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Determination of Extractives in Biomass

Laboratory Analytical Procedure (LAP)

Issue Date: 7/17/2005

A. Sluiter, R. Ruiz, C. Scarlata, J. Sluiter, and D. Templeton

Technical Report
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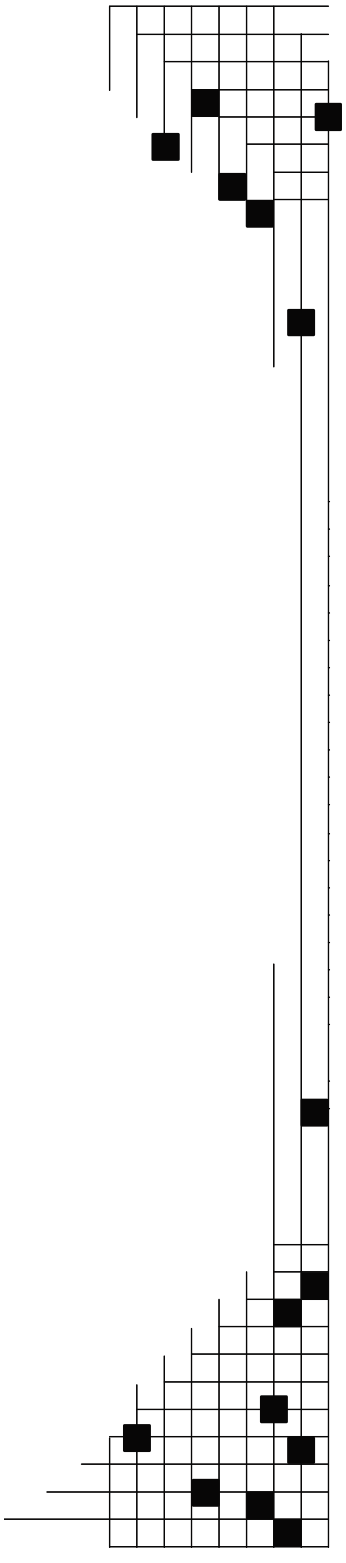

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Contract No. DE-AC36-99-GO10337

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Procedure Title: Determination of Extractives in Biomass

Laboratory Analytical Procedure

1. Introduction

1.1 It is necessary to remove non-structural material from biomass prior to analysis to prevent interference with later analytical steps. This procedure uses a two-step extraction process to remove water soluble and ethanol soluble material. Water soluble materials may include inorganic material, non-structural sugars, and nitrogenous material, among others. Inorganic material in the water soluble material may come from both the biomass and any soluble material that it is associated with the biomass, such as soil or fertilizer. No attempt is made to distinguish the source of the inorganic material. Ethanol soluble material includes chlorophyll, waxes, or other minor components. Some biomass may require both extraction steps, while other biomass may only require exhaustive ethanol extraction.

1.2 Refer to LAP-010 or ASTM Standard Test Method E 1690 "Determination of Ethanol Extractives in Biomass" for extraction procedures for isolation and characterization of extractives.

1.3 This procedure covers the determination of soluble non-structural materials in a biomass sample. The results are reported, on a dry weight basis, as a weight percentage of the biomass. Extractives percentages are measured and used to convert compositions from an extractives-free basis to and as-received basis. Determining the amount of water extractable sucrose is also covered. This LAP should be used in conjunction with other assays to determine the total composition of biomass samples as described in LAP "Summative Mass Closure for Biomass Samples". This procedure should be performed prior to LAP "Determination of Structural Carbohydrates and Lignin in Biomass"

2. Scope

2.1 This procedure has been optimized for the removal of extractives in biomass.

2.2 The decision to utilize a two-step or one step extraction should be made based on biomass type.

2.2.1 The two-step extraction should be used to quantify extractives in biomass samples that contain a significant amount of water soluble material, or biomass that has water soluble components of interest. Corn stover is an example of biomass that should be subjected to a two-step extraction process, as the water soluble portion of the biomass is significant and the water soluble material includes quantifiable carbohydrates.

2.2.2 Ethanol only extractions should be used for samples that have little or no water extractable material or when no water soluble components need to be quantified Examples include hardwoods and softwoods. These samples should be subjected to exhaustive ethanol extraction only.

2.2.3 This procedure should not be applied to other types of biomass without appropriate investigations as to the applicability of the method, including samples with partially ethanol-soluble fats.

3. Terminology

3.1 *Extractives*- The material in a biomass sample that is soluble in either water or ethanol during exhaustive extraction. Extractives include non-structural components of biomass samples that could potentially interfere with the down stream analysis of the biomass sample.

3.2 *Non-structural components*- Non-chemically bound components of biomass that include but are not limited to sucrose, nitrate/nitrites, protein, ash, chlorophyll, and waxes.
3.3 *Extractives-free biomass*- Biomass after exhaustive water and ethanol extraction.
3.4 *Soxhlet cycle*- One soxhlet cycle is an amount of time measured from the start of one siphoning until the start of the next siphoning from the extractor.
3.5 *Exhaustive extraction*- An exhaustive water extraction is complete when most or all of the water extractable material has been extracted from the biomass. For the Soxhlet method, this is usually 6-24 hours. An exhaustive ethanol extraction is usually complete in 24 hours using the Soxhlet method.
3.6 *Oven dry weight (ODW)*- The weight of biomass mathematically corrected for the amount of moisture present in the sample at the time of weighing, or the weight of glass or porcelain apparatus dried in a 105 $\pm$ 5 °C drying oven to a constant weight.

4. Significance and Use

4.1 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".
4.2 Removal of extractives prior to analysis for carbohydrates and lignin improves precision of those methods. Irreproducible partitioning of extractives introduces errors in those methods, see Milne et al. (1992)

5. Interferences

5.1 Samples that are moldy or aged may contain structural materials that have been modified and are now soluble in water or ethanol.
5.2 Failure to remove extractable materials may result in an error in structural sugar values. Hydrophobic extractives inhibit penetration of the sulfuric acid into the sample resulting in incomplete hydrolysis.
5.3 Failure to remove extractable material may result in falsely high lignin values when unhydrolyzed carbohydrates condense with the acid insoluble lignin.

6. Apparatus

6.1 Analytical balance, accurate to 1 mg or 0.1 mg
6.2 Medium to large capacity oven set to 105 $\pm$ 5 °C for glassware drying
6.3 Vacuum oven set to 40 $\pm$ 2 °C *or* drying oven set to 45 $\pm$ 2 °C
6.4 Apparatus for extraction, either Soxhlet or automatic
  6.4.1 Apparatus for Soxhlet extraction
    6.4.1.1 Glass Soxhlet extraction tubes of suitable size (capacity 85 mL)
    6.4.1.2 Heating mantles, suitable for 500 mL boiling flasks
    6.4.1.3 Condensers with appropriate fitting for Soxhlet tubes and a source of chilled water or other cooling system
    6.4.1.4 Single thickness cotton cellulose thimbles, 94 mm external length by 33 mm internal diameter (Alundum thimbles, medium porosity, of appropriate size for Soxhlet tube may also be used)
    6.4.1.5 250 mL glass rotary evaporator trap, optional
  6.4.2 Apparatus for automatic extraction

6.4.2.1 Dionex Accelerated Solvent Extractor, model 200
6.4.2.2 Extraction cells, 11ml
6.4.2.3 Appropriately sized glass or polypropylene filters and tamping rod. Polypropylene filter cloth may be used to cut filters, and is available from Sigma-Aldrich, catalog #Z557722, W 102 cm permeability factor activity 60 cfm. Polypropylene filters should not be used for incompatible solvents, such as hexane.

6.5 Evaporator, either apparatus listed or equivalent device suitable for evaporating water and ethanol

6.5.1 Rotary evaporator with trap and water bath set to 40 $\pm$ 5 °C

6.5.2 Automatic solvent removal system, such as TurboVap II, with appropriate tubes

6.6 YSI analyzer with appropriate membranes or equivalent sucrose quantification method

7. Reagents and materials

7.1 Reagents

7.1.1 Water, HPLC grade

7.1.2 Ethyl alcohol, 190 proof, USP grade

7.2 Materials

7.2.1 Materials necessary for Soxhlet extraction

7.2.1.1 Boiling flasks, round bottom, 500 mL capacity, 24/40 joint, equal to the number of extractions desired

7.2.1.2 Teflon boiling chips or stir bars (stir bars may only be used with heating mantles equipped with stirring capacity)

7.2.1.3 Cellulose filter paper, medium porosity, of appropriate size

7.2.1.4 Buchner funnels, for paper diameter 70 mm or larger

7.2.1.5 200 mL volumetric flasks, necessary for sucrose determination only

7.2.1.6 Desiccator(s) containing desiccant, of a volume large enough to accommodate appropriate glassware

7.2.2 Materials necessary for automatic extraction

7.2.2.1 Collection vials with lids and septa, sized to fit extraction cell volume

7.2.2.2 Extraction cell filters, either glass fiber or polypropylene, cut to fit if necessary

8. ES&H Considerations and Hazards

8.1 Ethyl alcohol is flammable.

8.2 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.

9.3 The test specimen should consist of 5-20 grams of milled sample obtained in such a manner as to ensure that the sample is representative of the entire lot of material. A minimum of 8 g of extracted sample is required for complete compositional analysis.

10. Procedure

10.1 Prepare the sample for extraction.

10.1.1 The moisture content of a biomass sample can change rapidly when exposed to air.

Weigh samples for total solids determination (LAP "Determination of Total Solids and Moisture in Biomass") at the same time as the samples for the extractives determination to avoid errors due to changes in humidity.

Extract the sample- Soxhlet method only

10.2 Prepare the apparatus for extraction.

10.2.1 Dry boiling flasks and other relevant glassware (bump traps and automatic evaporator glassware if desired) in a 105 $\pm$ 5 °C drying oven for a minimum of 12 hours. Remove the glassware and allow it to come to room temperature in a desiccator. Add boiling stones (or stir bars if using heating mantles with stirring capacity) to the flasks, label clearly, and record the oven dry weight (ODW) to the nearest 0.1 mg.. If significant foaming is expected during water extractions, the ODW of the bump traps may also be recorded.

10.2.2 Add 2-10 g of sample to a tared extraction thimble. Record the weight to the nearest 0.1 mg. The amount of sample necessary will depend on the bulk density of the biomass. The height of the biomass in the thimble must not exceed the height of the Soxhlet siphon tube. If the biomass height does exceed the siphon height, incomplete extraction will occur. Label the top edge of the thimble with a pencil.

10.2.3 Assemble the Soxhlet apparatus. Add a 250 mL bump trap between the receiving flask and the Soxhlet tube to control foaming if necessary. Insert the thimble into the Soxhlet tube.

10.3 Analyze the sample for water extractives (if necessary)

10.3.1 Add 190 $\pm$ 5 mL of HPLC grade water to the tared receiving flask. Place the receiving flask on the Soxhlet apparatus. Adjust the heating mantles to provide a minimum of 4-5 siphon cycles per hour.

10.3.2 Reflux for 6-24 hours. The reflux time necessary will depend on the removal rate of components of interest, the temperature of the condensers, and the siphon rate. In some biomass, such as corn stover, the reflux time is usually around eight hours, and any remaining water soluble material will be extracted during the ethanol extraction.

10.3.3 When reflux time is complete, turn off the heating mantles and allow the glassware to cool to room temperature.

10.3.4 If a successive ethanol extraction is to be performed, leave the thimble in the Soxhlet extractor, removing as much residual water from the Soxhlet tube as possible. If an ethanol extraction is not necessary, remove the thimble and transfer the extracted solids, as quantitatively as possible, onto cellulose filter paper in a Buchner funnel. Wash the solids with approximately 100 mL of fresh HPLC grade water. Allow the solids to dry using vacuum filtration or air dry.

10.3.5 Analyze the water extract for sucrose content if desired.

10.3.5.1 Once the water receiving flask has reached room temperature, transfer the water to a 200 mL volumetric flask. Bring to volume with HPLC grade water and mix well. Remove a 10.00 mL aliquot of the solution. Analyze the aliquot using a YSI analyzer equipped with appropriate membranes or equivalent sucrose quantification method. Replace the remaining 190.00 mL of water extract back into the water receiving flask. This removed volume must be compensated for during calculations.

10.4 Analyze the sample for ethanol extractives

10.4.1 Add 190 ± 5 mL 190 proof ethyl alcohol to the tared ethanol receiving flask. Place the receiving flask on the Soxhlet apparatus. Adjust the heating mantles to provide a minimum of 6-10 siphon cycles per hour.

10.4.2 Reflux for 16-24 hours. The reflux time necessary will depend on the removal rate of components of interest, the temperature of the condensers, and the siphon rate.

10.4.3 When reflux time is complete, turn off the heating mantles and allow the glassware to cool to room temperature.

10.4.4 Remove the thimble and transfer the extracted solids, as quantitatively as possible, onto cellulose filter paper in a Buchner funnel. Wash the solids with approximately 100 mL of fresh 190 proof ethanol. Allow the solids to dry using vacuum filtration or air dry.

Extract the sample- Automatic extraction method only

10.5 Prepare the apparatus for extraction.

10.5.1 Dry the collection tubes, but not plastic lids or septa, in a 105 ± 5 °C drying oven for a minimum of 12 hours. Remove the glassware and allow it to come to room temperature in a desiccator. Label each tube clearly and record the oven dry weight (ODW) to the nearest 0.1 mg.

10.5.2 Place two appropriately sized glass filters in the bottom of a labeled extraction cell, tamping down one at a time. A tamping rod is available to fit each extraction cell. Alternatively, a single polypropylene filter of appropriate size may be tamped into the bottom of the extraction cell. Polypropylene filter cloth may be used to cut filters, and is available from Sigma-Aldrich, catalog #Z557722,W 102 cm permeability factor activity 60 cfm. Polypropylene filters should not be used for incompatible solvents, such as hexane.

10.5.3 Add 1-10 g of sample to a tared extraction cell. Record the weight to the nearest 0.1 mg. The amount of sample necessary will depend on the bulk density of the biomass. Do not pack the biomass tightly in the thimble, as this can interfere with complete extraction. Do not use inert fillers to fill dead volume if further analysis of the biomass is required. Screw the ends of the cell on tightly. Place the cell in the automatic extractor, ensuring that the end with the filters is on the bottom.

10.6 Analyze the sample for water extractives (if necessary) and ethanol extractives

10.6.1 Create a method in the automatic extractor software. Using the Dionex ASE system, program the following parameters:

Pressure: 1500 PSI
Temperature: 100 ^{o}C
Preheat time: 0
Heat time: 5 minutes (automatic software default)
Static time: 7 minutes
Flush volume: 150%
Purge time: 120 seconds (optional)
Static cycles: 3

Note: If using 33 ml extraction cells, a flush volume of 150% and three static

cycles may overfill the collection vial. Programming one static cycle per collection vial may be necessary.

Save one program for 100% water, if desired, and another for 100% 190 proof ethanol.

10.6.2 Run each desired method on the sample. To avoid filling up the purge collection vials when running sequential water and ethanol extractions on a large number of samples, extract every sample with water, and then extract every sample with ethanol.

10.6.3 Allow the extraction cell to cool to room temperature. Remove the sample from the cell and allow the solids to air dry.

10.6.4 Analyze the water extract for sucrose content if desired.

10.6.4.1 Once the water collection tube has reached room temperature, transfer the water to a 50 mL volumetric flask. Bring to volume with HPLC grade water and mix well. Remove a 5.00 mL aliquot of the solution. Analyze the aliquot using a YSI analyzer equipped with appropriate membranes or equivalent sucrose quantification method. Replace the remaining 45.00 mL of water extract back into the water collection tube. This removed volume must be compensated for during calculations.

10.7 Remove solvent from the extractives

10.7.1 Combine any solvent from the Soxhlet tube with the solvent in the receiver flask if using Soxhlet method. For automatic extraction method, remove collection vials from instrument.

10.7.2 The solvent may be removed from the extract using either apparatus listed or an equivalent device suitable for evaporating water and ethanol.

10.7.2.1 To remove the solvent using a rotary evaporator, use a rotary evaporator equipped with a water bath set to 40 $\pm$ 5 °C and a vacuum source. Transfer the extract into a tared round bottom boiling flask. The vacuum source should be sufficient to remove solvent without extreme bumping. Continue to remove solvent until all visible solvent is gone.

10.7.2.2 To remove solvent using a TurboVapII, transfer the extract into a tared TurboVap tube if necessary, set the inlet pressure to 15 – 18 psi, and adjust the water bath to 40 °C. Continue to remove solvent until all visible solvent is gone.

10.7.3 Place the flask or tube in a vacuum oven at 40 $\pm$ 2 °C for 24 hours. Cool to room temperature in a desiccator. Weigh the flask or tube and record the weight to the nearest 0.1 mg. If necessary, this step may also be performed on the bump trap to quantify any extract remaining in the bump trap.

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".

$$ODW = \frac{(Weight_{\ thimble\ plus\ sample} - Weight_{\ thimble}) \ x\ \%\ Total\ solids}{100}$$

11.2 Calculate the amount of extractives in the sample, on a percent dry weight basis. If sucrose measurements were preformed, use the second equation to calculate extractives with a correction for the removed sample volume.

$$\%\ Extractives = \frac{Weight_{\ flask\ plus\ extractives} - Weight_{\ flask}}{ODW_{sample}}\ x\ 100$$

$$\%\ Extractives = \frac{Weight_{\ flask\ plus\ extractives} - Weight_{\ flask}}{ODW_{sample}}\ x\ \frac{200mL}{190mL} x100$$

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

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

Where:

X_1 and X_2 = measured values
X_{mean} = the mean of X_1 and X_2

11.4 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 the average percent extractives in the sample on a dry weight basis. Standard deviation and relative percent difference may also be reported.

13. Precision and Bias

13.1 Data obtained by replicate testing of a hybrid poplar sample in one laboratory, via Soxhlet, gave a standard deviation in extractive content of 0.15% and a CV% of 7.6%. Replicate testing of a National Institute of Standards and Technology (NIST) #8494 wheat straw gave a standard deviation of 0.20% and a CV% of 1.6% and NIST #8493 Pinus radiata gave a standard deviation of 0.20% and a CV% of 8.0%

13.2 Prolonged heating of the extractive residue may bias the reported results low because of evaporation of semivolatile constituents. Insufficient heating or using inadequate vacuum can bias the results high due to incomplete removal of solvent.

14. Quality Control

14.1 Reported Significant Figures: Report results as a weight percentage, with two decimal places. Standard deviation and relative percent difference may also be reported.

14.2 Replicates: Run all samples in duplicate.

14.3 Blank: Not applicable.

14.4 Relative percent difference criteria:

14.5 Calibration verification standard:

14.6 Sample size: The sample should be a minimum of three grams of sample per thimble, or the results should be flagged and the lack of precision noted.

14.7 Sample storage: If the extracted sample is greater than 90% solids, it may be stored at room temperature. If it is less than 90% solids, it should be stored in a refrigerator.

14.8 Standard storage: Not applicable.

14.9 Standard preparation: Not applicable.

14.10 Definition of a batch: Any number of samples that are analyzed and recorded together. The maximum size of a batch will be limited by equipment constraints.

14.11 Control charts: NIST standards or a QA/QC material should be control charted to verify reproducibility.

15. Appendices

15.1 None

16. References

16.1 ASTM Standard Test Method E 1690 “Determination of Ethanol Extractives in Biomass” in *2003 Annual book of ASTM Standards volume 11.05* Philadelphia, PA: American Society for Testing and Materials.

16.2 ASTM D1105-84, "Method for Preparation of Extractive-Free Wood." In *1993 Annual Book of ASTM Standards, Volume 04.09.* Philadelphia, PA: American Society for Testing and Materials.

16.3 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

16.4 NREL BAT Team Laboratory Analytical Procedure "Standard Method for the Determination of Total Solids in Biomass."

16.5 TAPPI Test Method T204, "Solvent Extractives of Wood and Pulp." *In Tappi Test Methods.* Atlanta, GA: Technical Association of the Pulp and Paper Industry

16.6 Milne, T. A.; Chum, H. L.; Agblevor, F. A.; Johnson, D. K. (1992). “Standardized Analytical Methods” Biomass & Bioenergy. Proceedings of International Energy Agency Bioenergy Agreement Seminar”, 2-3 April 1992, Edinburgh, U.K.. Vol. 2(1-6), 1992; pp. 341-366