

# Determination of Starch in Solid Biomass Samples by HPLC

## Laboratory Analytical Procedure (LAP)

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*Technical Report*

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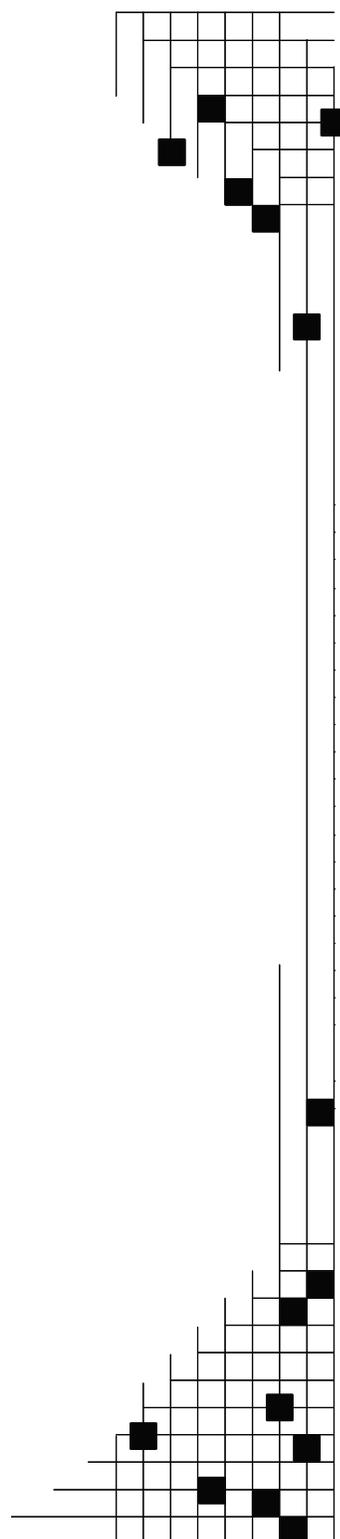
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### National Renewable Energy Laboratory

1617 Cole Boulevard, Golden, Colorado 80401-3393  
303-275-3000 • [www.nrel.gov](http://www.nrel.gov)

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# **Procedure Title: Determination of Starch in Solid Biomass Samples by HPLC**

## **Laboratory Analytical Procedure**

### **1. Introduction**

- 1.1 Carbohydrates make up a major portion of biomass samples. These carbohydrates are polysaccharides constructed primarily of glucose, xylose, arabinose, galactose, and mannose monomeric subunits. One such polysaccharide is starch, which is present in certain types of biomass. Starch will contribute to the overall glucose measurement when it breaks into the component sugar glucose. Starch is often a more readily accessible source of glucose than structurally bound glucose polymers. Due to the increased accessibility, it is often advantageous to determine the amount of starch present.
- 1.2 The amount of starch present in a solids biomass sample is determined by this procedure. This procedure is based on the Megazyme Total Starch Assay Procedure (amyloglucosidase /  $\alpha$ -amylase method). The Megazyme procedure is also listed as AOAC Method 996.11, AACC Method 76.13, and ICC Standard method No. 168.

### **2. Scope**

- 2.1 This procedure is used to measure the starch content in solid biomass samples.
- 2.2 This procedure is appropriate for solid biomass samples containing starch.
- 2.3 All analyses should be performed in accordance with an appropriate laboratory specific Quality Assurance Plan (QAP).

### **3. Terminology**

- 3.1 None

### **4. Significance and Use**

- 4.1 This procedure is used to determine the starch content of solid biomass samples. Other optional procedures can be used in conjunction with this procedure, to determine whole biomass composition.
- 4.2 This procedure is used, in conjunction with other procedures, to determine the chemical composition of biomass samples, see LAP "Summative Mass Closure for Biomass Samples". (Note: The amount of glucan determined in the sample will include a contribution of glucose from starch. The starch measurement should not be included as a separate category unless the starch contribution is first removed from the glucan value.)

### **5. Interferences**

- 5.1 Samples containing galacturonic acid, which elutes at the same time as glucose on the Aminex HPX-87H column, may have artificially high glucose peaks.

### **6. Apparatus**

- 6.1 Analytical balance, accurate to 0.1mg
- 6.2 Automatic moisture analyzer or 105°C drying oven

- 6.3 pH meter
- 6.4 Water bath, capable of maintaining boiling water and 50°C.
- 6.5 Vortex mixer
- 6.6 Centrifuge, capable of 3,000 rpm, with 15 ml centrifuge vial slots
- 6.7 HPLC system equipped with refractive index detector and the following columns:
  - 6.7.1 Biorad Aminex HPX-87H column (or equivalent) with corresponding guard column

## 7. Reagents and materials

### 7.1 Reagents

- 7.1.1 High purity D-(+)glucose standard
- 7.1.2 Second sample of high purity D-(+)glucose standard, as listed above, from a different source (manufacturer or lot), to be used to prepare calibration verification standards (CVS)
- 7.1.3 190 proof ethanol, reagent grade
- 7.1.4 Dimethyl sulfoxide (DMSO), reagent grade
- 7.1.5 Thermostable  $\alpha$ -amylase, available from Megazyme
- 7.1.6 Amyloglucosidase, available from Megazyme
- 7.1.7 MOPS buffer
  - 7.1.7.1 MOPS (sodium salt, 11.55g: Sigma Chemical Co.; cat. Number M9381)
  - 7.1.7.2 1M (10%) Hydrochloric acid
  - 7.1.7.3 Calcium chloride dihydrate
  - 7.1.7.4 Sodium azide (optional)
- 7.1.8 Sodium acetate buffer
  - 7.1.8.1 Glacial acetic acid
  - 7.1.8.2 1M sodium hydroxide solution
  - 7.1.8.3 Sodium azide (optional, see ES&H Considerations and Hazards, section 8.2)
- 7.1.9 Water, HPLC grade, 0.2  $\mu$ m filtered
- 7.1.10 Starch, chemical quality
- 7.1.11 0.01N sulfuric acid, or appropriate HPLC mobile phase

### 7.2 Materials

- 7.2.1 Volumetric flasks (2), 1 L
- 7.2.2 Autosampler vials with crimp top seals to fit and crimper
- 7.2.3 15 ml plastic tubes with tightly fitting screw caps
- 7.2.4 Disposable syringes, 3 mL, fitted with 0.2  $\mu$ m syringe filters
- 7.2.5 Volumetric pipets, class A, of appropriate sizes or corresponding pipettors
- 7.2.6 Volumetric flasks, class A, of appropriate sizes for standard and CVS dilution
- 7.2.7 Adjustable pipettors, covering ranges of 10  $\mu$ l to 10 ml

## 8. ES&H Considerations and Hazards

- 8.1 Sulfuric acid, hydrochloric acid, and glacial acetic acid are corrosive and should be handled with care
- 8.2 *Sodium azide is highly toxic and explodes when heated. It releases a poisonous gas when acidified. It is added to the buffers only as a preservative. The use of sodium azide can be avoided if the buffers are stored at 4°C.*
- 8.3 Follow all applicable NREL chemical handling procedures

## 9. Sampling, Test Specimens and Test Units

- 9.1 Follow LAP "Preparation of Biomass for Compositional Analysis" prior to analysis.
- 9.2 Care must be taken to ensure a representative sample is taken for analysis at each step. When measuring volumes for analysis, the liquid should be at room temperature.

## 10. Procedure

### 10.1 Prepare the buffers.

#### 10.1.1 MOPS buffer.

- 10.1.1.1 In a 1 L volumetric flask, add 11.55 g MOPS, sodium salt, to 900 ml of deionized water. Adjust the solution to pH7.0 using 1M (10%) HCl. Approximately 17 ml of 1M HCl will be required.
- 10.1.1.2 Add 0.74g calcium chloride dihydrate. If desired, add 0.2g sodium azide. The sodium azide can be omitted if the buffer is stored in the refrigerator. As sodium azide is highly toxic, omission is recommended. Bring the volume to 1 L with deionized water.

#### 10.1.2 Sodium acetate buffer

- 10.1.2.1 In a 1 L volumetric flask, add 11.8 ml glacial acetic acid to 900 ml of deionized water. Adjust the solution to pH 4.5 using a 1M (4g/100 ml) sodium hydroxide solution. Approximately 60 ml of 1M sodium hydroxide solution will be required.
- 10.1.2.2 If desired, add 0.2g sodium azide. The sodium azide can be omitted if the buffer is stored in the refrigerator. As sodium azide is highly toxic, omission is recommended. Bring the volume to 1 L with deionized water.

### 10.2 Prepare the sample for HPLC analysis.

- 10.2.1 Add 100 mg ( $\pm$  10mg) of sample to a tared 15 ml plastic centrifuge tube with a tightly fitting screw cap. Record the weight to the nearest 0.1 mg. LAP "Determination of Total Solids in Biomass" should be performed at the same time, to accurately measure the percent solids for correction. Each sample should be analyzed in duplicate, at minimum.
- 10.2.2 Repeat step 10.1.1 for a starch sample of known purity. The starch sample should be included throughout the analysis and treated as a sample.
- 10.2.3 Add 0.2 ml 190 proof ethanol and vortex vigorously. This will aid in sample dispersion.
- 10.2.4 Immediately add 2 ml of dimethyl sulfoxide (DMSO) and vortex vigorously.
- 10.2.5 Tightly cap the tube and place it in a briskly boiling water bath for five minutes. Use caution when removing the tubes, as they will be hot.
- 10.2.6 Immediately add 2.9 ml MOPS buffer and 0.1 ml thermostable  $\alpha$ -amylase (or 300 Units). Tightly cap the tube. Vortex vigorously. Incubate the tube in a boiling water bath for 6 minutes, vortex mixing every two minutes.
- 10.2.7 Place the tube in a 50°C water bath. Add 4 ml of the sodium acetate buffer, followed by 0.1 ml (20 units) amyloglucosidase. Vortex and incubate for 30 minutes at 50°C.
- 10.2.8 Remove the samples from the water bath and centrifuge the tubes for 10 minutes at 3,000 rpm.
- 10.2.9 If the sample has been capped tightly throughout the analysis, the solvent volume is

constant, and the volume of the solvent can be assumed to be 9.3 ml. Filter an aliquot through a 0.2 µm filter into an autosampler vial, seal and label. Prepare each sample in duplicate if desired.

### 10.3 Analyze the sample for glucose on an HPLC

- 10.3.1 Prepare 0.01 N sulfuric acid for use as a HPLC mobile phase. In a 2L volumetric flask, add 2.00 mL of 10 N sulfuric acid and bring to volume with HPLC grade water. Alternatively, add 278 µl concentrated sulfuric acid to a 1L volumetric flask and bring to volume with HPLC grade water. Filter through a 0.2 µm filter and degas before use.
- 10.3.2 Prepare a series of calibration standards containing D(+)-glucose. Suggested concentration range is 0.2 mg/ml to 15 mg/ml. Use a four point calibration. Filter the standard solutions through 0.2 µm filters into autosampler vials. Seal and label the vials.
- 10.3.3 Prepare an independent calibration verification standard (CVS) for each set of calibration standards. Use a reagent from a source or lot other than that used in preparing the calibration standards. Prepare the CVS at a concentration that falls in the middle of the validated range of the calibration curve, but is not equal to the concentration of any of the standards. The CVS should be analyzed on the HPLC after each calibration set and at regular intervals throughout the sequence, bracketing groups of samples. The CVS is used to verify the quality and stability of the calibration curve(s) throughout the run.
  - 10.3.3.1 A fresh set of standards is not required for every analysis. A large batch of standards may be produced, filtered through 0.2 µm filters into autosampler vials, sealed and labeled. The standards and CVS samples must be stored in a freezer and removed when needed. Thaw and vortex frozen standards prior to use. During every use, standards and CVS samples should be observed for unusual concentration behavior. Unusual concentrations may mean that the samples are compromised or components have been lost. Assuming sufficient volume, standards and CVS samples should not have more than 12 injections drawn from a single vial. In a chilled autosampler chamber, the lifetime of standards and CVS samples is approximately seven days.
- 10.3.4 Analyze the calibration standards, CVS, and samples by HPLC using a Biorad Aminex HPX-87H column.

HPLC conditions:  
Sample volume: 10 - 25 µL, dependent on sample concentration and detector limits  
Mobile phase: 0.01 N sulfuric acid, 0.2 µm filtered and degassed  
Flow rate: 0.6 mL / minute  
Column temperature: 55 – 65 °C  
Detector temperature: as close to column temperature as possible  
Detector: refractive index  
Run time: 50 minutes, or shorter if peak resolution is adequate  
Note: The retention time of DMSO should be verified prior to reducing the run time.
- 10.3.5 Calculate the amount of starch present in the sample. Correct for any starch loss

using the high purity starch sample as a reference. Use calculations 11.5.

## 11. Calculations

- 11.1 Calculate the oven dry weight (ODW) of the sample, using the average total solids content as determined by the LAP “Standard Method for the Determination of Total Solids in Biomass”.

$$ODW = \frac{\text{Weight}_{\text{air dry sample}} \times \% \text{ Total solids}}{100}$$

- 11.2 Create a calibration curve for each analyte to be quantified using linear regression. From these curves, determine the concentration in mg/mL of each component present in the samples analyzed by HPLC, correcting for dilution if required.
- 11.3 Calculate and record the amount of each calibration verification standard (CVS) recovered following HPLC analysis.

$$\% \text{ CVS recovery} = \frac{\text{conc. detected by HPLC, mg/mL}}{\text{known conc. of standard, mg/mL}} \times 100$$

- 11.4 For the reagent starch samples, calculate the amount of starch recovered after analysis. Average any replicate ( $\%R_{\text{starch}}$ ) values obtained for each individual starch sample and report  $\%R_{\text{avg, starch}}$ .

$$\% R_{\text{starch}} = \frac{\text{conc. detected by HPLC, mg/mL}}{\text{known weight of starch before analysis, mg/mL}} \times 100$$

- 11.5 Calculate the % starch for each sample.

$$\% \text{ Starch} = \left( \frac{C_{\text{HPLC}} \times \left( \frac{\text{Vol. solution, ml}}{\text{ODW}} \right)}{1.11 \times \% R_{\text{starch}}} \right) \times 100$$

Where:  $C_{\text{HPLC}}$  = conc. of glucose as determined by HPLC, mg/mL.  
 Vol. Solution = 9.3 ml, total volume of liquid added to solids  
 ODW = Oven dry weight of the sample, mg, as calculated in step 11.1.  
 “1.11” is the glucose to starch oligomer correction factor  
 $\%R_{\text{starch}}$  is the starch recovery as calculated in step 11.4

- 11.6 To report or calculate the relative percent difference (RPD) between two samples, use the following calculation

$$RPD = \left( \frac{(X_1 - X_2)}{X_{\text{mean}}} \right) \times 100$$

Where:  
 $X_1$  and  $X_2$  = measured values  
 $X_{\text{mean}}$  = the mean of  $X_1$  and  $X_2$

- 11.7 To report or calculate the root mean square deviation (RMS deviation) or the standard deviation (st dev) of the samples, use the following calculations.  
First find the root mean square (RMS), of the sample using

$$RMS = x_m = mean = \sqrt{\left(\frac{\sum_1^n x}{n}\right)^2}$$

Then find the root mean square deviation, or standard deviation, using

$$RMS\ deviation = \sigma = stdev = \sqrt{\frac{\sum_1^n (x_i - x_m)^2}{n}}$$

Where:

$x_m$ =the root mean square of all x values in the set  
 $n$ =number of samples in set  
 $x_i$ =a measured value from the set

## 12. Report Format

- 12.1 Report concentrations, in weight percent, of analytes of interest. RPD and RMS deviation may also be reported.

## 13. Precision and Bias

- 13.1 Determined by data quality objectives and laboratory specific Quality Assurance Plan.

## 14. Quality Control

- 14.1 Reported Significant Figures or Decimal Places: Determined by data quality objectives and laboratory specific Quality Assurance Plan.
- 14.2 Replicates: All analyses may be run in duplicate if desired.
- 14.3 Blank: An optional instrument blank may be run, using HPLC grade water analyzed by HPLC in the same manner as a sample.
- 14.4 Relative percent difference criteria: Determined by data quality objectives and laboratory specific Quality Assurance Plan.
- 14.5 Calibration verification standard: Calibration verification standards should be independently prepared and analyzed as described in the procedure section.
- 14.6 Sample size: 1.2 g minimum
- 14.7 Sample storage: Liquid samples and buffers should be stored in the refrigerator.
- 14.8 Standard storage: The standards should be stored in a freezer and removed when needed. Thaw and vortex frozen standards prior to use.
- 14.9 Standard preparation: Standards should be prepared as described in the procedure.

14.10 Definition of a batch: Any number of samples which are analyzed and recorded together. Samples within a batch should be of the same matrix. The maximum size of a batch will be limited by equipment constraints.

14.11 Control charts: All CVS and SRS recoveries should be control charted.

## **15. Appendices**

15.1 See Appendix 1 at the end of the document for a quick reference sheet for calculation 11.3.

## **16. References**

16.1 Megazyme “Total Starch Assay Procedure (Amyloglucosidase /  $\alpha$ -amylase method)” AA/AMG 11/01, AOAC Method 996.11, AACC Method 76.13, ICC Standard Method No. 168

16.2 NREL Laboratory Analytical Procedure, “Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction of Process Samples”.