

PROTOCOL: Carbon Extractions to Determine Hemicellulose, Cellulose and Lignin in Leaf Tissue

(i) Equipment

- Ankom 200/220 fiber analyzer
- Electric tea kettle
- Muffle furnace
- Crucibles and crucible tongs
- 1 L beakers
- 2 L beakers
- Heat sealer
- 1 gallon Ziploc bags
- Desiccant packets
- 100°C Oven
- Balance (0.0001g)

(ii) Consumable Materials

- Neutral detergent solution
- Acid detergent solution
- 72% sulfuric acid
- Acid resistant gloves, goggles, and apron
- Filter bags
- Acid resistant filter bag marker
- Metal test-tube racks
- Deionized (DI) water

(iii) Sample Preparation

- Label bags with marker and dry in oven at 100 °C for 30 min. Cool in desiccator for 30 min.
- Weigh each bag, record weight and tare bag.
- Weigh 0.5 g (\pm 0.005 g) of ground, dried sample into bag and record sample weight.
- Include a blank bag and an in-house standard reference material sample in each run (24 samples per run) for a blank bag correction and for ensuring consistency between runs.
- Seal the bag within 0.5 cm of the open edge using the heat sealer. Be sure to also seal the blank bag.
- Spread sample uniformly inside the filter bag by shaking and lightly flicking the bag to eliminate clumping.
- A maximum of 24 bags may be placed in the sample tray. All nine trays are used regardless of the number of bags being processed. Place three bags per tray and then stack trays on the center post with each level rotated 120 degrees to sit in the notches of the tray below it. The weight is placed on top of the empty 9th tray to keep the bag suspender submerged.

(iv) Measurement Procedure

- NDF (neutral detergent fiber) Extraction: Place the samples in the sample tray and insert it into the Ankom 200/220 Fiber Analyzer chamber. Fill the chamber with 1800-1900 ml neutral detergent solution and agitate with heat for 75 min to extract carbohydrates, lipids, pectin, starch, soluble proteins, and non-protein nitrogen. You

will be left with hemicellulose, proteins bound to the cell walls, cellulose, lignin, and recalcitrant materials. Drain the effluent and rinse the samples by agitating them for 5 minutes with 1800 ml of boiling DI water. Repeat 2-3 times for a total of 3-4 rinses. Once rinsing is complete, place sample bags in a metal test tube rack to dry overnight to prepare for ADF extraction.

- ADF (acid detergent fiber) Extraction: Remove the bags from the oven and place them in a Ziploc bag with desiccant to let them cool. Record the weights. Place samples in the sample tree, then into the chamber and add 1800-1900 ml ADF solution. Heat and agitate the samples for 60 minutes to extract hemicellulose and membrane-bound proteins. Drain the effluent and rinse the samples by agitating them for 5 minutes with 1800 ml of boiling DI water. Repeat twice for a total of 3 rinses. Once complete, place sample bags in a metal test tube rack to dry overnight to prepare for ADL extraction.
- ADL (acid determined lignin) Extraction: Remove the bags from the oven and place them in a Ziploc bag with desiccant to let them cool. Record the weights. Add ~ 400 ml 72% sulfuric acid to a 2L beaker containing 48 completely dried sample bags. Place a 1 L beaker on top to keep the bags submerged. Manually agitate the bags once submerged and every 30 minutes thereafter by lifting the 1 L beaker up and down 30 times. Repeat for 3 hours. Pour the concentrated sulfuric acid into a properly labeled waste container, add 1000 ml boiling DI water and agitate to rinse the samples. Rinse the samples 7-8 times until the pH is neutral. Dry samples overnight at 100° C. This final extraction removes cellulose and leaves lignin and recalcitrant materials. Weigh the bags after they've dried and record the final weights.
- Ashing: Rinse the crucibles with DI water and dry them in the furnace for an hour at 150°C. Remove the crucibles from the oven and place them in the desiccator for an hour to cool. Weigh the crucibles once they've reached room temperature. Fold and place the bags in the crucibles once everything is weighed. Place a lid on the crucibles and insert them in the muffle furnace at 500 °C for 5.5 hours. After 5.5 hours, reduce the temperature to 150 °C and leave the samples in the furnace overnight. The next day, remove the crucibles from the furnace using crucible tongs and cool in a desiccator for 2 hours. Turn off the muffle furnace. Weigh the crucibles and record their weight.

(v) Data Preparation and Finalization

Note: blank bag correction (C) = blank bag wt. after extraction / blank bag tare wt.

NDF Calculations

Soluble cell contents =

$$\frac{((\text{Tare wt. sample bag} + \text{sample wt.}) - \text{bag wt. after NDF}) \times 100}{\text{sample wt.}}$$

Hemicellulose, bound proteins, cellulose, lignin, recalcitrants =

$$\frac{(\text{bag wt. after NDF} - (\text{tare wt. sample bag} \times C_{\text{NDF}})) \times 100}{\text{sample wt.}}$$

ADF Calculations

Hemicellulose and bound proteins =

$$\frac{((\text{bag wt after NDF} - (\text{tare wt sample bag} \times C_{\text{NDF}})) - (\text{bag wt after ADF} - (\text{tare wt. sample bag} \times C_{\text{ADF}})) \times 100}{\text{sample wt.}}$$

Cellulose, lignin, and recalcitrants =

$$\frac{(\text{bag wt. after ADF} - (\text{tare wt. sample bag} \times C_{\text{ADF}})) \times 100}{\text{sample wt.}}$$

ADL Calculations

Cellulose =

$$\frac{((\text{bag wt after ADF} - (\text{tare wt sample bag} \times C_{\text{ADF}})) - (\text{bag wt after ADL} - (\text{tare wt. sample bag} \times C_{\text{ADL}})) \times 100}{\text{sample wt.}}$$

Lignin and recalcitrants =

$$\frac{(\text{bag wt. after ADL} - (\text{tare wt. sample bag} \times C_{\text{ADL}})) \times 100}{\text{sample wt.}}$$