



*Chemical Analysis and Testing Task
Laboratory Analytical
Procedure*

LAP-015

Procedure Title: HPLC Analysis of Liquid Fractions of Process Samples for Byproducts and Degradation Products

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HPLC Analysis of Liquid Fractions of Process Samples for Byproducts and Degradation Products

Laboratory Analytical Procedure #015

1. Introduction

1.1 During processing of biomass samples, such as in acid pretreatment of biomass, a liquid portion is produced which may contain carbohydrate degradation products, such as HMF and furfural, as well as other components of interest, such as organic acids and sugar alcohols. These components are analyzed by HPLC with refractive index detection to determine optimal production process parameters or to monitor ongoing processes.

2. Scope

2.1 This procedure is used to determine the concentration of carbohydrate degradation products as well as selected organic acids and sugar alcohols present in the liquid fractions of biomass to ethanol process streams. These process streams include 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 This procedure is used to determine the amount of ethanol, selected organic acids and sugar alcohols, and carbohydrate degradation products (such as HMF and furfural) in the liquid fraction of biomass to ethanol process streams. Several of the compounds being measured are potential inhibitors of the process, and are therefore important to monitor.

- 4.2 The concentrations of these byproducts and degradation products are used in conjunction with other assays to determine the total composition of process stream samples.

5. Interferences

- 5.1 Arabitol coelutes with xylitol. If the sample is thought to contain arabitol, the experimentally determined xylitol concentration should be flagged as potentially being biased high due to the suspected arabitol component.
- 5.2 The HPLC column used in this protocol is only partially capable of resolving the monomeric sugars of importance in biomass analysis. Glucose, xylose, and arabinose will be resolved, but galactose and mannose will coelute with xylose. If monomeric sugars are present in concentrations far exceeding the concentrations of the analytes to be quantified by this protocol, some of these analytes will appear as small humps on the shoulders of larger peaks, leading to difficulties when integrating.
- 5.3 In addition to the glycerol, arabitol, and xylitol, some samples may contain sorbitol. This sugar alcohol elutes about a minute earlier than xylitol on the Aminex HPX-87H column, and will appear as a peak in between the xylose and arabinose peaks.

6. Apparatus

- 6.1 Analytical balance, accurate to 0.1 mg.
- 6.2 HPLC system equipped with a refractive index detector and a Biorad Aminex HPX-87H analytical column (or equivalent) with corresponding guard column.

7. Reagents and Materials

- 7.1 High purity standards - including xylitol, succinic acid, lactic acid, glycerol, formic acid, acetic acid, ethanol, 5-hydroxy-2-furaldehyde (HMF), and furfural.
- 7.2 Second set of the high purity standards listed above, from a different source (manufacturer or lot), to be used to prepare calibration verification standards (CVS).
- 7.3 Sulfuric acid, concentrated, ACS reagent grade.
- 7.4 Water, HPLC grade or better.
- 7.5 0.2 μm syringe filters.

- 7.6 Disposable syringes, 3 mL.
- 7.7 Solvent filtration system equipped with 0.2 μm filter.
- 7.8 Autosampler vials with crimp top seals to fit.
- 7.9 Volumetric pipettes, class A, of appropriate sizes.
- 7.10 Volumetric flasks, class A, of appropriate sizes.
- 7.11 Adjustable pipettors, covering ranges of 10 to 1000 μL .

8. ES&H Considerations and Hazards

- 8.1 Sulfuric acid is corrosive and should be handled with care.
- 8.2 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

9. Sampling, Test Specimens and Test Units

- 9.1 Care must be taken to ensure a representative sample is taken for analysis.
- 9.2 Store sample in a sealed container to ensure the concentration of its volatile components do not change.

10. Procedure

- 10.1 Prepare 0.01N sulfuric acid for use as mobile phase in this analysis. In a one liter volumetric flask, add 278 μL concentrated sulfuric acid and bring to volume with HPLC grade water. Filter through a 0.2 μm filter and degas thoroughly before use.
- 10.2 Prepare the sample for HPLC analysis by passing it through a 0.2 μm syringe filter into an autosampler vial. Seal and label the vial. Prepare each sample in duplicate.
- 10.3 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 components 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.
- 10.4 Prepare a series of calibration standards containing the compounds that are to be quantified, referring to Table 1 for suggested concentration ranges and special considerations. If standards are prepared outside of the suggested ranges, the new range for these calibrations curves must be validated. Since the retention times of two

pairs of components, xylitol/succinic acid and glycerol/formic acid, are so close, before standards are prepared the column should be tested to verify adequate separation and quantification can be achieved. If the separation is not adequate, either replace or regenerate the column and then confirm improved separation has been achieved. If separation is still not adequate, prepare standards without one of the components from each pair (succinic acid for the first case, formic acid for the second). An second set of standards must then be prepared containing the deleted component(s).

Table 1.

Component	Expected retention time	Suggested concentration range	*Special considerations
xylitol	11.6 min	0.2 - 6.0 mg/mL	a
succinic acid	12.0 min	0.2 - 10.0 mg/mL	a
lactic acid	13.2 min	0.2 - 12.0 mg/mL	
glycerol	14.2 min	0.2 - 8.0 mg/mL	a
formic acid	14.4 min	0.2 - 6.0 mg/mL	a
acetic acid	15.5 min	0.2 - 12.0 mg/mL	b
ethanol	22.7 min	1.0 - 15.0 mg/mL	b
HMF	29.4 min	0.02 - 5.0 mg/mL	c
furfural	42.8 min	0.02 - 5.0 mg/mL	b,c
calib. verification	--	middle of linear range	--
meth. verification	--	within linear range	d

*Special considerations:

a = confirm xylitol/succinic acid and glycerol/formic acid can be adequately separated before preparing standards.

b = samples containing volatile components must be submitted in sealed containers.

c = the linear range for HMF and furfural is limited by their solubility; when preparing a standard, add these components after the ethanol has been added to make it easier to solubilize the HMF and furfural.

d = representative sample(s) should be chosen for spiking.

- 10.5 Prepare an independent calibration verification standard (CVS) for each set of calibration standards, using components obtained from a source other than that used in preparing the calibration standards. This CVS must contain precisely known amounts of each compound contained in the calibration standards, at a concentration that falls in the middle of the validated range of the calibration curve (refer to Table 1). The CVS is to be analyzed after each calibration curve and at regular intervals in the HPLC sequence, bracketing groups of samples. The CVS is used to verify the quality of the calibration curve(s) throughout the HPLC run.
- 10.6 Analyze the calibration standards, calibration verification standards, method verification standard, and the samples by HPLC using a Biorad Aminex HPX-87H column. The following instrumental conditions are to be used for the HPX-87H column:
- Sample volume: 50 μ L.
 - Eluant: 0.2 μ m filtered and degassed 0.01 N sulfuric acid.
 - Flow rate: 0.6 mL/min.
 - Column temperature: 55°C.
 - Detector: refractive index.
 - Run time: 50 minutes.
- 10.7 If an analyzed sample or method verification standard (spiked sample) falls outside the validated calibration range, dilute as needed and rerun the sample. The value can then be reported after correcting for dilution.

11. Calculations

- 11.1 Create a calibration curve for each analyte to be quantitated 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.
- 11.2 Calculate and record the amount of each calibration verification standard (CVS) recovered following HPLC analysis.

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

- 11.3 Calculate and record the percent spike recoveries (% recovery MVS) for each component used to prepare the method verification standards analyzed by HPLC.
- 11.3.1 Correct the initial sample concentration for the dilution resulting from the addition of a known volume of spike solution.

$$C_{corrected} = \frac{V_{sample}}{V_{final}} \times C_{sample}$$

Where: V_{sample} = volume of sample prior to spiking, in mL.
 V_{final} = final volume of solution (spike plus sample), in mL.
 C_{sample} = initial concentration of sample prior to spiking in mg/mL, as determined by HPLC.
 $C_{corrected}$ = concentration of sample after being corrected for dilution, in mg/mL.

11.3.2 Calculate the percent recovery of the spike.

$$\% Recovery MVS = \frac{C_{actual} - C_{corrected}}{C_{spike}} \times 100$$

Where: C_{actual} = actual concentration of spiked sample, as determined by HPLC, in mg/mL.
 $C_{corrected}$ = concentration of sample after correcting for dilution, in mg/mL, as calculated above.
 C_{spike} = known concentration of spike solution added to sample prior to analysis, in mg/mL.

12. Precision and Bias

- 12.1 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:
- glycerol, 5.52%; acetic acid, 4.58%;
 - HMF, 6.15%; furfural, 5.61%;
 - lactic acid, 5.85%; and succinic acid, 3.18%.

Analytes at or near the detection limit could have significantly higher precision errors.

13. Quality Control

- 13.1 *Reported significant figures:* All results are reported in mg/mL, with two decimal places. Also report the standard deviation and relative percent difference.
- 13.2 *Replicates:* All samples are run in duplicate.

- 13.3 *Relative percent difference criteria:* The maximum RPD for duplicate samples is as follows: glycerol, 8.8%; acetic acid, 7.1%; HMF, 9.3%; furfural, 8.9%; lactic acid, 9.4%; and succinic acid, 4.5%. If the RPD exceeds the stated value, the sample should be rerun. However, analytes at or near the detection limit could have significantly higher RPDs.
- 13.4 *Blank:* The only requirement is an instrument blank, consisting of the HPLC grade water analyzed by HPLC in the same manner as a sample.
- 13.5 *Method verification standard:* This method will utilize a matrix spike as the method verification standard, as indicated in the procedure.
- 13.6 *Calibration verification standard:* Calibration verification standards shall be independently prepared and analyzed as described in the procedure.
- 13.7 *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.
- 13.8 *Sample size:* 5 mL minimum.
- 13.9 *Sample storage:* Samples should be refrigerated. Those containing volatile components must be stored in sealed containers.
- 13.10 *Standard storage:* Standards should be frozen in sealed containers or vials. Shake vigorously upon thawing.
- 13.11 *Standard preparation:* Standards are prepared as described in the 'Procedure' section of this protocol.
- 13.12 *Control charts:* All spike recoveries and calibration verification standards are control charted.