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Chapter 22

Extraction and Analysis of Soluble Carbohydrates

Niels Maness

Abstract

Soluble sugars are a universal component of most living organisms and a fundamental building block in biosynthetic processes. It is no wonder that both qualitative and quantitative changes in carbohydrates often accompany plant's responses to stress. Depending on the speed of onset of stress, plant tissues can exhibit rapid and very site-specific shifts in their soluble carbohydrate pool – rapid and precise tissue collection and stabilization are necessary if analytical results are to truly represent the sugar composition at the instant of harvest. Since soluble carbohydrates are, by definition, soluble in the cell's aqueous environment, they may be analyzed directly from liquids obtained from plants or they may require extraction from the plant matrix. During extraction and prior to analysis, steps should be taken to avoid change in form or quantity of sugars by endogenous active enzyme conversion or by contaminating microbial growth. Many procedures for soluble sugar analysis exist; the choice of the most appropriate analytical protocol is ultimately dictated by the depth of information required to substantiate findings for a particular purpose.

Key words: Colorimetric sugar assay, glucose, GC, fructose, fructan, HPLC, soluble sugars, sucrose, starch.

1. Introduction

Soluble carbohydrates are perhaps the most fundamental metabolic pool in plants. Derived from carbon dioxide during the dark reaction of photosynthesis and utilized as precursors for numerous compounds required to maintain plant health and even signaling molecules for stress-induced source–sink response (1), soluble carbohydrates indeed play a substantial role in higher plant development. Localized changes in quantity and form of soluble carbohydrates are often associated with plant stress. These changes may be due to increased or decreased sugar biosynthesis, conversion of starch or other storage forms to soluble sugars, breakdown of cell wall polysaccharides, and/or changes in the

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rate of sugar transport. Some transgenic plants accumulate sugar alcohols which are thought to increase cell osmotic potential and increase stress tolerance (2).

In order to validate and document the interaction of soluble sugar content with plant stress interaction, plant tissues must first be stabilized so that further changes in sugar quantity or identity are not imposed during tissue harvest. Sugars must then be extracted from the plant tissue in a stable form, at high enough concentration to accommodate the analytical procedure. Sugar identity and quantity can then be determined by a number of analytical techniques. This chapter will attempt to outline various methods used for soluble sugar determination from a variety of plant tissue types, highlighting techniques applicable for sample acquisition and stabilization, sugar extraction, and sugar analysis. Although the focus of this chapter is on soluble sugars, some methods for starch and fructan extraction and analysis are included since these oligomers/polymers may be hydrolyzed in response to stress and thus change the soluble sugar pool in given higher plant tissues.

2. Materials

2.1. Sample Acquisition and Stabilization

1. Liquid samples can be obtained directly from very high water tissues (fruits and fruit vegetables) by homogenizing or otherwise expressing liquid from a pre-cooled sample (*see* **Note 1**). Common homogenizers include Waring® blenders (Waring Corp., Torrington, CT), Omni Mixer homogenizers (Omni International, Marietta, GA), and Polytron probe (Brinkmann Instruments, Inc., Westbury, NY). Ground samples may be filtered through one to several layers of Miracloth® (Calbiochem; EMD Chemicals, Inc., La Jolla, CA) or through a clean sintered glass funnel, or may be obtained as a supernatant following centrifugation. Low-volume juice samples (vascular exudates, etc.) may be collected into pulled borosilicate glass capillary tubes (*see* **Note 2**), sealed, and frozen with liquid nitrogen (3).
2. Common lyophilizers for medium to large samples include cabinet-type systems with shelf temperature control (Millrock Technology, Inc., Kingston, NY) or manifold systems (Labconco Corp., Kansas City, MO). Cabinet-type systems are best suited for drying tissues, while manifold systems are best suited for drying liquids. Small-volume liquid samples may be lyophilized using a centrifugal lyophilizer such as a Speed Vac® (Savant, Farmingdale, NY).
3. Mechanical convection ovens should maintain high air flow for rapid temperature transfer into a tissue at 90°C. Oven

temperature should not exceed 95°C. Samples should be placed inside a labeled heat-resistant container (paper bags are appropriate) and then immediately into the forced draft oven at 90°C (*see* **Note 3**) for no longer than 90 min.

2.2. Sample Homogenization and Sugar Extraction

1. Liquid samples may not require further treatment prior to analysis except appropriate dilution and filtration, or lyophilization if further stabilization is desired. If enzyme inactivation is desired, heat a known volume of the sample to not higher than 95°C for no longer than 60 min.
2. If tissue samples preserved by freezing are to be processed without lyophilization, they may be pre-processed for extraction by powdering or directly homogenized with a wet grinder. Tissue powdering is done using a ceramic mortar and pestle (Coors Tek, Golden, CO) with the mortar bowl on dry ice or in a shallow dry ice:acetone bath. Frozen tissues may be directly homogenized in extraction solvent [water (*see* **Note 4**) or ethanol (*see* **Note 5**)] with one of the wet grinders indicated in **Section 2.1**, step 1. Sample:solvent ratio should be 1:10 (w/v) to accommodate adequate grinding. Grinding may be done directly in the same centrifuge tube used for extraction. Sonication can be achieved by placing the grinding vessel into a sonication water bath or by placing a clean sonication probe into the ground sample (Branson, Danbury, CT) while it is held on ice.
3. Dry tissues are typically ground to a fine powder prior to extraction. If tissue pieces are small enough (or can be readily broken by hand to obtain smaller pieces) to pass through the opening of a UDY cyclone mill (UDY Corp., Fort Collins, CO), they can be ground in one step to pass a 1-mm screen (*see* **Note 6**). If samples are too large to pass through the cyclone mill opening, they may need to be reduced in particle size. Waring-type blenders (Waring Corp., Torrington, CT) or a Wiley mill (Thomas Scientific, Swedesboro, NJ) may be used for small sample quantities; Hammer mills (Schutte-Buffalo Hammer Mill, LLC, Buffalo, NY) may be used for larger sample quantities.
4. If the sugar content of a high oil substrate [10% (w/w) oil or higher] is to be measured, oil should first be removed with diethyl ether (*see* **Note 7**) and then sugars can be extracted quantitatively with the solvent of choice. Use a Waring blender (Waring Corp., Torrington, CT) to pre-grind the sample in three bursts of not more than 20 s each.
5. A refluxing tube (pulled glass or a long Pasteur pipette) can be inexpensively fit into a rubber stopper to fit a 50-mL centrifuge tube or through a silicon insert to fit the inside of an open top, screw-on lid for a 2 dram vial to

accommodate ethanol vapor re-condensation during boiling ethanol extraction (*see* **Note 8**). Vapors from boiling ethanol are cooled during travel up the tube enough to re-condense into a liquid. Liquid ethanol then drips back into the extraction system.

6. If determination of both soluble sugar and starch concentration is desired on the same sample, the pellet from ethanol sugar extraction (but not water extraction) can be used for starch determination (*see* **Note 9**).
7. A mixture of 2% (w/v) amyloglucosidase and 0.5% (w/v) α-amylase (Sigma Chemical Co., St. Louis, MO) is prepared in 0.1 M sodium acetate buffer (pH 4.5). If stored at 4°C, this reagent is stable for at least 1 week. Commercial amyloglucosidase preparations may contain substantial glucose which necessitates co-incubation and analysis of a blank with each set of starch hydrolysates to correct for the contaminating glucose in each determination.

2.3. Sample Analysis

1. Soluble sugars may be concentrated by evaporation of a known volume of the extraction solvent. This is done typically in vacuo with a Speed Vac® (Savant, Farmingdale, NY) but can be done under a steam of nitrogen gas. Typically the sample is brought to dryness and may be stored in a freezer prior to analysis (*see* **Note 10**).
2. Sample dilution should be done just prior to analysis (*see* **Note 10**). Assure that liquids are equilibrated to room temperature prior to dilution since liquid density is impacted by temperature. Following dilution in water, samples should not be stored for more than 5 days at 4°C prior to analysis.
3. If a non-hydrolyzing colorimetric reducing sugar assay via Nelson–Somogyi reagent or a similar assay is used and both reducing and non-reducing sugars are to be quantitated, the assay should be performed on one sample without pretreatment to account for resident reducing sugars, then non-reducing sugars (disaccharides, oligosaccharides, or polysaccharides) are converted to monomeric reducing sugars, and the assay is redone (*see* **Note 11**). Nelson–Somogyi reagents are prepared as follows:
   A) Prepare copper reagent in three steps:
      Reagent A: Dissolve 100 g sodium sulfate, 12.5 g anhydrous sodium carbonate, 10 g sodium bicarbonate, and 12.5 g sodium potassium tartrate in 400 mL water and bring to 500 mL volume. If the solution is cloudy, filter through Whatman no. 1 filter paper.

      Reagent B: Dissolve 3 g copper sulfate pentahydrate in 5 mL water, add one drop of concentrated sulfuric acid, and bring to 20 mL volume.

Copper working solution: Add 4 mL of reagent B to 96 mL of reagent A. This solution is stable for at least 3 weeks at room temperature.

B) Prepare arsenomolybdate reagent in three steps:
Dissolve 25 g ammonium molybdate in 400 mL water and add 25 mL concentrated sulfuric acid and mix.

Dissolve 3 g sodium arsenate heptahydrate in 25 mL water.

Add the sodium arsenate heptahydrate solution to the ammonium molybdate solution and bring to 500 mL volume. Heat at 50–60°C to dissolve completely, if necessary. This stock solution is stable for at least 2 weeks at room temperature.

Arsenomolybdate working solution: Dilute the above stock solution 1:5 with water just prior to use. This working solution is stable for 1 week at 4°C.

4. If a hydrolyzing colorimetric assay is used (anthrone), no distinction between monomeric reducing sugars and other linked sugars can be made; results reflect total sugar and approximate weight of linked carbohydrates cannot be determined.

   Prepare anthrone reagent by mixing 100 mg anthrone with 100 mL ice-cold 72% sulfuric acid. Store anthrone reagent in the dark at 4 °C. This solution is stable for up to 2 months.

5. Enzymatic sugar assays give more specific information about reducing and non-reducing sugars because enzymes are specific to individual sugars (*see* **Note 12**). Like colorimetric assays, they usually require only a spectrophotometer as analytical equipment. If disaccharides, oligosaccharides, or polysaccharides are of interest, in most cases they must be hydrolyzed to monosaccharides to accommodate analysis (*see* **Note 11**). The enzymatic analytical method described in **Section 3** uses a Boehringer Mannheim/R-Biopharm test combination for sucrose/glucose/fructose for approximately 20 assays.

   Enzyme substrates and buffers required for analysis are prepared as follows:

   a) Solution 1 contains 0.5 g lyophilisate of citrate buffer and 720 U of invertase (β-fructosidase); once diluted with 10 mL redistilled water (*see* **Note 18**), the solution should have a pH of 4.6 and is stable for 4 weeks at 2–8°C. Solution should be brought to room temperature before use.

   b) Solution 2 contains about 7.2 g of powder comprised of triethanolamine buffer, 110 mg NADP, 260 mg ATP, and magnesium sulfate; once diluted with 45 mL

redistilled water, the solution should have a pH of 7.6 and is stable for 4 weeks at 2–8°C. Solution should be brought to room temperature before use.

c) Suspension 3 contains about 1.1 mL suspension of 320 U of hexokinase and 160 U of glucose-6-phosphate dehydrogenase. This suspension is used undiluted and is stable for 3–4 weeks at 4°C.

d) Suspension 4 contains about 0.6 mL suspension of 420 U of phosphoglucose isomerase. This suspension is used undiluted and is stable for 3–4 weeks at 4 °C.

6. Gas chromatographic sugar assays require that monosaccharides be first derivatized to make them volatile. Common procedures are trimethylsilylation (TMS) (4) or formation of alditol acetates (5). TMS sugars chromatograph as multiple peaks of repeatable relative responses; alditol acetates chromatograph as single peaks. For most plant tissues inositol is an acceptable internal standard. Disaccharides, oligosaccharides and polysaccharides must first be methanolized to monosaccharide constituents and then derivatized. Polysaccharide sugar linkages vary in susceptibility to cleavage; conditions which result in quantitative conversion for α-1,4-glucose may not be sufficient for quantitative conversion of β-1,4-glucose or of uronic acid linkages.

A) Methanolic HCl preparation: To 10 mL HPLC grade methanol, add 2.1 mL acetyl chloride drop-wise with vigorous stirring. Store in securely capped vials in 2–5 mL aliquots in a freezer; allow solution to warm to room temperature before opening. Solution is stable for 3 weeks stored frozen or 1 week at room temperature.

B) Trisilylation reagent: Prepare a 1:1:5 (v/v/v) mixture of hexamethyldisilazane:trimethylchlorosilane:pyridine [or dilute TriSil (Pierce Chemical Co., Rockford, IL) with dry pyridine].

7. The various high-performance liquid chromatography (HPLC) procedures used for sugar analysis can be categorized by separation and/or detection requiring moderate pH versus those which require high pH (pH 11 or higher). HPLC is suitable for monomeric to polymeric sugar analysis. Most sugar columns operating at moderate pH are packed with cation exchange resin and may use water, acetonitrile:water, or dilute buffer as mobile phase. Guard columns typically last for up to 300 injections for most sample types (6). Ethanol extracts may be injected directly onto these columns after appropriate dilution. Bio-ethanol yields can be assessed along with soluble sugar residue in a single injection. Most detectors are non-selective (refractive

index or light scattering), vary in sensitivity, and may not be suitable for mobile phase gradients. Sugar columns operating at high pH via varying sodium hydroxide concentrations in the mobile phase (*see* **Note 13**) separate sugars based on ion exchange. The molarity of sodium hydroxide impacts mobile phase pH, which in turn impacts the degree of protonation of sugar hydroxyl groups. Guard columns tend to last for over 300 injections unless samples contain a high amount of particulates. Ethanol extracts must be dried and re-dissolved in water or mild buffer solution prior to injection (*see* **Notes 10** and **14**). Detection is typically by pulsed electrochemical detection (PED). With the appropriate wave form, PED can be selective for sugars and is more sensitive than refractive index. Gold working electrodes may be purchased in disposable or non-disposable styles – non-disposable electrodes are most economical for common sugar analyses. Most monosaccharide/disaccharide separations can be done isocratically; larger oligomeric or polymeric carbohydrates usually require a gradient of either increasing sodium hydroxide concentration or increasing buffer salt concentration, with sodium hydroxide concentration held constant.

A) Removal of phenolic compounds with a C18 Sep-Pak® (Waters Associates, Milford, MA) syringe cartridge:
   a) Precondition cartridge by applying at least 2 mL of HPLC methanol through the cartridge.
   b) Equilibrate cartridge with at least 4 mL water, then blow out remaining water with air from an empty syringe.
   c) Apply extract in water and rinse with one to two additional volumes of water.
   d) Utilize the cartridge effluent for sugar analysis.

B) Potential waveform for pulsed electrochemical detection of carbohydrates:

Time (s)	Potential (V)	Integration
0.00	0.05	
0.20	0.05	Begin
0.40	0.05	End
0.41	0.75	
0.60	0.75	
0.61	−0.15	
1.00	−0.15	

3. Methods

Within any given plant tissue, soluble sugars are in a state of flux with various metabolic cycles and with storage pools. It is important to separate the tissue of interest and stabilize it as rapidly as possible to preserve the sugars in the form and quantity they were in at the instant of harvest. Metabolism may be suspended by freezing or stopped by heat inactivation; dense tissues may need to be dissected to allow rapid thermal penetration. Soluble sugars in certain high water tissues or plant exudates may be directly analyzed following particulate removal via centrifugation or filtration. More frequently, sugars are extracted from frozen or dried tissues using water or ethanol. Tissues are first ground (non-dried tissues are powdered while still frozen or ground with wet grinders in extraction buffer; pre-dried tissues are ground with dry mills prior to extraction) and then extracted with the appropriate solvent. Multiple extractions are necessary for quantitative sugar recovery. If sugar content of high oil tissues [5–10% (w/w) oil or higher] is to be determined, lipids can first be extracted with diethyl ether (*see* **Note 7**) and then sugars can be extracted. Extraction with boiling 95% (v/v) ethanol allows extraction of soluble sugars and subsequent hydrolysis and analysis of starch content. For extraction of oligomeric sugars such as fructans, ethanol concentration should be reduced to no more than 70% (v/v) ethanol.

The depth of information needed in the determination of soluble sugars impacts the choice of an analytical protocol. Relatively simple colorimetric assays can be used to evaluate concentration of reducing and non-reducing sugars, but these assays do not provide information regarding individual sugar concentration and tend to be less sensitive than other assays. Enzymatic assays provide specific sugar concentration and require the use of relatively inexpensive laboratory instrumentation but require multiple readings to evaluate sugar concentration and can be labor intensive. Gas chromatographic assays provide information regarding numerous sugars in a single run and are sufficiently sensitive for most applications but require sample derivatization prior to analysis and may not be appropriate for quantitative analysis of oligomeric sugars. Absolute quantization of all sugars may be difficult to achieve because sugar derivatization may be incomplete for certain sugars. HPLC allows sensitive characterization of the presence and quantity of numerous sugars in a single run; most detectors do not require derivatization of sugars prior to injection and separations may be optimized to evaluate concentration of monomeric to oligomeric sugars if the appropriate extraction solvent was used.

3.1. Sample Acquisition and Stabilization

1. Samples acquired in liquid form should be cooled on ice (*see* **Note 1**) prior to juice acquisition to slow metabolic conversions. To assure homogeneity in sugar content, pre-cooled tissue samples of adequate size to be representative of the tissue in question can be weighed and then ground with a blender to release cellular liquids. Maintenance of cold temperatures during grinding is essential to avoid excessive sugar metabolic conversion; a Waring® blender must be placed inside a cold room, whereas the container with sample for Omni Mixer or Polytron grinding may be placed into an ice bath to maintain cold sample temperature. Juice samples of a known volume can be obtained as filtrates or as supernatants after centrifugation (3,000–10,000×*g* is sufficient) and should be either immediately analyzed or placed inside a sealable plastic ampoule and frozen with liquid nitrogen, then stored inside a freezer (–20°C minimum, –80°C recommended). Frozen liquid samples can be further stabilized by heat treatment or with lyophilization. If lyophilization is to be conducted, shell freezing increases surface area and decreases drying time.
2. Samples acquired in solid form should be excised with a clean, sharp instrument and then stabilized by rapid freezing with liquid nitrogen and stored in a freezer as indicated for liquid samples or heat inactivated, dried (*see* **Note 3**), and then stored inside a freezer to await dry grinding and extraction. Tissues should be dissected into smaller pieces (1–3 cm) to allow rapid thermal transmission through the tissue. Frozen tissue samples may be directly homogenized and extracted or lyophilized and stored frozen to await dry grinding and extraction. Prior to extraction, samples should be removed from the freezer and allowed to thaw to room temperature prior to opening of the storage container to prevent moisture condensation onto the tissue surface. Since enzymes may still be active, tissue processing is continued soon after thawing.
3. Non-liquid samples to be preserved by heat should be held at 90°C for a maximum of 90 min (minimum of 60 min) to stop enzymatic sugar conversion in the tissue and then dried at 70°C or lower for an additional 12–36 h to a constant weight (*see* **Note 3**). Prolonged tissue exposure to 90°C beyond 90 min can cause complexation reactions of sugar with other tissue components (especially proteins and phenolic compounds) and should be avoided. Samples should be stored in a freezer after drying to await dry grinding and extraction.

3.2. Sample Homogenization and Sugar Extraction

1. Frozen samples can be pre-ground to a powder in the frozen state or they may be ground in extraction buffer with a wet homogenizer. Tissue powdering is conducted on a cold surface to maintain the tissue in a frozen state throughout the powdering process to prevent excessive tissue water thaw, which would cause a change in the form of the powder to a gum. If the entire sample were weighed just prior to freezing, it can be quantitatively transferred into an extraction container using a small volume of extraction buffer. Frozen tissues may also be placed into extraction solvent [water (*see* **Note 4**) or ethanol (*see* **Note 5**)] and homogenized with a wet grinder. The homogenizer should be rinsed with a minimal volume of grinding solvent back into the ground sample to accommodate quantitative sugar recovery and prevent contamination with the next sample. If water is used as extraction solvent, an additional rinse with 95% ethanol helps prevent protein or microbial contamination between samples.
2. Pre-dried samples are ground to a fine powder with a UDY mill (UDY Corp., Fort Collins, CO) prior to extraction. After grinding, the samples are either immediately extracted or stored frozen inside a brown bottle.
3. For "typical" reducing and non-reducing soluble sugars (monomeric and most dimeric sugars), boiling 95% (v/v) ethanol serves as the extraction solvent. Accurately weigh 100–400 mg dry ground or frozen wet sample into a round–bottom, plastic 50-mL centrifuge tube. Add 15–20 mL 95% (v/v) ethanol, cap with a one-hole rubber stopper equipped with a glass reflux tube (*see* **Note 8**), mix and place into a water bath set at 85°C. Observe liquid for initiation of boiling and incubate in boiling ethanol for 20 min. Uncap tubes and centrifuge at 10,000×*g* for 10 min. Decant the supernatant into a volumetric flask and repeat extraction three more times. If determination of starch content is desired, after decanting the fourth 95% (v/v) ethanol supernatant into the volumetric flask, proceed with starch digestion or overlay the centrifugal pellet with 95% (v/v) ethanol, cap the tubes with a rubber stopper, and store at 4°C for up to 2 weeks. The ethanol extracts can be held at room temperature for up to 2 weeks prior to analysis; storage at 4°C helps prevent ethanol evaporation during storage. This process can be easily scaled down to a 2 dram vial (*see* **Note 8**), using 20–80 mg of sample and 4 mL extraction solvent and decanting the extraction supernatant into a 20- or 25-mL volumetric flask. Water can be used for extraction of soluble sugars using the same basic procedure outlined above except that the samples are incubated at 60°C rather

than 85°C, centrifugal speeds of 20,000–30,000×*g* (rather than 10,000×*g* for the ethanol procedure) are necessary for an equivalent pellet, and samples should be stored for no more than 5 days at 4°C prior to analysis. Since starch may be partially solubilized during water extraction, the pellet after water extraction is not suitable for subsequent starch determination.

4. Starch determinations should be done on samples after soluble sugar removal [sugar removal should have been done with 95% (v/v) ethanol]. If samples were overlaid with ethanol, uncap and evaporate the ethanol prior to proceeding (*see* **Note 9**). Add 10 mL water to samples in a 50-mL centrifuge tube (2 mL water for samples in 2 dram vials) and mix. Include two or three blank tubes, handled exactly the same as those containing sample. Incubate the samples at 90°C with intermittent mixing for 30 min to gelatinize starch. Allow samples to cool to room temperature, then add 10 mL of 0.2 M sodium acetate, pH 4.5 (2 mL for 2 dram vials). Add 1 mL of amyloglucosidase and α-amylase (200 μL for 2 dram vials), mix well, and stopper tubes. Incubate at room temperature for 1–2 h to allow α-amylase to pre-digest the gelatinized starch, then incubate at 55°C for 16–24 h (*see* **Note 15**). Allow tubes to cool and then centrifuge at 30,000×*g* for 10 min. Decant supernatant into a volumetric flask (volume may be the same as for sugar extraction or it may differ depending on the amount of starch in the tissue), add 10 mL water (2 mL for 2 dram vials) to the pellet, mix well, and incubate at 60°C for 10 min. Repeat centrifugation and re-extract with water two more times. Bring volumetric flasks to volume with water and store the starch extracts and blanks at 4°C for up to 5 days to await analysis.
5. If co-analysis of fructans with soluble sugars is desired, use of 70% (v/v) ethanol with 0.05% calcium carbonate (to prevent pH drop during extraction) as extraction buffer is common. Perform extractions while boiling as indicated in **Section 3.2**, step 3 for 95% (v/v) ethanol extraction. Bring solution to volume with 70% (v/v) ethanol.
6. If the sugar content of a tissue containing more than 10% (w/w) of oil is desired, lipid extraction of the tissue should precede sugar extraction (*see* **Note 7**). Grind with a Waring® blender, place the ground material on a clean, flat surface, and separate out any particles greater than 1 mm. The remainder of the sample can then be accurately weighed (target weight depends on expected sugar content) and extracted with diethyl ether (ratio of 1:10 for sample weight versus diethyl ether volume) with stirring for 20 min.

Centrifuge at between 3,000 and 10,000×*g* and decant supernatant into a clean, pre-weighed container. Repeat the extraction three more times for quantitative oil removal. Evaporate diethyl ether from the extract and weigh the container for gravimetric oil content determination. Completely evaporate diethyl ether from the sample residue and proceed with sugar extraction as described in step 3 of this section. If boiling 95% (v/v) ethanol is used as extraction solvent, starch analysis can proceed following sugar extraction, as described in step 4 of this section.

3.3. Sample Analysis

3.3.1. Colorimetric Non-hydrolyzing Assay (Nelson–Somogyi Procedure)

There are a number of procedures used for colorimetric determination of reducing sugars which do not hydrolyze sugar linkages in the sample. A very common procedure is the Nelson–Somogyi procedure (7). Since no sugar linkages are hydrolyzed, the procedure will not detect non-reducing sugars such as sucrose, but sucrose and like sugars can be hydrolyzed with acid or preferably enzymatic treatment; the yield of monomeric reducing sugars can then be detected and quantitated using the procedure. Since different sugars react with the reagent to give differing absorbance and thus different standard curves, a standard containing the same sugar(s) expected in the sample should be used and base results relative to the sugar(s) chosen as standard. This procedure has been scaled for use of microplates, which greatly increases the number of samples that can be run (8).

1. Prepare a sugar standard solution at 100 μg/mL water (*see* **Note 16**). This solution is stable for 1 week at 4°C.
2. Add 1 mL of aqueous sample (or of appropriate dilutions of the sugar standard solution) to a test tube. Add 1 mL water (or appropriate sample buffer) to triplicate tubes to serve as reagent blank.
3. Add 0.5 mL copper working solution and mix.
4. Heat at 100°C for 10 min and cool to room temperature.
5. Mix well, then add 3 mL arsenomolybdate working solution and allow to stand at room temperature for 10 min.
6. Mix well and measure absorbance of blue to dark blue samples, blanks, and standards at 520 nm.
7. Calculate reducing sugar concentration relative to the sugar(s) chosen as standard, from the standard curve for each run. Subtract the average of blank absorbance from samples and standards.

3.3.2. Colorimetric Hydrolyzing Assay (Anthrone Procedure)

As with non-hydrolyzing colorimetric sugar assays, many procedures exist for colorimetric determination of sugars which do hydrolyze sugar linkages in the sample. The anthrone procedure (9) can be used to determine total sugars but will not yield

specific information on the amount of reducing sugars in a sample if other sugars are hydrolyzed and subsequently contribute to the colorimetric response. The anthrone procedure has found utility for determination of starch content (if starch is present in a relatively pure form) (10). Choose sugars for use in standards which are representative of those expected in samples and present results relative to the sugar(s) chosen for standards (*see* **Note 16**).

1. Prepare a sugar standard solution at 100 μg/mL or a starch standard solution at 1 mg/mL (*see* **Note 16**). This solution is stable for 1 week at 4°C.
2. Add 1 mL of aqueous sample (or of appropriate dilutions of the sugar standard solution) to a test tube. Add 1 mL water (or appropriate sample buffer) to triplicate tubes to serve as reagent blank.
3. Add 4 mL ice-cold anthrone reagent to all tubes (*see* **Note 17**), vortex, and place at 100°C for exactly 10 min.
4. Place tubes into an ice bath and rapidly cool.
5. Mix well and read absorbance of green to dark green blanks, standards, and samples at 630 nm.
6. Calculate total sugar concentration relative to the sugar(s) chosen as standard, from the standard curve for each run. Subtract the average of blank absorbance from samples and standards.

3.3.3. Enzymatic Sugar Determination

Enzymatic sugar determinations have an advantage over colorimetric assays in being sugar specific and may be more sensitive than standard colorimetric assays. In most cases a stoichiometric enzyme product other than the sugar is measured spectrophotometrically. Enzyme substrates are provided in excess to assure completion of enzyme reaction. Once the user has confirmed quantitative conversion of a standard within a range of concentrations, standards need not be run with each set of samples. Samples must contain sugars within the specified range of concentration; too much sugar may overwhelm substrate concentration and cause deviation in the stoichiometric relationship required for quantification. A number of enzymatic sugar assays are available. The assay demonstrated here is from Boehringer Mannheim/R-Biopharm and is taken from the R-Biopharm Enzymatic Bioanalysis and Food Analysis handbook using test kits. A number of sugar assays are available; glucose, fructose, and sucrose quantization via stoichiometric NADPH formation by glucose-6-phosphate dehydrogenase will be demonstrated. Glucose is converted to glucose-6-phosphate by hexokinase and then glucose-6-phosphate is converted to gluconate-6-phosphate with stoichiometric production of NADPH from NADP, which is measured against a co-incubated

blank solution spectrophotometrically at 340 nm. Fructose is converted to fructose-6-phosphate by hexokinase and then isomerized to glucose-6-phosphate by phosphoglucose isomerase and quantified in the same reaction mixture as above. Sucrose is hydrolyzed into glucose and fructose by invertase and then glucose is quantified as above in a separate cuvette.

1. Warm sample extracts (ethanol or water) to room temperature and mix well before pipetting.
2. Conduct glucose/fructose assay in the same spectrophotometer cuvette as follows (*see* **Note 12**):
   a) Add 1.9 mL redistilled water, followed by 1.0 mL solution 2 and mix (*see* **Note 19**).
   b) Add 0.100 mL sample or blank [95% (v/v) ethanol or water, depending on the extraction solvent] and mix.
   c) Read and record the time zero absorbance of sample and blank solutions at 340 nm.
   d) Start glucose reaction by adding 20 μL of suspension 3 to sample and blank cuvettes and mix.
   e) Read and record absorbance after 15 min or after sample absorbance becomes static.
   f) Start fructose conversion to glucose and coupled glucose reaction by adding 20 μL of suspension 4 to sample and blank cuvettes and mix.
   g) Read and record absorbance after 15 min or after sample absorbance becomes static.
3. Conduct sucrose assay independent of the glucose/fructose assay by first mixing 0.2 mL solution 1 (invertase in citrate buffer) with 0.100 mL sample or blank, then follow steps a–e outlined in step 2 above.
4. Calculations are based on ΔA values, where $\Delta A = (A_{\text{after enzyme addition}} - A_{\text{prior to enzyme addition}})$ for cuvettes containing sample $- (A_{\text{after enzyme addition}} - A_{\text{prior to enzyme addition}})$ for cuvettes containing blank solution. For determination of glucose, this would be A from step e of step 2 above – A from step c of step 2 above; for fructose this would be A from step g of step 2 above – A from step e of step 2 above.

The general calculation for sugar concentration (c) is as follows:

$$c = ((V \times \text{MW})/(\varepsilon \times d \times v \times 1{,}000)) \times \Delta A$$

where

V is the final reaction volume (mL),

v is the sample volume (mL),

MW is the molecular weight of sugar being assayed (180.16 g/mol for glucose or fructose; 342.3 g/mol for sucrose),

D is the light path length (1 cm),

ε is the extinction coefficient for NADPH at 340 nm = 6.3 l/mmol/cm.

For glucose, $c = ((3.020 \times 180.16)/(6.3 \times 1.00 \times 0.100 \times 1{,}000)) \times \Delta A = 0.864 \times \Delta A$.

For fructose, $c = ((3.040 \times 180.16)/(6.3 \times 1.00 \times 0.100 \times 1{,}000)) \times \Delta A = 0.869 \times \Delta A$.

For sucrose, $c = ((3.020 \times 342.3)/(6.3 \times 1.00 \times 0.100 \times 1{,}000)) \times \Delta A = 1.641 \times \Delta A$.

3.3.4. Gas Chromatographic Sugar Determination

Sugar determination by gas chromatography requires that the sugars in extracts first be dried and then derivatized to make the sugars volatile enough for travel down a gas chromatography column. The two most common derivatives used for sugar analysis are trimethylsilylation (TMS; 4) or alditol acetates (11). Although TMS derivatives for most sugars chromatograph as multiple peaks, which makes data entry more complicated, the ratio of peak area units from each sugar is constant and can thus be used as a qualitative identification of sugars within a sample. Sugar-reducing ends are first methanolized with methanolic HCl and the monomeric sugars are then trimethylsilylated. If dimeric, oligomeric, or polymeric sugars are present, they will be converted into their monomeric constituents and information regarding their concentration will be lost. The TMS procedure is thus not a method of choice for quantifying sucrose or maltose in the presence of glucose and fructose, but it can be useful for quantifying starch if soluble sugars were removed in a prior step and glucose-containing oligomeric or polymeric carbohydrates which are susceptible to cleavage by methanolic HCl are substantially absent (*see* **Note 5**). The TMS procedure provides sugar composition information but does not yield the confirmation from which they originated.

1. To a 1 or 2 dram screw cap glass vial, add a volume of sugar extract containing approximately 50 μg sugar to a volume of inositol containing 100.0 nmol inositol (used as internal standard) and completely dry under a stream of nitrogen gas (*see* **Note 20**).
2. Place vials into a vacuum desiccator over P_2O_5 for at least 3 h at room temperature.
3. Add 0.40 mL methanol, that is, 1.5 M HCl and 0.10 mL methyl acetate, to each vial and securely cap vials with a Teflon-lined screw cap (*see* **Note 21**).

4. Place vials into a dry block heater at 80°C for 15 min, retighten screw caps, and incubate overnight (*see* **Note 22**).
5. Allow vials to cool, remove the screw caps (*see* **Note 22**), then add 3–6 drops of *t*-butanol, and evaporate their contents under a gentle stream of nitrogen gas at room temperature (*see* **Note 23**). Dry vial contents to one concentrated spot in the vial versus many spattered dry spots to accommodate the TMS step.
6. Add 25 μL of trimethylsilylation reagent to each vial, making sure that the reagent comes in contact with the dry sample.
7. Securely cap each sample and incubate at room temperature for at least 15 min but no more than 2 h.
8. Dry the reagent with a gentle stream of nitrogen gas at room temperature and add 100 μL isooctane just when dryness is achieved.
9. Inject 1 μL sample via cool, on-column injection onto a DB-1 fused silica capillary column (0.25 mm × 30 m, 0.25 μm film thickness; J and W Scientific, Folsom, CA) equipped with an FID detector using helium as a carrier gas. Oven temperature program is as follows:
   a) Start at 105°C for 2 min.
   b) Increase temperature to 160°C at 10°C/min and hold for 4 min.
   c) Increase temperature to 200°C at 1°C/min and hold for 5 min.
   d) Increase temperature to 290°C at 10°C/min and hold for 5 min to clean contamination.
   e) Cool to 105°C and hold for 10 min prior to a subsequent injection.
10. Sugar peaks are identified by co-elution with authentic standards and calculations are based on sugar responses (area units) relative to inositol response (area units) using an internal standard procedure and compared to previously run sugar standards:
    ((area units of sugar in sample/area units of 100 nmol inositol in sample)/(area units of 100 nmol of sugar in standard/ area units of 100 nmol inositol in standard)) × 100 nmol = nanomoles of sugar in sample.

The nanomoles of sugar in the sample is equivalent to the nanomoles of sugar per unit volume initially dried down prior to methanolysis and can be calculated back to concentration of sugar per extract volume or per unit weight of the original sample (*see* **Note 24**).

3.3.5. HPLC Sugar Determination

HPLC is perhaps the most popular and most powerful means for soluble sugar analysis. Most procedures do not require derivatization of sugars and many allow direct injection of extracts. Procedures differ based on the mode of sugar separation and detection. Two differing protocols will be presented: one based on weak ionic interaction using water or acetonitrile:water as mobile phase and refractive index as detection and the other based on relatively strong ion exchange using an isocratic or a gradient of sodium hydroxide as mobile phase and pulsed electrochemical detection (gold working electrode).

HPLC can be used for quantification of simple monomeric sugars but is also capable of yielding quantitative and qualitative information for dimeric sugars and up to polysaccharides. While polysaccharide analysis is beyond the scope of this chapter, procedures do exist for their analysis via HPLC.

The Biorad Aminex® (Biorad Laboratories, Hercules, CA) series of HPLC columns have been in use for sugar analysis since the early 1980s and are still popular for certain uses today. Two of the more popular sugar columns today are the HPX 87C and HPX 87P. A third column the HPX 87H is popular for combined organic acid, ethanol, and sugar separations. Aminex® columns are usually stainless steel in construction and have resin-based packings, usually require elevated temperatures (up to 85°C) for optimum peak resolution, and have moderate maximum pressure limits (1,500 psi). The typical mobile phase for the sugar columns is adequately degassed HPLC water (*see* **Note 4**) and the HPX 87C column can be used for direct injection of either water or ethanol extracts (*see* **Note 25**). They should be protected with the appropriate pre-column(s) which will generally withstand 300–350 injections before they need to be replaced. Detection can be done with various methods, the most simple of which is refractive index (RI).

1. Appropriately dilute extract with extraction solvent or reconstitute a dried sample in degassed water.
2. Remove particulates by filtration through a 0.45-μm filter or by centrifugation. If extract has excessive quantities of phenolics of other dissolved impurities, preprocess by flowing through a C18 Sep-Pak (or equivalent) syringe cartridge.
3. Place sample into an autosampler or use a manual injector with a 20-μL injection loop.
4. Overfill the injection loop by applying at least 50 μL of sample prior to injection onto an HPX 87P (or HPX 87H; 300 mm × 7.8 mm; BioRad Laboratories, Hercules, CA) column contained inside a heated jacket at 85°C for the HPX 87P or 60°C for the HPX 87H.

5. Inject the sample onto the column with water as eluent for the HPX 87P and 0.05 M sulfuric acid as eluent for the HPX 87H columns at a flow rate of 0.6 mL/min, using a refractive index detector with column eluent in the reference cell (*see* **Note 26**). Larger sugars elute first with the HPX 87C (6), HPX 87P (**Fig. 22.1**), and HPX 87H (**Fig. 22.2**) columns in the order of sucrose (or cellobiose), glucose, then fructose.

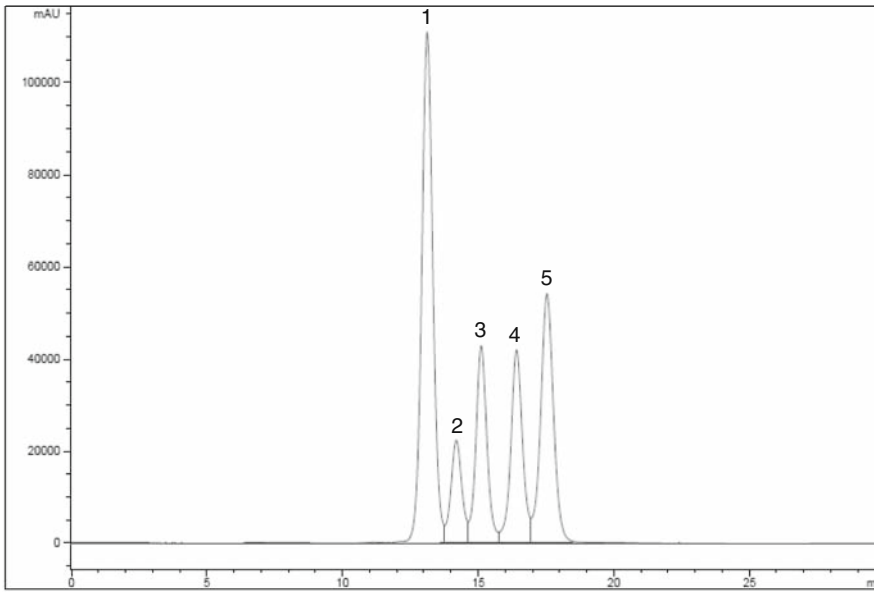

Fig. 22.1. BioRad HPX 87P HPLC chromatogram of standards for glucose (1), xylose (2), galactose (3), arabinose (4), and fructose (5). Sugars were injected in 20 μL (1.12 μmol glucose, 0.33 μmol xylose, 0.46 μmol galactose, 0.53 μmol arabinose, and 0.67 μmol fructose) and chromatography was conducted at 85°C using degassed water as elution buffer at a flow rate of 0.6 mL/min. Sugars were detected using a refractive index detector with water as reference. (Chromatogram courtesy of Mark Wilkins, Biosystems and Agricultural Engineering Dept., Oklahoma State University.)

6. Note pressure periodically and change guard columns when pressure starts to climb 200–300 psi above nominal operating pressure. Guard column(s) should last for 300–350 injections. If guard columns must be replaced substantially more often, filter or pre-clean samples as indicated in **Section 2**.
7. Certain samples may be very high in one sugar (sucrose, for example) and low in other sugars (glucose and/or fructose, for example). If quantification of all sugars is of interest, run the sample in different dilutions in order to prevent exceeding the linear range of quantification for each sugar.
8. If collection of fractionated sugars is of interest, attach a fraction collector with the smallest length of tubing practical. Use a low internal diameter (i.d.) tubing (usually matching the i.d. of the tubing running between the column and the

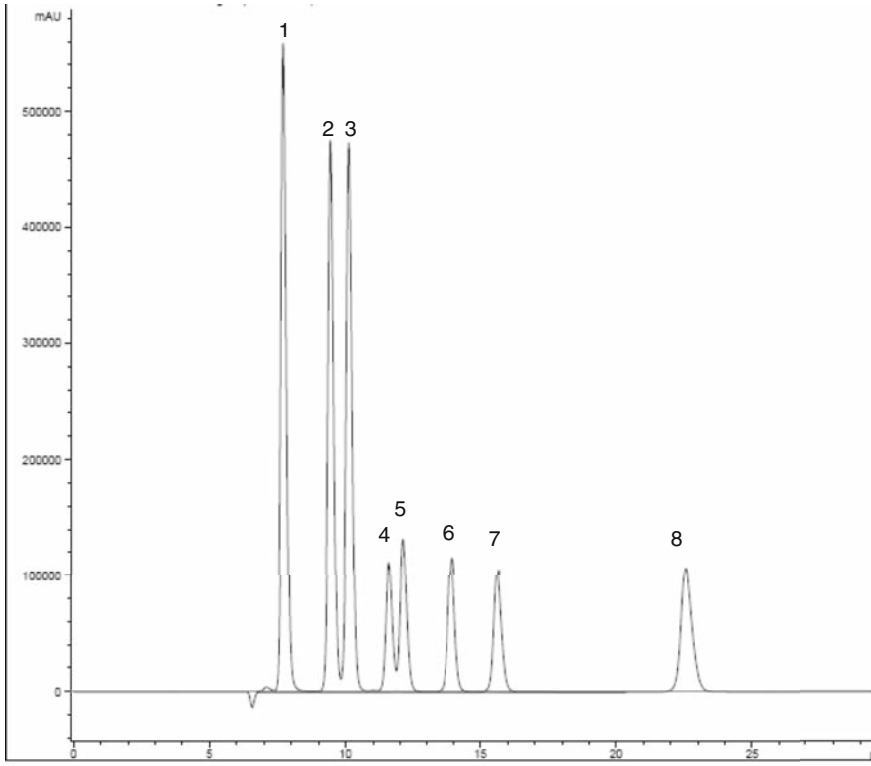

Fig. 22.2. BioRad HPX 87H HPLC chromatogram of standards for cellobiose (1), glucose (2), xylose (3), succinic acid (4), xylitol (5), glycerol (6), acetic acid (7), and ethanol (8). Sugars and organic acids were injected in 20 μL (1.23 μmol cellobiose, 2.40 μmol glucose, 3.20 μmol xylose, 1.71 μmol succinic acid, 0.81 μmol xylitol, 1.76 μmol glycerol, 5.04 μmol acetic acid, and 10.2 μmol ethanol) and chromatography was conducted at 60°C using 0.05 M sulfuric acid as elution buffer at a flow rate of 0.6 mL/min. Peaks were detected using a refractive index detector with 0.05 M sulfuric acid as reference. Although sucrose may be partially hydrolyzed in the elution buffer, if it were present, it would essentially co-elute with cellobiose. (Chromatogram courtesy of Mark Wilkins, Biosystems and Agricultural Engineering Dept., Oklahoma State University.)

detector is sufficient). Calculate the volume contained in the tubing to estimate when sugars will reach specific tubes in the fraction collector.

9. Calculations are usually done using the external standard procedure and utilizing standard sugar responses run with each batch of samples. A new standard set should be run at least daily (if not more frequently) to account for any drift in detector response. From the appropriate standard runs, calculate results for each sugar as

   (Area units of sugar in sample / concentration of sugar in sample) = (area units of sugar in standard/known concentration of sugar in standard)

   Concentration of sugar in sample = ((area units of sugar in sample × known concentration of sugar in standard)/area units of sugar in standard)

Note that units of concentration of sugar in the standard are used for the sample. Account for sample dilution and initial sample weight to calculate the quantity of sugar in the original sample prior to extraction.

The Dionex CarboPac® (Dionex Corp., Sunnyvale, CA) PA series of columns (PA1, PA10, PA100, PA200) are popular for many sugar separation applications, ranging from simple sugars to polysaccharides. The MA1 column is more useful for sugar alcohol and some pentose sugar separations. CarboPac® columns are made from a polymer material similar to Peak® and packings are resin based and are especially well suited for high pH. Most applications do not require elevation of column temperature, flow rates are typically 1.0 mL/min (MA1 is typically 0.6 mL/min), and maximum pressures range from 2,000 to 4,000 psi. Elution buffer contains variable concentrations of sodium hydroxide (*see* **Note 13**) and is thus very alkaline in pH. The high pH causes hydroxyl groups on sugars to be variably de-protonated, which induces a negative charge for the sugar. Reproducibility in degree of charge is regulated by sodium hydroxide ionic strength of the elution buffer, which is more commonly applied during a run of glucose, fructose, and sucrose isocratically (**Figs. 22.3** and **22.4**). Reproducibility in elution time is better accommodated using a gradient of increasing sodium hydroxide concentration to prevent carbonate buildup on the column (*see* **Note 13**). More complex carbohydrate separations (fructans, inulins, etc.) can be accommodated with sodium acetate gradient, usually with sodium hydroxide concentration held constant (**Figs. 22.5** and **22.6**). The mode of separation for the CarboPac® columns is ion exchange. For simple sugars, ionic strength of the elution buffer sodium hydroxide is usually relative low, in the range of 20–100 mM and most separations can be achieved under isocratic conditions. Most gradients of sodium hydroxide do not exceed 200 mM, and most

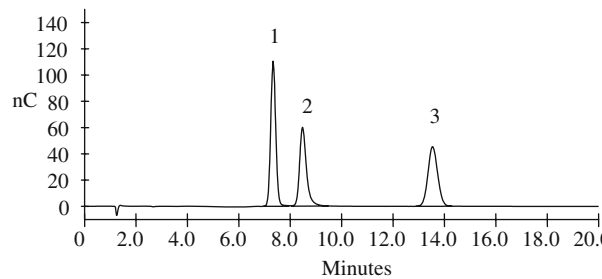

Fig. 22.3. Dionex PA1 HPLC chromatogram of standards for glucose (1), fructose (2), and sucrose (3). Sugars were injected in 50 μL (4 nmol each) and chromatography was conducted at ambient temperature using 20 mM NaOH as isocratic elution buffer at a flow rate of 1 mL/min. Sugars were detected using a pulsed electrochemical detector using the standard potential waveform indicated in **Section 2**.

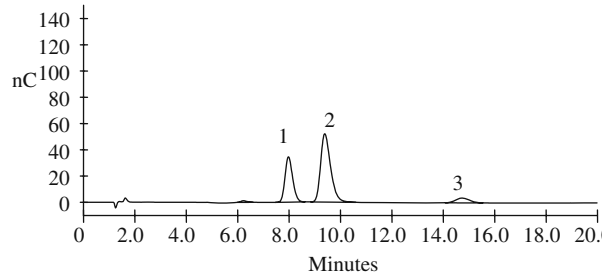

Fig. 22.4. Dionex PA1 HPLC chromatogram of mature "Sugartime" watermelon juice. Watermelon mesocarp tissue was ground with a Polytron blender and centrifuged for 10 min at 3,000 × *g*. Juice (50 μL) was diluted to 100 mL with HPLC water. Run conditions and sugar peak elution patterns were identical to those described for the standards in **Fig. 22.3**. Note that the elution of sugars in this figure is earlier than that in the standard run shown in **Fig. 22.3** due to carbonate buildup on the column.

gradients involving sodium acetate are conducted in 100 mM sodium hydroxide. Samples should be in water prior to injection. Pre-columns should be used, packed with the same resin as the analytical column. Typically, the pre-columns last for up to 600 injections; at times the inlet frit can become clogged but can be easily replaced. Detection is typically by pulsed electrochemical detection (PED), using a gold working electrode. Sugars at high pH are oxidized at the surface of the gold electrode and detected by a difference in potential caused by the oxidation. The oxidized material is cleaned from the gold surface by changing the electrical potential. The sequence of potentials can be changed and optimized for the sugars of interest under set operating conditions and is referred to as the detector "waveform." Probably the most universal CarboPac® column is the PA1, and the following procedure will include its use.

1. Appropriately dilute extract with water or reconstitute a dried sample in degassed water.
2. Remove particulates by filtration through a 0.45-μm filter or by centrifugation. If extract has excessive quantities of phenolics of other dissolved impurities, preprocess by flowing through a C18 Sep-Pak (or equivalent) syringe cartridge.
3. Place sample into an autosampler or use a manual injector with a 20-μL injection loop.
4. Overfill the injection loop by applying at least 50 μL of sample prior to injection onto a CarboPac® PA1 column (4 mm × 250 mm; Dionex Corp., Sunnyvale, CA) at ambient temperature.
5. Inject the sample onto the column with 20 mM sodium hydroxide as eluent for glucose/fructose/sucrose separations or a gradient from 40 mM sodium hydroxide to 500 mM sodium acetate in 100 mM sodium hydroxide for

fructan separations, at a flow rate of 1.0 mL/min, using pulsed electrochemical detection. For most applications, the monosaccharide/disaccharide recommended waveform is sufficient for simple sugar detection. Smaller (less charge dense) sugars elute first with the PA1 column in the order of glucose, fructose, lactose, sucrose, and higher degree of polymerization (DP) sugars (**Figs. 22.3**, **22.4**, **22.5**, and **22.6**; melezitose may be used as an internal standard for plant fructan extracts).

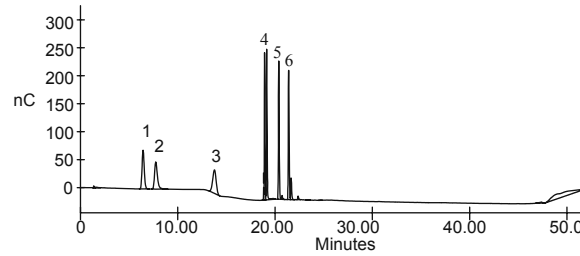

Fig. 22.5. Dionex PA1 HPLC chromatogram of standards for glucose (1), fructose (2), sucrose (3), raffinose, and 1-kestose (4) [degree of polymerization (DP) = 3; raffinose is the first twin peak and 1-kestose is the second twin peak in peak 4], nystose (5) (DP = 4), and 1-fructofranosylnystose (6) (DP = 5). Sugars were injected in 50 μL (4 nmol each for glucose, fructose, and sucrose; 12 nmol each for raffinose, 1-kestose, nystose, and 1-fructofranosylnystose) and chromatography was conducted at ambient temperature using a gradient as follows: 40 mM NaOH from 0 to 10 min, 40–100 mM NaOH from 10 to 11 min, 100 mM NaOH to 100 mM NaOH plus 500 mM sodium acetate from 11.5 to 41.5 min, 100 mM NaOH plus 500 mM sodium acetate to 100 mM NaOH from 45 to 46 min, 100 mM NaOH back to 40 mM NaOH from 46.5 to 47 min. Flow rate was 1 mL/min. Sugars were detected using a pulsed electrochemical detector using the standard carbohydrate potential waveform indicated in **Section 2**.

6. Run standards before and after every 12–15 sample injections for isocratic glucose, fructose, and sucrose separations. Following the last standard run, remove any carbonate buildup from the column with a sodium hydroxide gradient as follows (*see* **Note 13**):
   a) Increase sodium hydroxide concentration from 20 to 500 mM in 5 min.
   b) Hold for 5 min.
   c) Decrease sodium hydroxide concentration from 500 to 20 mM in 2 min.
   d) Hold for at least 5 min prior to starting the next series of sugar injections.
7. Note pressure periodically and change guard column when pressure starts to climb from 500 to 800 psi above nominal operating pressure. Oftentimes the guard column inlet frit becomes clogged and its replacement can bring pressure back to nominal operating conditions. Guard column

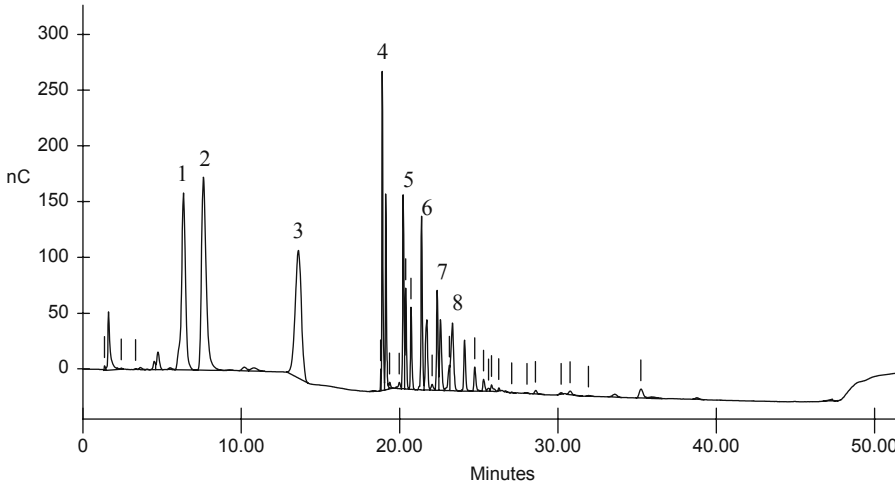

Fig. 22.6. Dionex PA1 HPLC chromatogram of fructans extracted from a short-day onion. Lyophilized onion tissue was homogenized with 70% ethanol plus 0.05% (w/v) sodium carbonate and extracted with boiling. A 1 mL aliquot was dried and reconstituted with 100 μL water and chromatography was conducted at ambient temperature at a flow rate of 1 mL/min. Glucose (1), fructose (2), sucrose (3), melezitose and raffinose (4) (first large twin peak is melezitose, added at 6.2 nmol as internal standard, and second smaller twin peak is raffinose), degree of polymerization (DP) 4 peaks (5), DP 5 peaks (6), DP 6 peaks (7), and DP 7 peaks (8). Other peaks in the series were of sequentially increasing DP. Chromatographic and gradient elution conditions were identical to those indicated in **Fig. 22.5**. Sugars were detected using a pulsed electrochemical detector using the standard carbohydrate potential waveform indicated in **Section 2**.

should last for at least 600 injections. If the inlet frit for the guard column must be replaced substantially more often, filter samples or centrifuge at a higher speed/longer duration to remove particulates. If degradation in performance beyond nominal operation is noted too frequently, it means that the packing has been prematurely fouled by sample contaminants which should be removed prior to injection with an appropriate Sep-Pak or equivalent syringe cartridge.

8. Certain samples may be very high in one sugar (sucrose, for example) and low in other sugars (glucose and/or fructose, for example). If quantification of all sugars is of interest, run the sample in different dilutions in order to prevent exceeding the linear range of quantification for each sugar.
9. Calculations are similar to those presented for the Aminex® columns. Even if internal standard procedure (calculated as outlined for GC sugars) is used, run a new set of standards often to account for changes in performance during the course of that sugar series. This is especially important if excessive column carbonate buildup occurs since peak retention time and resolution may be impacted enough to shift standard peak response (compare **Fig. 22.3** with **Fig. 22.4**).

4. Notes

1. Never place sample directly onto ice since water from ice can cause excessive sample contamination. Instead, place samples into a clean labeled water-resistant, non-insulating container (freezer bag or ampoule with a sealable lid), seal it, and then place into ice. Layer the container with ice to assure most rapid cooling.
2. Glass capillary tubes can be heated by the flame from a Bunsen burner or a soldering torch, pulled, and the glass broken to a fine point to allow juice collection from a very small area or used to puncture into and collect juice from a liquid-containing tissue zone (3). The fine point also accommodates rapid juice flow into the capillary tube by capillary action.
3. Preservation of samples by heat killing is acceptable for simple sugar (glucose, fructose, sucrose, etc.) and starch stabilization but high temperatures may be detrimental to heat-labile sample components such as enzymes, antioxidants, pigments, and low molecular weight volatile compounds. If co-determination of these compounds is anticipated, preservation by freezing is recommended. If preservation with heat is acceptable, cut bulky or high water-containing tissues (stems, fruits, woody tissues) into smaller pieces to accommodate rapid heat transfer and enzyme inactivation in the tissue.
4. Unless stated otherwise, water used for extraction or mixing of reagents has a resistivity of 18.2 MΩ cm and total organic content of less than five parts per billion. If water is used for extraction, inclusion of 0.05% (w/v) of sodium azide will help prevent microbial contamination. If water is used as HPLC elution solvent, degas with vacuum or by extensive sparging with chromatographic grade helium.
5. Non-denatured ethanol should be used for sugar extraction. For glucose, fructose, and sucrose determination, extraction with 95% (v/v) boiling (solution held at above 78°C; 80–85°C is typical) ethanol prevents solubilization of starch and many other oligomeric or polymeric sugars and allows separate extraction of soluble sugars from starch. For extraction of fructans and other oligomeric sugars, reducing ethanol concentration to 70% (v/v) may be necessary to accommodate quantitative extraction. Use of the highest concentration of ethanol deemed appropriate for extraction of sugars of interest is advisable to denature enzymes which could otherwise metabolize the sugars

and alter their concentration. Higher ethanol concentration also inhibits microbial growth and thus prevents sugar use by contaminating microorganisms.

6. The size of the round inlet for a cyclone mill is typically less than 2–3 cm in diameter, and a spring-loaded rubber ball may be depressed over the inlet to halt air flow through the mill during sample bottle detachment and reattachment. Clear sample bottles are typically supplied with the mill; brown screw top glass bottles (120 mL) fit the mill and can be used in place of the clear bottles. Brown glass has an added advantage of discouraging microbial growth. Since bottles are typically stored in a freezer to await sample extraction, tape labels should slightly overlap to avoid label loss caused by water condensation and loss of adhesion to the glass surface during re-warming.
7. Oils can interfere with sugar extraction and cause incomplete extraction as well as a non-volatile oily residue which can interfere with subsequent sugar analysis. Since oils and many other lipids are essentially universally soluble in diethyl ether, and sugars and other hydrophilic substances are essentially universally insoluble in diethyl ether, oil content can be determined and the extraction residue can then be used for quantitative sugar extraction. In our experience a sample can be pre-extracted with diethyl ether [sample:extractant ratio of 1:10 (w/v)] four consecutive times, followed by extraction with boiling 95% (v/v) ethanol [sample:extractant ratio of 1:10 (w/v)] four consecutive times to yield >98% recoveries of both oil and sugar.
8. Reflux tubes should extend prior to tapering about 10–12 cm past the top of the tube stopper or vial closure to allow enough distance for vapor cooling and recondensation. The bottom of the tube should extend about 2 cm into the extraction vessel to allow dripping of fresh ethanol into the extraction system without contacting either the bottom of the closure or the sides of the extraction vessel. A tapered end provides resistance to free vapor exchange into the atmosphere and thus encourages condensation upon cooling. The tapered end should be otherwise open to the atmosphere to allow pressure release and prevent pressurization of the vessel during extraction. Pasteur pipettes are typically about the right length to allow cooling/re-condensation of ethanol vapors if used with a 2 dram vial. These pipettes are insufficient in length to fit through a standard rubber stopper into a 50-mL centrifuge tube and are too fragile to be pushed through the hole in the stopper. Thicker glass tubing of about the same outer dimension as a Pasteur pipette can be used if tapered at a

sufficient length to accommodate the added length of the single-hole stopper.

9. If immediate processing for starch determination is not conducted, overlay the top of the pellet with 95% ethanol, seal, and place samples at 4°C. This step helps prevent microbial contamination; storage at 4°C also reduces evaporation of the ethanol. Just prior to starch processing, warm samples at 80°C to allow ethanol to vaporize.
10. Prior to drying, record the total extract volume and the aliquot volume used for drying. Sugars are most stable for storage in a dry form but should be stored in subdued light in a freezer. Certain analytical protocols [high-performance anion exchange pulsed electrochemical detection (HPAE-PED), for example] are not compatible with ethanol and ethanol must be evaporated to accommodate the analytical protocol. During re-hydration with water, samples may be warmed to 60°C and vortexed to assure total solubilization prior to quantitative transfer into a volumetric flask.
11. The Nelson–Somogyi reducing sugar assay, and most of the derivations thereof, reacts with the sugar-reducing end and results in a change in absorbance or transmittance of the assay solution. The chosen standard should be representative of sugars to be assayed and standard curves should be checked for linearity within the range of concentrations to be assayed. This type of assay does not accurately quantitate most dimeric (sucrose, maltose), oligomeric (fructans, small starch or cell wall fragments), or polymeric (starch, cell walls, etc.) sugars. These sugars must be degraded to their monomeric forms (enzymatic hydrolysis results in definable end products; acid hydrolysis is non-specific, may degrade the sugars, and some sugar linkages may be more resistant to hydrolysis than others) and then the reducing sugar assay redone. To convert the results from monomeric sugars back to a weight of oligo- or polymeric sugars, the weight of water added when the sugar was hydrolyzed to its monomeric form must be subtracted. For starch, multiply the weight of glucose by 0.90 to account for its dehydrated form in the polymeric molecule.
12. Enzymatic sugar assays depend on completion of reaction with specific sugars, which can be checked with an assay for linearity within the range of sugar concentrations to be analyzed. Since some procedures allow determination of multiple sugars in the same test solution (such as glucose/fructose/sucrose test combinations), use of disposable cuvettes allows for multiple readings of the same solution and saves time. Check that the cuvette is

recommended for use at the wavelength of your assay and be sure during placement of the cuvette into the spectrophotometer that it is aligned properly within the light path. Plastic ordure swords fit nicely into most square cuvettes which can be used for mixing the reaction solution after component additions.

13. Dissolved carbonates in the mobile phase will bind to the chromatographic column and cause a loss of chromatographic resolution and efficiency. Water used for the mobile phase and for sample dilution prior to injection should be sparged exhaustively with chromatographic grade helium to displace dissolved carbon dioxide and should be stored under a blanket of helium to avoid any additional exposure to carbon dioxide. Sodium hydroxide solutions for the mobile phase should be prepared using carbonate-free water from commercially available 50% sodium hydroxide (w/w) solutions rather than from sodium hydroxide pellets since the pellets are commonly covered with a layer of sodium carbonate which would serve as a source of contamination. Depending on how often the 50% sodium hydroxide solution is opened to the atmosphere, replace it at regular intervals (monthly under moderate use) to avoid introduction of substantial quantities of carbonate into the mobile phase.
14. Direct injection of samples with high ethanol concentration causes a large and tailing peak at the beginning of the run which may mask sugar alcohol and certain monosaccharide response. Ethanol can be dried relatively quickly using vacuum-assisted drying or under a stream of nitrogen gas. Reconstitute the sample just prior to dilution and HPLC injection in carbonate-free water, prepared as directed in **Note 13**.
15. During incubation at 55°C, gas pressure inside the centrifuge tube may build up to a sufficient level to pop the rubber stopper off the tube. Securing the stoppers with a rubber band around a single tube or a wooden stick across several tubes will prevent stopper loss during incubation.
16. Colorimetric procedures such as Nelson–Somogyi or anthrone give varying results depending on the sugar being analyzed and absorbance values can change from run to run. For preparation of a standard curve, use the reducing sugar most prevalent in the samples to be analyzed and prepare standards in the same buffer as used for samples. Run a new standard curve with each set of samples. If the Nelson–Somogyi assay is used to quantitate enzyme activity, it is common to terminate the reaction by addition of

the copper working solution immediately followed by incubation at 100 °C. If the anthrone procedure is used for starch determination, use either glucose (multiply results by 0.9 to account for the dehydrated form of glucose in the starch molecule) or starch as standard (no correction needed).

17. Since the anthrone reagent contains a very high concentration of sulfuric acid, it is quite viscous and may be difficult to pipette. Exercise care when adding high acid concentrations to water. The solution will become very hot and may react violently upon vortexing.
18. Redistilled water can be prepared by distilling house reverse osmosis or distilled water. Distilling particularly removes any trace metal contamination which can interfere with enzyme sugar assays. Collect and store redistilled water in glass or sturdy plastic containers and avoid conveyance or storage in metallic pipes or containers.
19. While the Boehringer–Mannheim procedure published by Rhone-Biopharm suggests addition of sample and buffer prior to redistilled water addition, we have found that for certain samples, absorbance values deviated from linearity and gave elevated sugar results unless water was added first to dilute buffer prior to sample addition.
20. Drying solvents under nitrogen gas should be conducted inside an appropriate fume hood to prevent organic vapors from entering the laboratory air.
21. During methanolysis, methanol, catalyzed by HCl, is added across glycosidic bonds and reducing ends of free sugars to form methyl glycosides. Methyl acetate is added to scavenge any water formed during the reaction from the solution to prevent hydrolysis of the methyl glycosides.
22. As the vials heat up, the screw caps will sometimes loosen and allow methanol to evaporate. This would cause HCl to increase in concentration and damage the sugars as well as remove methanol needed for the formation of methyl glycosides. Retightening of the screw caps helps to prevent evaporation of the methanolysis solution; use of heat-resistant gloves protects hands from burns and affords some protection in case vials break during tightening. If vials have dried during incubation, discard the sample.
23. *t*-Butanol should be stored in a warm place to ensure that it remains liquid. It is added to co-evaporate with HCl, helping to remove HCl during evaporation without degrading the sugars.

24. Absolute recovery of sugars in TMS results cannot always be assured – to verify sugar recovery and correct for any sugar losses, either include a monomeric sugar during extraction which is not present in the sample and correct for recovery or conduct a recovery trial by spiking a sample prior to extraction with approximately 25% (w/w) of the sugar expected in the sample and compare analytical results to unspiked samples as follows:
((Sugar concentration in spiked sample – sugar concentration in native sample)/concentration of sugar added as spike) × 100 = percentage recovery.
25. Column re-equilibration for subsequent injections is substantially less if samples are injected in water versus 95% (v/v) ethanol. For determination of glucose, fructose, and sucrose, system re-equilibration time was 17 min for extracts in water versus 40 min for extracts in 95% (v/v) ethanol (6).
26. Baseline stability is optimized when the reference cell liquid is changed on a regular basis. Usually changing the reference solution every 3 days to each week is sufficient; if excessive baseline drift is noted, change the reference solution more often with well-equilibrated column effluent.

Acknowledgments

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