STANDARD OPERATING PROCEDURE
STAN MAYFIELD BIOREFINERY PILOT PLANT

TITLE: HPLC Calibration

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DATE: January23rd, 2012

APPROVALS: Process Change Committee

DATE:

A. Scope

This procedure describes how to perform the calibration of sugars, organic acids and inhibitors for High Pressure Liquid Chromatography (HPLC) and their determination.

B. Safety and Training Requirements

Refer to UF Environmental Health and Safety (EH&S) policies when handling chemicals and disposing of waste.

Review the Material Safety Data Sheets (MSDS) for each material listed in section D below before starting any process work.

Review the location of fire extinguishers, fire blankets, safety showers, spill cleanup equipment and protective gear before beginning any process work.

During operations in the laboratory, the following safety gear will be utilized at all times:

- Safety Goggles
- Protective Gloves (nitrile, neoprene)
- Lab coat

C. Related Documents and SOPs

1. Agilent Technologies HPLC System operating manual (1260-1, 1260-2)
2. Sampling SOP-0511
3. Microcentrifuge manual (XXXX)
4. UF Chemical Waste Management Guide
5. Sugars, Organic Acids, and Inhibitors Concentration SOP-0505

D. Preparation/Materials/Equipment

1. Analytical Balance

2. National Scientific vial crimper
3. Pipette tips (200-1000 µl range)
4. Automatic pipette (200-1000µL)
5. 13X100mm glass tubes for making serial dilutions of stock solutions
6. National Scientific HPLC sample vials
7. National Scientific HPLC sample crimper vial caps
8. 3 mL syringes (Non sterile)
9. 0.45 µm syringe filters
10. Volumetric flasks 100-250mL
11. Nanopure water
12. Sulfuric acid solution (4 mM)
13. Computer with Chemstation Software
14. Blast proof refrigerator for volatile compounds (HMF, Furufral)
15. Aminex HPX-87P, 300 mm x 7.8 mm, Catalog #125-0098
16. Aminex HPX-87H, 300 mm x 7.8 mm, Catalog #125-0140

E. Detailed Procedure

HPLC # 1: sugars and inhibitors calibration

1. Using a volumetric flask, prepare 0.1-0.25 L 1 M standard stock solutions of xylose, glucose, arabinose, galactose, hydroxymethylfufural, furfural, and mannose with nanopure water. Cellobiose will not completely dissolve at a 1 M concentration, so prepare at 200 mM stock concentration.
    a. Standards should be diluted to concentrations that bracket the concentration of the unknown (when possible)
        i. Xylose calibration range should be 10 mM to 500 mM
        ii. Cellobiose calibration range should be 10 mM to 100 mM
        iii. Glucose calibration range should be 10 mM to 250 mM
        iv. Galactose, Manose, Arabinose, should be between 1 mM and 50 mM
        v. Furans (HMF, Fufural) should be between 5 mM and 50 mM
    b. Prepare 0.1 to 0.25 liter 1 M stock solutions by weighing and recording to the nearest 0.1 mg the desired HPLC grade reagent into the volumetric flask and bring to volume using nanopure water.
    c. Aliquot by syringe filtration, 10 mL of each stock solution in 15 mL Nalgene sterile conical tubes
    d. Place stock solutions in the -80C freezer and remove and thaw in warm water as needed.

3. Make sure the four nanopure water bottles on top of the HPLC are full (~1 L each one).
4. Verify that the liquid waste container for the HPLC # 1 has sufficient space to accommodate the volume of waste to be generated in the current run.
5. Turn on the HPLC # 1 and the respective computer and start the Chemstation software (On line).
6. Open the purge valve in Chemstation (on line) and turn on the HPLC # 1 quaternary pump at a flow of 0.2 mL/min of nanopure water at least one hour before starting the measurements to make sure you have a stable baseline.
7. In Chemstation (on line), verify that the flow rate for sugars column is 0.2 mL/min. Turn on the column thermostat and the refraction index detector thermostat for heating the column and the refraction index detector up to 80 °C and 35 °C respectively.
8. Once an hour has passed and the temperature of the column has equilibrated (80 °C), set the flow rate of nanopure water to 0.6 mL/min in Chemstation (On line) for the sugars column.
9. Record the column pressure in the HPLC log book once a stable baseline is achieved. The column pressure must not exceed 80 PSI once temperature in the column is equilibrated.
10. Prepare the standard samples to be analyzed by HPLC by filtering the samples into an HPLC sample vial:
    a. Use a 1 mL pipette and pipette tips (200-1000 µL range) to transfer the standards to a 3 mL syringe attached with a 0.45 µm syringe filter
    b. Filter the sample into a labeled HPLC sample vial (the sample needs to be at least 300 µL). Label the samples according to batch number and date.
    c. Seal the vial with a crimper using the crimper vial caps.
11. Run the samples in the HPLC by putting all the samples into the HPLC tray in the same order of the sequence of samples and following the instructions given in the HPLC System Operating Manual (1260-1).
12. Once the samples have run, obtain the results from the Chemstation Software (Off line) by:
    a. Verify the key peaks are integrated properly in the Integration Mode.
    b. Make a calibration curve of each component in the Calibration Mode obtaining the slope and correlation coefficient that must be close to 1 for a good determination of the components.
    c. Save the calibration curve as a new Method in Chemstation Software (Off line).
13. Run and integrate the peaks of each process sample and determine the concentration of sugars, Furfural and HMF using the corresponding calibration

curve in the Chemestation Software (off line) according to the Sugars, Organic Acids, and Inhibitors Concentration SOP-0505.

HPLC # 2: organic acids calibration

1. Using a volumetric flask, prepare 0.1-0.25 L 1 M standard stock solutions of acetic acid, formic acid, levulinic acid, lactic acid, and succinic acid with nanopure water.
2. Dilute the stock solutions using nanopure water into microcentrifuge tubes to achieve 25 mM, 50 mM and 100 mM concentrations of each standard. Only about 1 mL is needed for the calibration curve.
3. Prepare 4 L of 4 mM Sulfuric Acid (H_2SO_4) solution with nanopure water and fill the four bottles on the top of HPLC # 2 (~1 L each one).
4. Verify that the liquid waste container for the HPLC # 2 has sufficient space to accommodate the volume of waste to be generated in the current run.
5. Turn on the HPLC #2 and the respective computer, and open the Chemstation software (On line).
6. Open the purge valve in Chemstation (On line) and turn on the HPLC # 2 quaternary pump at a flow of 0.2 mL/min of H_2SO_4 4 mM at least one hour before starting the measurements to make sure you have a stable base line.
7. In the Chemstation software (On line), verify that the flow rate of the acids column is 0.2 mL/min.
8. Turn on the column and the refraction index detector thermostat in order to heat the column and the refraction index detector to 45°C.
9. Once an hour has passed and the temperature has equilibrated (45 °C), set the flow rate of 4 mM H_2SO_4 to 0.4 mL/min in Chemstation (On line) for the acids column.
10. Record the column pressure in the HPLC log book once a stable baseline is achieved. The column pressure must not exceed 60 PSI.
11. Prepare the standard samples to be analyzed by HPLC by filtering the samples into an HPLC sample vial:
    a. Use a 1 mL pipette and pipette tips (200-1000 µL range) to transfer the standards (1 mL) to a 3 mL syringe attached with a 0.45 µm syringe filter
    b. Filter the sample into a labeled HPLC sample vial (the sample needs to be at least 300 µL). Label samples according to batch number and date.
    c. Seal the vial with a crimper using the crimper vial caps.
12. Run the samples in the HPLC by putting all the samples into the HPLC tray in the same order of the sequence of samples and following the instructions given in the HPLC System operating manual (1260-2).

13. Once the samples have run, obtain the results from the Chemstation Software (Off line) by:
    a. Verify the key peaks are integrated properly in the Integration Mode.
    b. Make a calibration curve of each component in the Calibration Mode obtaining the slope and correlation coefficient that must be close to 1 for a good determination of the components.
    c. Save the calibration curve as a new Method in Chemstation Software (Off line).
14. Run and integrate the peaks of each process sample and determine the concentration of each organic acid using the corresponding calibration curve in the Chemstation Software (Off line) according to the Sugars, Organic Acids, and Inhibitors Concentration SOP-0505.

F. Data Archival and Analysis

Record all measurements in the laboratory notebook including the date, time, and batch number of the sample.