



*Chemical Analysis and Testing Task
Laboratory Analytical
Procedure*

LAP-014

Procedure Title: Dilute Acid Hydrolysis Procedure for Determination of Total Sugars in the Liquid Fraction of Process Samples

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Dilute Acid Hydrolysis Procedure for Determination of Total Sugars in the Liquid Fraction of Process Samples

Laboratory Analytical Procedure #014

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. During dilute acid pretreatment of biomass, a portion of these polysaccharides are hydrolyzed and soluble sugars released into the liquid stream. These sugars, if present in oligomeric form, cannot easily be quantified without further processing into their monomeric units.

2. Scope

- 2.1 This procedure is used to determine the total sugar content, including both monosaccharides plus oligosaccharides, of the liquid fractions of biomass to ethanol process streams, including pretreatment liquors, liquid fermentation time point samples, and the liquid fraction of fermentation residues.
- 2.2 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

- 3.1 Ehrman, C.I., and M.E. Himmel. 1994. "Simultaneous Saccharification and Fermentation of Pretreated Biomass: Improving Mass Balance Closure." *Biotechnology Techniques*, 8(2):99-104.
- 3.2 Moore, W., and D. Johnson. 1967. Procedures for the Chemical Analysis of Wood and Wood Products. Madison, WI: U.S. Forest Products Laboratory, U.S. Department of Agriculture.

4. Significance and Use

- 4.1 The total sugar content is used in conjunction with other assays to determine the total composition of process stream samples.

- 4.2 The difference between the total sugar content determined by this procedure and the monosaccharide content, determined by LAP #013, can be used to assess the competence of specific process steps and is an indicator of the amount of non-fermentable sugars present at that specific phase of the process.

5. Apparatus

- 5.1 Analytical balance, accurate to 0.1 mg.
- 5.2 pH meter, readable to 0.01 pH unit.
- 5.3 Pan balance, accurate to 0.01 g.
- 5.4 Autoclave, set to $121^{\circ} \pm 3^{\circ}\text{C}$.
- 5.5 HPLC system equipped with refractive index detector.
- 5.6 Biorad Aminex HPX-87C with corresponding guard column and/or HPX-87P column with deashing guard column (or analytical HPLC column shown to give equivalent separations as the Biorad columns).

6. Reagents and Materials

- 6.1 High purity sugars for standards - glucose, xylose, arabinose, galactose, and mannose.
- 6.2 Second set of the high purity sugars listed above, obtained from a different source (manufacturer or lot) for preparation of calibration verification standards.
- 6.3 Sulfuric acid, 72% w/w (specific gravity 1.6389 at $15.6^{\circ}\text{C}/15.6^{\circ}\text{C}$).
- 6.4 Calcium carbonate, ACS reagent grade.
- 6.5 Water, HPLC grade or better, 0.2 μm filtered.
- 6.6 Erlenmeyer flasks, 25 mL.
- 6.7 Glass bottles, crimp top style, with rubber stoppers and aluminum seals to fit.
- 6.8 Pasteur pipettes.
- 6.9 pH paper (range 2-9).

- 6.10 0.2 μm syringe filters.
- 6.11 Disposable syringes, 3 mL.
- 6.12 Autosampler vials with crimp top seals to fit.
- 6.13 Volumetric pipets, class A, 20 mL.
- 6.14 Volumetric flasks, class A, of appropriate sizes.
- 6.15 Adjustable pipettors, coverings ranges of 10 to 1000 FL.

7. ES&H Considerations and Hazards

- 7.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

8. Calibration and Standardization

- 8.1 This analysis uses a multipoint calibration as described in the procedure.

9. Procedure

- 9.1 Thoroughly mix and then accurately measure out duplicate 20.0 mL portions of each sample into labeled crimp-top bottles. If the available amount of sample is limited, this procedure can be scaled back by using 10.0 mL portions. Other procedural steps must then be scaled accordingly.

Note: If the specific gravity of the sample is close to 1.0, the sample can be measured out by accurately weighing 20.00 g portions on a pan balance instead of by pipetting.

- 9.2 Dispense a separate aliquot of each sample into an Erlenmeyer flask, measure and record the pH of each sample to the nearest 0.01 pH unit.
- 9.3 Prepare method verification standards (MVS) by selecting representative samples to be used to determine spike recoveries. Spike an accurately measured volume of each selected sample with a known amount of the sugars of interest, such that the final concentrations of each component still falls within the linear range of the analysis. Process these spiked samples along with the rest of the samples.

- 9.4 Calculate the amount of 72% w/w sulfuric acid required to bring the initial acid concentration of each sample to a 4% final acid concentration (refer to the example presented in the calculation section). Swirl the sample while carefully adding the required amount of acid.
- 9.5 Stopper the sample bottles and crimp aluminum seals into place.
- 9.6 Prepare a set of sugar recovery standards (SRS) that will be taken through the complete analytical procedure to correct for losses due to the destruction of sugars during the dilute acid hydrolysis. Weigh out the required amounts of each sugar to the nearest 0.1 mg, transfer the sugars to a crimp-top bottle, and add 20.00 g HPLC grade water. A typical protocol for preparing the necessary sugar recovery standards is presented in the following table:

Sugar recovery standard (SRS)	Column	Target weights (for 20.00 mL total volume)				
		glucose	xylose	galactose	arabinose	mannose
G/X/A low	either column	25 mg	25 mg	---	25 mg	---
G/X/A mid		100 mg	100 mg	---	100 mg	---
G/X/A high		200 mg	200 mg	---	200 mg	---
G/X/Gal/A/M low	HPX-87P only	25 mg	25 mg	25 mg	25 mg	25 mg
G/X/Gal/A/M mid		100 mg	100 mg	100 mg	100 mg	100 mg
G/X/Gal/A/M high		200 mg	200 mg	200 mg	200 mg	200 mg

- 9.7 To each sugar recovery standard, add 697 μ L of 72% sulfuric acid (refer to example in the calculation section). Stopper the bottles, label, and crimp aluminum seals into place.
- 9.8 Autoclave the sealed samples, method verification standards, and sugar recovery standards for one hour at 121°C. After completion of the autoclave cycle, allow the hydrolyzates to cool somewhat before removing the seals and stoppers.

9.9 Neutralize the hydrolyzates with calcium carbonate to a pH of 5 to 6. Add the calcium carbonate slowly with frequent mixing to avoid problems with foaming. Monitor the pH of the solutions with pH paper and, as the pH begins to approach 4.0, slow down the rate of calcium carbonate addition and swirl frequently to avoid over-neutralizing. Keep these neutralized samples cold.

9.10 Dilute the neutralized samples as needed, so the concentration of each sugar falls within the validated range of the analytical method.

Note: It may be useful to determine initial glucose concentrations of the samples using an alternative technique, such as YSI glucose analyzer, in order to predict whether or not the sugars in the sample will fall within the linear range of the analysis. The results from the LAP-013 analysis will also be helpful in determining if dilution will be needed. In samples such as pretreatment liquors, xylose typically is present at high concentrations, often three to five times the level of glucose. In other samples, such as the liquid fractions of fermentation samples, the levels of all the soluble sugars can be relatively low, and the samples may not require dilution.

9.11 A portion of each appropriately diluted hydrolyzate is prepared for HPLC analysis by passing the sample through a 0.2 μm syringe filter into an autosampler vial. The vial is then sealed and labeled.

9.12 Prepare a series of sugar standards in HPLC grade water at concentrations appropriate for creating a calibration curve for each of the sugars of interest. A suggested scheme for the HPX-87C column is to prepare a set of multi-component standards containing glucose, xylose, and arabinose in the range of 0.2 -12.0 mg/mL. For the HPX-87P column, galactose and mannose should be included as additional components in the standards.

Note: Extending the range of the calibration curves beyond 12.0 mg/mL will require validation.

9.13 Prepare an independent calibration verification standard (CVS) for each set of calibration standards. This CVS should contain precisely known amounts of each sugar contained in the calibration standards, at a concentration that falls in the middle of the validated range of the calibration curve. The CVS is to be analyzed at regular intervals during the HPLC sequence and is used to verify the validity of the calibration curves throughout the HPLC run.

- 9.14 Analyze the calibration standards, the calibration verification standards, the hydrolyzed sugar recovery standards, the hydrolyzed samples, and the hydrolyzed method verification (spiked) samples by HPLC using an Biorad Aminex HPX-87C or HPX-87P column for glucose, xylose, and arabinose. If mannose and galactose are also to be determined, only the Biorad Aminex HPX-87P column should be used. For many analyses it is useful to run the same samples on both columns and compare the results. The following instrumental conditions are used for both the HPX-87C and the HPX-87P columns:

Sample volume: 50 μ L.
 Eluant: 0.2 μ m filtered and degassed HPLC grade water.
 Flow rate: 0.6 mL/min.
 Column temperature: 85°C.
 Detector: refractive index.
 Run time: 20 minutes data collection plus a 15 minute post-run.

- 9.15 Samples containing sugar levels falling outside the validated range of the calibration curves must be rerun after appropriately diluting.

10. Calculations

- 10.1 For each sample and standard, calculate the volume of 72% sulfuric acid required to bring the acid concentration to a 4% final acid concentration. The molar concentration of hydrogen ions, $[H^+]$, in a sample can be calculated from its pH:

$$pH = -\log[H^+], \text{ therefore, } [H^+] = \text{antilog}(-pH).$$

The volume of 72% sulfuric acid to be added is then calculated from the following

$$V_{72\%} = \frac{[(C_{4\%} \times V_s) - (V_s \times [H^+] \times 98.08g \text{ H}_2\text{SO}_4 / 2 \text{ moles H}^+)]}{C_{72\%}}$$

equation:

where: $V_{72\%}$ is the volume of 72% acid to be added, in mL
 V_s is the initial volume of sample or standard, in mL, which includes the volume of spike added (if applicable)
 $C_{4\%}$ is the concentration of 4% w/w H_2SO_4 , 41.0 g/L
 $C_{72\%}$ is the concentration of 72% w/w H_2SO_4 , 1176.3 g/L
 $[H^+]$ is the concentration of hydrogen ions, in moles/L

Example #1: Calculate the amount of 72% H₂SO₄ needed to prepare a sample with a pH of 2.41 for 4% acid hydrolysis. If the pH is 2.41, then [H⁺]=0.00389 M. Therefore:

$$\frac{[(41.0 \text{ g/L})(20 \text{ mL}) - (20 \text{ mL})(0.00389 \text{ moles/L})(98.08 \text{ g/2 moles})]}{1176.3 \text{ g/L}} = 0.694 \text{ mL}$$

Example #2: Calculate the amount of 72% H₂SO₄ needed to prepare a sugar recovery standard for 4% acid hydrolysis. The standard itself is prepared in water with no added acid, so the pH can be assumed to be about 7. Therefore [H⁺]=0.0000001 M, a value small enough to be ignored in the following calculation.

$$(41.0 \text{ g/L} \times 20 \text{ mL}) / 1176.3 \text{ g/L} = 0.697 \text{ mL}$$

- 10.2 Create a calibration curve for each sugar to be quantified using linear regression. From these curves, determine the concentration in mg/mL of the sugars present in each solution analyzed by HPLC.
- 10.3 Calculate and record the amount of each calibration verification standard (CVS)

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

recovered following HPLC analysis.

- 10.4 For all three sugar recovery standards (SRSs), calculate the amount of each component sugar recovered after being taken through the dilute acid hydrolysis procedure. Average the % R_{sugar} values obtained for each individual sugar and report as % R_{ave, sugar}.

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

- 10.5 Use the percent hydrolyzed sugar recovery values calculated in the previous step to correct the corresponding sugar concentration values obtained by HPLC for each of the hydrolyzed samples (C_{cor. sample}) and each of the hydrolyzed spiked samples (C_{cor. spiked sample}), accounting for any dilution made to the sample prior to HPLC analysis.

$$C_x = \frac{C_{HPLC} \times \text{dilution factor}}{\% R_{ave. sugar} / 100}$$

Where: C_{HPLC} = conc. of a sugar as determined by HPLC, mg/mL.
 $\% R_{ave. sugar}$ = average recovery of a specific SRS component.
 $C_x = C_{cor. sample}$ or $C_{cor. spiked sample}$, concentration in mg/mL of a sugar in the hydrolyzed sample or spiked sample after correction for loss on 4% hydrolysis.

10.6 Calculate and record the percent spike recoveries (% MVS recovery) for each sugar used to prepare the method verification standards analyzed by HPLC.

$$\% MVS \text{ recovery} = \frac{C_{cor. spiked sample} - C_{cor. sample} \times \frac{V_{sample}}{V_{final}}}{C_{spike}} \times 100$$

Where: $C_{cor. spiked sample}$ = concentration of the hydrolyzed spiked sample after being corrected for loss on 4% hydrolysis, in mg/mL.
 $C_{cor. sample}$ = concentration in mg/mL of the hydrolyzed sample after correction for loss on 4% hydrolysis.
 V_{sample} = volume of sample prior to spiking, in mL.
 V_{final} = final volume of solution (spike plus sample), in mL.
 C_{spike} = known concentration in mg/mL of the spike solution added to a sample prior to analysis.

11. Precision and Bias

11.1 In the determination of the total sugar contents of process samples, the neutralized hydrolyzates are routinely analyzed using the HPX-87P column. When these samples are known not to contain galactose and mannose, the HPX-87C column may be used instead. Based on a root mean square evaluation of duplicate data, there is a 95% certainty that the "true value" will be within the range of the average plus or minus:
 - glucose 4.86% (HPX-87C) and 2.90% (HPX-87P),
 - xylose 2.73% (HPX-87C) and 3.09% (HPX-87P).
 Analytes at or near the detection limit could have significantly higher precision errors.

12. Quality Control

- 12.1 *Reported significant figures:* All results are reported in mg/mL with two decimal places. The standard deviation or relative percent difference are also to be reported.
- 12.2 *Replicates:* All samples are to be run in duplicate. For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).
- 12.3 *Relative percent difference criteria:* The maximum RPD for duplicate samples is as follows: glucose, 7.5%, and xylose, 5.0%. If the stated RPD is exceeded, the sample should be rerun. However, analytes at or near the detection limit could have significantly higher RPDs.
- 12.4 *Blank:* The only requirement is an instrumental blank, consisting of the HPLC grade water analyzed by HPLC in the same manner as the samples.
- 12.5 *Method verification standard:* This method will utilize a matrix spike as the method verification standard, as indicated in the procedure.
- 12.6 *Calibration verification standard:* Calibration verification standards shall be independently prepared and analyzed as described in the procedure.
- 12.7 *Definition of a batch:* Any number of samples which are analyzed together and recorded together. Samples within a batch must be of the same matrix. The maximum size of a batch would be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.
- 12.8 *Sample size:* 50 mL per sample.
- 12.9 *Sample storage:* Samples should be refrigerated.
- 12.10 *Standard storage:* Standards should be frozen and then shaken vigorously upon thawing.
- 12.11 *Standard preparation:* Standards are prepared according to instructions given in the Procedure section of this protocol.
- 12.12 *Control charts:* All spike recoveries and calibration verification standards are control charted.