CHAPTER 12

DETERMINATION OF SOLUBLE CARBOHYDRATES

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1. INTRODUCTION

Leaf litter and other plant detritus consist primarily of structural polysaccharides and lignin, neither of which are readily accessible to stream invertebrates (Chapters 11, 12 and 17). However, more easily digestible soluble carbohydrates, such as sucrose or glucose are also present in notable concentrations. Initially, these compounds may account for up to 16% of total dry mass (e.g. in hickory leaves; Suberkropp et al. 1976), while leaching can reduce this value by ≥80% within a few days (Gessner 1991). The rate of leaching may be significantly influenced by treatment of the leaves before immersion in the stream (Chapter 5; Gessner 1991, Bärlocher 1997).

During decomposition, microbial enzymes attack detrital polymers, releasing a mixture of oligomeric and monomeric carbohydrates, which again are more accessible to invertebrates than the original polymers (Bärlocher & Porter 1986). In addition, fungi colonizing leaves (which can account for up to 17% of detrital dry mass at intermediate stages of decay; Gessner 1997) contain soluble carbohydrates in their mycelia.

Analysis of total soluble carbohydrates facilitates the quantification of nutritionally valuable carbon fractions of leaf material. Identification of the individual compounds, combined with analyses of hydrolyzed polysaccharides allows characterization of the course of enzymatic breakdown of these leaf constituents. The same methods can also be modified to measure activities of selected degradative enzymes present in microorganisms or invertebrates (Chapter 32).

Two approaches can be taken to analyze soluble carbohydrates. One involves determining total available carbohydrates (Method A) and ignores their composition.

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The procedure we present follows a modified method of White & Kennedy (1986). The second approach (Method B) is more specific. It quantifies individual monosaccharides, which can then be used to calculate the total amount of soluble carbohydrates present in a sample (Mansfield et al. 1997). Both methods require that the soluble sugars first be extracted from the lignocellulosic material; the procedure described below follows a modified protocol of Guy et al. (1984).

2. EQUIPMENT, CHEMICAL AND SOLUTIONS

2.1. Equipment and Material

- Freeze-drier
- Analytical balance
- Spectrophotometer
- Rotavap evaporator
- Desiccators (containing phosphorus pentoxide)
- Mortar and pestle
- Test tubes
- Acid-washed glass test tubes (10 ml; wash with 10% nitric acid overnight, then rinse thoroughly with distilled water)
- Test tube rack
- Hot water bath or heated test tube reactor
- Thermometer
- Ice water bath
- Vortex
- Freezer (–20 °C)
- Micropipettors
- Cuvettes (disposable ones are suitable)
- Spectrophotometer (set at 540 nm)
- Laboratory timer or stop watch
- Separatory funnel
- Aluminium foil
- High Performance Liquid Chromatograph (HPLC) with electrochemical detector using pulsed amperometry
- HPLC filters (0.45 µm pore size)
- HPLC vials and caps
- Disposable syringes

2.2. Chemicals

- Glucose
- Sucrose
- Fructose

- Liquid nitrogen
- Methanol:chloroform:water (12:5:3; v:v:v) solution
- Distilled water (degassed)
- 1 M NaOH solution (degassed)
- Internal standard: Fucose (10 mg ml^{-1})
- 10% (w:w) nitric acid
- Sugar standard: Glucose in water or appropriate buffer (e.g. 50 mM sodium phosphate or sodium acetate) with a concentration ranging from 10 to 120 µg ml^{-1}
- Freshly prepared 0.2% orcinol reagent (2 g l^{-1} orcinol dissolved in concentrated sulphuric acid); this reagent can be stored for up to one week at 4 °C

3. EXPERIMENTAL PROCEDURES

3.1. Sample Preparation

1. Wash lignocellulosic material (e.g. a leaf sample) thoroughly with distilled water.
2. Freeze-dry lignocellulosic material overnight.
3. Using a mortar and pestle grind freeze-dried material in liquid nitrogen into a powder.
4. Store ground material in sample vials in a desiccator (with phosphorus pentoxide) until extraction.
5. Weigh out 50 mg of pulverized sample into a test tube, and record weight to nearest 0.01 mg. This should be done in at least duplicate for each sample.
6. To each test tube with sample add 50 µl of fucose internal standard.
7. Add 5 ml of methanol:chloroform:water (12:5:3) solution to each test tube, mix, cover with aluminium foil, and place in a freezer for at least 12 h.
8. Remove samples from freezer and mix well with a vortex.
9. Centrifuge samples (5000 *g*) for 10 min at 4 °C, then pipet supernatant into separatory funnel.
10. Add 4 ml of methanol:chloroform:water (12:5:3) solution to the pellet, vortex and centrifuge for 10 min. Remove supernatant and pool with sample in separatory funnel. Repeat a second time, and pool.
11. Add 5 ml of distilled water to the separatory funnel, cap, and mix thoroughly.
12. Allow for phase separation (this could take up to 2 h).
13. Discard lower layer, and dispense aqueous top layer to evaporating flask.
14. Evaporate all solvent from flask under vacuum in a rotary evaporator at 40 °C (ensure that the water bath does not exceed 40 °C).
15. Re-suspend dried sample in 1 ml of distilled water.
16. Remove sample from evaporating flask with a disposable syringe.
17. When using the HPLC method for quantification, pass sample through 0.45 µm filter into HPLC vial and cap.

3.2. Method A – Total Soluble Carbohydrate Analysis

1. Add 0.5 ml each of the carbohydrate standard, water (blank), and/or sample to separate 10-ml test tubes with a pipettor.
2. Cover each tube with aluminium foil.
3. Immerse test tubes in ice bath (~4 ºC) for 15 min.
4. Add 2 ml of orcinol reagent to the test tubes (start timer with first sample and proceed with each subsequent sample at 1 min intervals).
5. Vortex reaction mixtures vigorously and incubate in an 80 ºC water bath for exactly 15 min.
6. Terminate the reaction by rapid cooling in an ice bath for 5 min.
7. Equilibrate tubes to room temperature.
8. Measure absorbance of the reaction mixture with a spectrophotometer at 540 nm (the water blank can be used for zeroing the spectrophotometer).
9. Determine total carbohydrates in the samples by reference to an appropriate standard curve generated from a standard solution (i.e. glucose).

3.3. Method B – Soluble Carbohydrate Determination by High Performance Liquid Chromatography (HPLC)

1. Set HPLC to the conditions indicated in Table 12.1.
2. Prepare sugar standards (glucose, fructose, sucrose etc.) at concentrations ranging from 0.1—2 mg ml^{-1}. A range of sugars, including sugar alcohols, can be used as standards, depending on the lignocellulosic material being analyzed or specific carbohydrates of interest.
3. Run standards and samples on HPLC to obtain chromatogram as in Fig. 12.1.
4. Normalize peaks for internal standards.
5. Prepare standard curves by plotting areas under the peaks versus concentrations for each sugar in the standards mixture.
6. Calculate concentration of each monomeric sugar in sample dry mass. The sum of all individual monomers gives the total soluble carbohydrate concentration.

Table 12.1. HPLC conditions for soluble carbohydrate determination

Parameter	*Condition*
Mobile phase	200 mM NaOH for 52 min; gradient from 200—420 mM NaOH from 58—80 min; 420—180 mM from 80—84 min; 180 mM from 84—100 min
Flow rate	0.4 ml min^{-1}
Column	Dionex MA-1
Column temperature	Ambient room temperature
Detection	Pulsed amperometry (using gold electrode)
Injection volume	20 µl

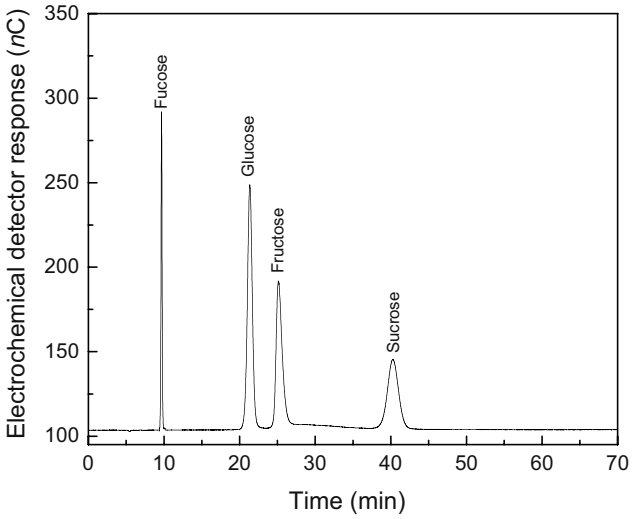

Figure 12.1. Chromatogram of soluble carbohydrate standards obtained with a Dionex HPLC system equipped with an electrochemical detector (pulsed amperometry) and the conditions described for Method B.

4. FINAL REMARKS

Samples analyzed by either method may require dilution if the unknown samples give absorbance or detection readings greater than the highest value obtained while generating the standard curve. Should this occur, dilute samples, record dilution volume, and repeat the analysis.

As a spectrophotometric assay, Method A is subject to interference from particles or air bubbles in sample or reaction solutions. Different sugars give a different quantitative response (e.g. glucose ≠ xylose). Therefore, choice of the standards will depend on the carbohydrate composition of the sample. Sensitivity of Method A is approximately 5—10 μg ml^{-1} carbohydrate (1 ml sample required); that of Method B ≥1 μg ml^{-1} carbohydrate.

Orcinol is a harmful substance and special care needs to be taken when handling it, especially when it is made up as a solution in concentrated sulphuric acid. As such, laboratory coats, protective eyewear and gloves are required.

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