DATE: MAY 2007

Total Starch in Forages and Grains

1. Application

This procedure covers the determination of starch in biomass samples. The percent starch content is used in conjunction with other assays to determine the total composition of biomass samples.

2. Summary of Methods

3. Safety

All chemicals should be considered a potential health hazard. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material handling data sheets should be made available to all personnel involved in the chemical analysis.

4. Interferences

This procedure is suitable for air-dried biomass samples, as well as for samples that have been oven dried at a temperature of 45°C or less. The assay results will be biased slightly low for samples dried at 105°C. If sample availability is limited, it may be necessary to run this analysis on a 105°C dried sample but the results must be flagged as being biased low. The assay is also suitable for wet samples if the particle size is known to be small and if the moisture content of the sample can be estimated accurately enough to predict the amount of sample needed to give 0.5 g of solids. Interferences by free glucose and cellobiose present in samples are not a problem because both glucose and cellobiose are destroyed during the NaOH solubilization step.

5. Sample Collection, Preservation, and Handling

All samples are dried at 55°C in a cabinet-type forced air dryer for 12-18 hrs. After drying the sample is ground to pass through a 1 mm forage mill. A sub-sample is then dried at 105°C for 3 hours to determine laboratory dry matter content.

6. Apparatus and Materials

6.1 Analytical balance, accurate to 0.1 mg.
6.2 YSI 2700 Select Biochemistry Analyzer – equipped with a YSI 2365 dextrose membrane and YSI 2357 buffer and calibrated with YSI 2776 2.5 g/L calibrator solution.
6.3 Hot plate or water bath set at 90°C $\pm$ 2°C.

6.4 Graduate cylinders of appropriate sizes.
6.5 100 ml, 500 ml, and 1000 ml volumetric flasks, class A.
6.6 5 and 10 ml pipets or adjustable pipettor.
6.7 Timer.
6.8 Water bath set at $40^{o}C \pm 1^{o}C$.
6.9 Erlenmeyer flasks, 125 ml.
6.10 Test tubes, 10 ml.

7. Reagents

7.1 Glucose calibration verification standards, such as YSI 2.0 and 9.0 mg/ml glucose standards.
7.2 Amyloglucosidase (suggested source, Sigma A-3042).
7.3 Corn Starch (Spectrum S1552)
7.4 Methanol, ACS reagent grade.
7.5 2N NaOH
  7.5.1 Weigh 40 g of sodium hydroxide pellets into a 500 ml volumetric flask.
  7.5.2 Add 300 ml of reagent grade water and mix.
  7.5.3 Cool, dilute to volume and mix.
7.6 2N HCl
  7.6.1 Measure 82.4 ml of concentrated hydrochloric acid and transfer to a 500 ml volumetric flask.
  7.6.2 Let cool, dilute to volume with reagent grade distilled water and mix.
7.7 Acetate buffer (pH 4.2)
  7.7.1 Weigh 9.1 g of sodium acetate into 500 ml volumetric flask.
  7.7.2 Add about 300 ml of reagent grade distilled water and mix until all solid is dissolved.
  7.7.3 Add 22.3 ml (23.4 g) of glacial acetic acid.
  7.7.4 Dilute to volume with distilled water and mix.
7.8 Amyloglucosidase working solution
  7.8.1 Prepare a fresh working solution of the enzyme such that it contains 60 units of activity per milliliter.
  7.8.2 If using the Sigma A-3042 amyloglucosidase, dilute the solution one hundred-fold into cold reagent grade water. Prepare daily and store in the refrigerator.
7.9 25% TCA – dissolve 50.0 g trichloracetic acid in 200 ml reagent grade water.
7.10 Phosphate buffer
  7.10.1 Dissolve 40 g NaH_2PO_4 and 10 g Na_2HPO_4 in reagent grade water
  7.10.2 Bring to volume in a 1000 ml volumetric flask

8. Methods

8.1 Determine the total dry matter content of each sample. Record the total dry matter value as T_{final}.
8.2 Weigh out approximately 0.500 g of sample to the nearest 0.0001 g and transfer to a 125 ml Erlenmeyer flask. Record as $W_{sample,}$ the initial sample weight.

8.3 Weigh a 0.500 g portion of pure corn starch to the nearest 0.0001 g and transfer to an Erlenmeyer flask. Record the weight as $W_{standard}$, the initial standard reference material weight. As with the unknown samples, the total dry matter content, T_{final}, of the standard reference material must also be determined.

8.4 Add 25 ml of reagent grade water to each flask. Swirl to ensure the sample is wetted and evenly dispersed. Note: A few drops of methanol may be used to pre-wet the sample which will aid in its dispersion once the water is added.

8.5 Add 10 ml of 2N NaOH to the solution in each flask. Place flasks on a heating unit or in a water bath preheated to 90°C. Heat for 20 minutes, swirling periodically to wet any sample that may be clinging to the side of the flask. A glass stirring rod may be needed to break up clumps of material.

8.6 Add 10 ml of 2N HCl to each flask and swirl to mix. Cool the flasks to below 50°C, which takes about 20 minutes.

8.7 Add 10 ml of acetate buffer to each flask and swirl to mix.

8.8 Add 5.0 ml amyloglucosidase working solution to each flask. Mix well and place the flasks in a 40°C water bath for 60 minutes.

8.9 After 60 minutes incubation, remove the flasks from the water bath. Immediately add 5 ml of 25% TCA to each flask to stop hydrolysis.

8.10 Pipette 6.5 ml of hydrolyzate into a 10 ml test tube. Add 3.5 ml phosphate buffer to each tube.

8.11 Since the enzyme solution may contain free glucose, an enzyme blank must be run in parallel with the samples. Dilute duplicate 5.0 ml portions of the amyloglucosidase working solution to 100 ml with reagent grade water in a volumetric flask. These enzyme blanks will be analyzed in the same manner as the sample, with the averaged results used to correct the glucose contents of the samples.

8.12 The sample itself may contain free glucose, which normally would be analyzed as starch. However in this procedure the glucose, and also cellobiose, is destroyed in the NaOH solubilization step. Therefore no correction for free glucose is needed when calculated the total starch content of a sample.

8.13 Set up and calibrate the YSI as described in the manufacturer's manual using the dextrose membrane, YSI 2357 system buffer, and YSI 2776 2.5 g/L calibrator solution. Program the instrument to auto calibrate every fourth sample or every fifteen minutes, set the sample size to 25µL, and use the following probe parameters:

- 8.13.1 Chemistry – dextrose
- 8.13.2 Units – g/L
- 8.13.3 Calibrator – 2.50 g/L
- 8.13.4 End point – 30 seconds
- 8.13.5 Cal Station # - 1

8.14 Verify the calibration of the YSI using the glucose calibration verification standards before starting the run. Re-verify the calibration periodically during the analysis and at the end of the run.

8.15 Measure the glucose levels in the enzyme blanks and in all the samples. The validated linear range of the instrument is 0 – 9.0 g/L dextrose. If the value

reported exceeds the validated range, the hydrolyzate must be diluted appropriately and re-run.

9. Calculations

9.1 Calculate the amount of starch recovered from each analysis of the amylopectin standard reference material as follows (on a 105°C dry weight basis) and then average the recoveries:

9.1.1 % Standard recovered = [{($YSI_{standard}$, g/L – $YSI_{enzyme\ blank,}$ g/L) * total volume, L} / {standard weight, g, $W_{standard}$ * (% total solids, T_{final} / 100)}] * 0.9 * 100%

9.1.2 Note: Amylopectin recoveries of 93 to 95% have routinely been achieved with this protocol. Recoveries less than 90% indicate the data generated for the batch of samples should be rejected and the analysis repeated.

9.2 Calculate the amount of starch present in each sample, on a 105°C dry weight basis:

9.2.1 % Starch = [{(YSI_{sample}, g/L – $YSI_{subenzyme\ blank,}$ g/L) * total volume, L} / {standard weight, g, $W_{standard}$ * (% total dry matter, T_{final} / 100)}] * 0.9 * 100%

9.2.2 Note: The factor 0.9 converts grams of glucose to grams of the anhydrosuger (starch, in this case). The factor can be calculated by dividing the molecular weight of glucose less one molecule of water (180-18) by the molecular weight of glucose.

9.3 The calculated percent starch in each sample can be correct for assay losses using the percent recovery of the standard reference material, amylopectin, as follows:

9.3.1 % Starch, corrected = (% starch / average % standard recovered) * 100%

10. Quality Control

A standard reference material, corn starch, is run in parallel with each batch of samples.

11. Reporting

Report the percent starch present in the sample, to two decimal places, on a 105°C dry weight basis. If duplicate samples are run, report the average.

12. References

12.1 NREL Ethanol Project Laboratory Analytical Procedure #001, "Standard Method for the Determination of Total Solids in Biomass."

12.2 Solvay Enzymes. 1996. "Fungal Glucoamylase for Starch Hydrolysis." Diazyme L-200 Technical Notes.

12.3 YSI Incorporated. 12994. "Determination of Cook in Extruded Cereal Products." Application Note #319.