chlorophylls, pigments, lipids, phenolics, proteins, etc. that interfere with absorbance readings at 490 nm. A parallel sugar assay of the same sample solution is necessary in which the phenol step is skipped. (k) Instead of yellow, the color developed in the sample solutions is pale green to purple, and very light yellow for the GFG standard solutions. (l) Put waste into chemical disposal as it contains sulfuric acid.
A11	**Values obtained**: (note (m)) Build the GFG standard curve in linear regression, set the y-intercept to zero: $A490'_{mean\ GFG} = a1' \times [GFG]$ Where: $A490'_{mean\ GFG}$ = average of the absorbance readings, **A490'$_{mean}$**, of GFG standard, without the use of phenol a1' = absorption coefficient of GFG standard, without the use of phenol [GFG] = concentration of GFG standard solutions, in µg/mL Total sugar concentration in the sample extract, corrected for interference, expressed as glucose-equivalent, in µg/mL: $[sugar, total]_{corrected} = ((A490_{mean\ sample} - A490_{mean\ blank}) - (A490'_{mean\ sample} - A490'_{mean\ blank})) / (a1 - a1') \times (V5 + V11) / V5 \times D1$ Where: $A490_{mean\ sample}$ = average of the absorbance readings, **A490$_{mean}$**, of unknown sample (see Step 8) $A490_{mean\ blank}$ = average of the absorbance readings, **A490$_{mean}$**, of assay blank (see Step 8) a1 = absorption coefficient of GFG standard (see Step 8) $A490'_{mean\ sample}$ = average of the absorbance readings, **A490'$_{mean}$**, of unknown sample, without the use of phenol $A490'_{mean\ blank}$ = average of the absorbance readings, **A490'$_{mean}$**, of the assay blank, without the use of phenol a1' = absorption coefficient of GFG standard, without the use of phenol D1 = dilution factor on extract solution = 10	(m) For final calculations see Protocol 6.

A12	Since the amount of interfering substances appears to be dependent on the plant material type, the correction for interfering substances (Steps 9 and 10) can be skipped after an equation for the interference correction has been developed for each material type that is being analyzed. Using 10 representative samples of the same material type and from the same species, plot a graph of $[sugar, total]_{corrected}$ against $[sugar, total]_{uncorrected}$ and determine the equation using linear regression. For the rest of analyses on the same material type, measure only the uncorrected sugar concentration $[sugar, total]_{uncorrected}$ using phenol. The total sugar concentration $[sugar, total]_{corrected}$ can then be calculated using the regression equation. (note (n))	(n) **CAUTION!** Also make sure to build separate interference correction equations even within the same species and material type if you anticipate differences in composition due to experimental conditions (e.g. significant differences in specific leaf area or nutrient status).
A13	**Values obtained:** (note (o)) Build the sugar interference correction curve using the result from the 10 representative samples of the same material type: $[sugar, total]_{corrected} = [sugar, total]_{uncorrected} \times m + b$	(o) For final calculations see Protocol 6.

Part B. Measurement of glucose hydrolysate in sample solutions after starch digestion (from Protocol 2)

Summary: Step numbers refer to those described in the Details table below



Details:

<table>
<tr><th>Step</th><th>Procedure</th><th>Notes</th></tr>
<tr><td>B1</td><td>Obtain the tube containing the 0.6 mL sample solution (from Step 7 of Protocol 2, including the assay blank), warm up to room temperature if required. Mix thoroughly by vortex. Centrifuge at 13,000 g for 1 min.</td><td>CAUTION! Phenol-sulfuric acid method described in Part A cannot be used to measure the glucose hydrolysate because the residual enzyme proteins present in the sample solution interfere.</td></tr>
<tr><td>B2</td><td>Carefully transfer 0.25 mL of the supernatant into a glass test tube and dilute it 10× (D2) by adding 2.25 mL of dH_2O. Do the same for the unknown samples and the assay blank. Mix thoroughly by vortex twice. (note (a))</td><td>(a)) This dilution factor is suitable to analyze plant samples of starch concentration 0.5 to 35% dry weight.</td></tr>
<tr><td>B3</td><td>Prepare glucose standard solutions for the construction of the standard curve. In separate glass test tubes, add the solutions as shown in the table below.
<table>
<tr><th>Concentration of glucose standard solution (µg/mL)</th><th>Volume of 1mg/mL glucose standard stock solution (mL)</th><th>Volume of dH_2O (mL)</th><th>Total volume of glucose standard solution (mL)</th></tr>
<tr><td>0</td><td>0.0</td><td>5.0</td><td>5.0</td></tr>
<tr><td>50</td><td>0.25</td><td>4.75</td><td>5.0</td></tr>
<tr><td>100</td><td>0.5</td><td>4.5</td><td>5.0</td></tr>
<tr><td>150</td><td>0.75</td><td>4.25</td><td>5.0</td></tr>
<tr><td>200</td><td>1.0</td><td>4.0</td><td>5.0</td></tr>
</table>
Mix thoroughly by vortex twice.</td><td></td></tr>
<tr><td>B4</td><td>Each sample is run in duplicates, pipet 0.2 mL each of the diluted sample solutions (Step 2) and the glucose standards (0 to 200 µg/mL) into two glass test tubes (A and B).</td><td></td></tr>
<tr><td>B5</td><td>Add 2.0 mL of PGO-color reagent solution into all tubes. Mix by vortex. (note (b))</td><td>(b) An orange color gradually develops.</td></tr>
<tr><td>B6</td><td>Keep the tubes in dark at room temperature for 45 min.</td><td></td></tr>
<tr><td>B7</td><td>Under the fume hood, add 0.4 mL of 75% H_2SO_4 solution directly to the liquid inside the tube. (note (c)) Mix by vortex twice.</td><td>(c) It stabilizes the color that turns pink.</td></tr>
<tr><td>B8</td><td>Let cool in dark at room temperature for 20 min. (note (d))</td><td>(d) The color is stable for a few hours.</td></tr>
<tr><td>B9</td><td>Pour the whole solution into a 1.5 mL disposable cuvette and read absorbance at 525 nm (A525) using a spectrophotometer. Zero with the solution developed from the 0 µg/mL glucose standard. Finish the readings within 30 min after transferred to the cuvette. (notes (e), (f), (g))

Alternatively, the absorbance can also be read using microplates. Transfer the sample solutions to 96-well microplates, 3 wells for test tube A and 3 wells for test tube B of each sample, 200 µL per well. Read the absorbance using a microplate reader.</td><td>(e) The sample solution will generate small bubbles if left in the cuvette for a long time and affects the reading. Alternatively, the bubbles can be removed by tapping on the cuvette. Absorbance reading will not be affected.

(f) If the absorbance readings from test tubes A and B differ by more than 5 %, repeat the assay for that particular sample solution (steps 4 to 9).</td></tr>
</table>

	Average value of the absorbance readings (**$A525_{mean}$**) of the 6 wells is used for the calculation of that sample solution.	(g) Put waste into chemical disposal as it contains o-dianisidine dihydrochloride and sulfuric acid.
B10	**Values obtained:** (note (h)) Build the glucose standard curve in linear regression, set the y-intercept to zero: $A525_{mean\ glucose} = a2 \times [glucose]$ Where: $A525_{mean\ glucose}$ = average of the absorbance readings, **$A525_{mean}$**, of glucose standard a2 = absorption coefficient of glucose standard [glucose] = concentration of glucose standard solutions, in µg/mL Concentration of glucose hydrolysate in the solution after starch digestion, in µg/mL: $[glucose\ hydrolysate] = (A525_{mean\ sample} - A525_{mean\ blank}) / a2 \times D2$ Where: $A525_{mean\ sample}$ = average of the absorbance readings, **$A525_{mean}$**, of unknown sample $A525_{mean\ blank}$ = average of the absorbance readings, **$A525_{mean}$**, of the assay blank a2 = absorption coefficient of glucose standard D2 = dilution factor on digested sample solution = 10	(h) For final calculations see Protocol 6.

Protocol 6: Final calculations of NSC concentrations

This protocol summarizes the calculations for NSC concentrations (sugar and starch separately) in plant samples with sugar extracted by ethanol and starch digested enzymatically and quantified by one of the three methods: IC, enzyme, or acid and PGO. All formula abbreviations refer to those used in Protocols 1 to 5.

ATTENTION! For verification of the measurements also calculate the sugar and starch concentrations of the two NSC standards (synthetic and plant material). If the measured values exceed ± 5% of the values for the synthetic standard and/or the running average of the plant standard, the entire batch needs to be redone starting from the beginning (i,e, extraction of samples starting with Protocol 1).

Quantified by IC (Protocol 3)

Percentage of sugar content (glucose + fructose + galactose + sucrose) in plant sample, in weight of sugar to dry weight of sample, expressed as glucose-equivalent:

$$\%\ sugar_{glu+fru+gal+suc}\ (w/w) = (([glucose] + [fructose] + [galactose] + [sucrose] \times 360.3 / 342.3) \times V_{extract}) / (W \times 10^3) \times 100\%$$

Where:
[glucose], [fructose], [galactose], [sucrose] = Concentration of glucose, fructose, galactose and sucrose, respectively, in the sample solution, in µg/mL (Protocol 3, Step 14)
$V_{extract}$ = Extract volume, in mL = 1.5 mL (Protocol 1, Step 11)
W = Sample input weight, in mg. Use $\mathbf{W_{sample}}$ for unknown plant samples and $\mathbf{W_{control}}$ for NSC control standards (Protocol 1, Step 11).

Percentage of starch content in plant sample, in weight of starch to dry weight of sample:

$$\%\ starch\ (w/w) = ([glucose\ hydrolysate] \times V_{starch}) / (W \times 10^3) \times 0.9 \times 100\%$$

Where:
[glucose hydrolysate] = Concentration of glucose hydrolysate in the solution after starch digestion, in µg/mL (Protocol 3, Step 14)
V_{starch} = Equivalent volume of digested sample, in mL (Protocol 2, Step 8)

Quantified by enzyme method (Protocol 4)

Part A - Glucose

Percentage of glucose content in plant sample, in weight of glucose to dry weight of sample:

$$\%\ \text{sugar}_{glu}\ (w/w) = ([\text{sugar, glu}] \times V_{extract}) / (W \times 10^3) \times 100\%$$

Where:
[sugar, glu] = Concentration of glucose in the sample solution, in µg/mL (Protocol 4 – Part A, Step A8)
$V_{extract}$ = Extract volume, in mL = 1.5 mL (Protocol 1, Step 11)
W = Sample input weight, in mg. Use $\mathbf{W_{sample}}$ for unknown plant samples and $\mathbf{W_{control}}$ for NSC control standards (Protocol 1, Step 11).

Part B – Free sugar (glucose + fructose)

Percentage of sugar content (glucose + fructose) in plant sample, in weight of sugar to dry weight of sample, expressed as glucose-equivalent:

$$\%\ \text{sugar}_{glu+fru}\ (w/w) = ([\text{sugar, glu+fru}] \times V_{extract}) / (W \times 10^3) \times 100\%$$

Where:
[sugar, glu+fru] = Concentration of glucose and fructose combined in the sample solution, in µg/mL (Protocol 4 – Part B, Step B3)

Part C - Sucrose

Percentage of sucrose content in plant sample, in weight of sucrose to dry weight of sample:

$$\%\ \text{sugar}_{suc}\ (w/w) = ([\text{sugar, suc}] \times V_{extract}) \times (342.3 / 360.3) / (W \times 10^3) \times 100\%$$

Where:
[sugar, suc] = Concentration of sucrose in the sample solution, in µg/mL as glucose-equivalent (Protocol 4 – Part C, Step C5)

Parts B & C: Total Sugar

Percentage of sugar content (glucose + fructose + sucrose) in plant sample, in weight of sugar to dry weight of sample, expressed as glucose-equivalent:

$$\%\ \text{sugar}_{glu+fru+suc}\ (w/w) = \%\ \text{sugar}_{glu+fru}\ (w/w) + \%\ \text{sugar}_{suc}\ (w/w) \times (360.3 / 342.3)$$

Part D: Starch

Percentage of starch content in plant sample, in weight of starch to dry weight of sample:

$$\%\ \text{starch}\ (w/w) = ([\text{glucose hydrolysate}] \times V_{starch}) / (W \times 10^3) \times 0.9 \times 100\%$$

Where:
[glucose hydrolysate] = Concentration of glucose hydrolysate in the solution after starch digestion, in µg/mL (Protocol 4 – Part D, Step D4)
V_{starch} = Equivalent volume of digested sample, in mL = 6 mL (Protocol 2, Step 8)

Quantified by acid and PGO (Protocol 5)

Percentage of total sugar content in plant sample, in weight of sugar to dry weight of sample, expressed as glucose-equivalent:

$$\%\ \text{sugar}_{\text{total}}\ (\text{w/w}) = ([\text{sugar, total}]_{\text{corrected}} \times V_{\text{extract}}) / (W \times 10^3) \times 100\%$$

Where:
$[\text{sugar, total}]_{\text{corrected}}$ = Total sugar concentration in the sample solution, in µg/mL, corrected for interference, expressed as glucose-equivalent (Protocol 5 – Part A, Step A11 or A13)
V_{extract} = Extract volume, in mL = 1.5 mL (Protocol 1, Step 11)
W = Sample input weight, in mg. Use $\mathbf{W_{sample}}$ for unknown plant samples and $\mathbf{W_{control}}$ for NSC control standards (Protocol 1, Step 11).

Percentage of starch content in plant sample, in weight of starch to dry weight of sample:

$$\%\ \text{starch}\ (\text{w/w}) = ([\text{glucose hydrolysate}] \times V_{\text{starch}}) / (W \times 10^3) \times 0.9 \times 100\%$$

Where:
[glucose hydrolysate] = Concentration of glucose hydrolysate in the solution after starch digestion, in µg/mL (Protocol 5 – Part B, Step B10)
V_{starch} = Equivalent volume of digested sample, in mL (Protocol 2, Step 8)