A & L GREAT LAKES LABORATORIES, INC.
Standard Operating Procedure

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TITLE: Acid Detergent Fiber and Lignin Analysis in Feeds - Filter Bag Technique (for A200 and A200I)

AUTHOR / JOB TITLE: Amanda Krey / Quality Chemist
Austin Schlarb / Quality Assurance Coordinator

PURPOSE OR SUMMARY: This method determines Acid Detergent Fiber, which is the fiber cellulose and lignin residue remaining after digesting with H_2SO_4 and CTAB. The fiber residues are predominantly cellulose and lignin. Acid Detergent Lignin (ADL) can then be measured using sulfuric acid extraction and ashing.

SCOPE / APPLICATION: This method is applicable for the analysis of Acid Detergent Fiber and Lignin in routine and non-routine Feed samples in the Agricultural Division

DISTRIBUTION: Quality Assurance Officer, Agriculture Department Manager, four (4) copies for Ag Division Manuals

APPROVAL		
Name	Job Title	Date
Linda S. Senger	President	01 / 07 / 2025
[signature]	Quality Assurance Coordinator	01 / 07 / 2025
Amanda K	Quality Chemist	01 / 07 / 2025

HISTORY	
Supersedes	Reason for change
0	Updates to sections **II.A, V.B, VII.A, X.T., XI.D., XII. A., B., XV., XVI., and XIX. Form A.**
1	Updates to sections **V.A, IX.A, XI.,XII.B. and Form A.**
2	Updates to sections: **V.D, VIII.B, X.A., D., XII., XV.B, XVI.B., C., D. and XIX. Form A.**
3	Updates to sections:**VII.B.**
4	Update language on Lignin check sample.

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I. DETECTION LIMIT

A. 0.01% Reporting Limit

II. APPLICABLE MATRIX OR MATRICES

A. This method is applicable to grains, feeds, forages, plant foliage, and all fiber-bearing material

III. INTERFERENCES

A. N/A

IV. SAFETY

A. Follow normal laboratory safety guidelines.

B. Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. Always add acid to water and not the reverse.

C. CTAB will irritate mucous membranes. A dust mask and gloves should be worn when handling this chemical.

D. Acetone is extremely flammable. Avoid static electricity and use a fume hood when handling.

V. EQUIPMENT AND SUPPLIES

A. Analytical Balance—capable of weighing 0.1 mg.

B. Oven—capable of maintaining a temperature of 104 ± 2°C.

C. Digestion instrument—capable of performing the digestion at 100 ± 0.5°C and maintaining a pressure of 10-25 psi. The instrument must be capable of creating a similar flow around each sample to ensure uniformity of extraction (ANKOM200 with 65 rpm agitation, ANKOM Technology).

D. **F57 Filter Bags**—constructed from chemically inert and heat resistant filter media, capable of being heat sealed closed and able to retain 25-micron particles while permitting rapid solution penetration (F57 ANKOM Technology). **Note: Do not use F58 filter bags. F58 filter bags deteriorate in 72% Sulfuric acid (H_2SO_4) during the acid detergent lignin (ADL) procedure.**

E. Heat sealer—sufficient for sealing the filter bags closed to ensure complete closure (1915, ANKOM Technology).

F. Desiccant Pouch—collapsible sealable pouch with desiccant inside that enables the removal of air from around the filter bags (MoistureStop weigh pouch, ANKOM Technology).

G. Marking pen—solvent and acid resistant (F08, ANKOM Technology).

H. NIST SRM 1575a Pine Needles reference material for use as ADF and lignin check sample.

VI. REAGENTS

A. Acid Detergent Solution — Add 20 g cetyl trimethylammonium bromide (CTAB) to 1 L 1.00 N H_2SO_4 previously standardized (premixed chemical solution available from ANKOM). Agitate and heat to aid solution. Or, premixed solution.

B. Sulfuric acid (H_2SO_4) (72% by weight) — 672 mL concentrated sulfuric acid in 328 mL DIW.

C. Acetone purified

VII. SAMPLE COLLECTION, PRESERVATION, SHIPMENT AND STORAGE

A. Samples will be ground (in-house or prior to delivery) in a centrifugal mill with a 10 mesh (2 mm) screen or cutter type (Wiley) mill with a 20 mesh (0.841 mm) screen. Samples ground finer may have particle loss from the filter bags and result in erroneous values.

B. NEON ground samples in the analysis batch will be dried at 70°C for 2 hours, and stored in a desiccator until weighing. When NEON ground samples are placed in the 70°C oven, the caps on scintillation vials must be loosened or removed first.

VIII. QUALITY CONTROL

A. A blank and NIST SRM 1575a pine needle check sample are run every 18 samples or once per batch if the batch
Is less than 18 samples.

B. NIST pine needle check lignin is required to be within 15% of the known value. Otherwise the set must be repeated.

IX. CALIBRATION AND STANDARDIZATION

A. N/A

X. PROCEDURE – ACID DETERGENT FIBER (ADF) BY BAG TECHNIQUE

A. Use a solvent resistant marker to label the F57 filter bags to be used in the analysis.

B. Weigh and record the weight of each empty filter bag (**W1**). **NOTE:** Do not pre-dry filter bags. Any moisture will be accounted for by the blank bag correction.

C. Place 0.45 – 0.50 g of prepared sample in up to 23 of the bags and record the weight (**W2-sample + bag**) of each. Avoid placing the sample in the upper 4 mm of the bag.

D. Include at least one empty bag in the run to determine the blank bag correction (**CB1-for ADF, CB2 for ADL, and CB3-Lignin (LOI)**) of that batch.

NOTE: A running average blank bag correction factor (**C1 for ADF, C2 for ADL, and C3-Lignin (LOI)**) should be used in the calculation of fiber. The inclusion of at least one blank bag in each run is mainly used as an indicator of particle loss. A **CB1, CB2, or CB3** larger than 1.0000 indicates that sample particles were lost from filter bags and deposited on the blank bag during the extraction. Any fiber particle loss from the filter bags will generate erroneous results. If particle loss is observed, then the grinding method needs to be evaluated.

E. Using a heat sealer, completely seal each filter bag closed within 4 mm of the top to encapsulate the sample. **NOTE:** Use sufficient heat to completely seal the filter bags and allow enough cool time (2 sec) before raising the heat sealer arm to remove each bag from the heat sealer.

F. Pre-extract only samples containing >5% fat: Extract samples by placing bags with samples into a container with a top. Pour enough acetone into the container to cover the bags and secure the top. Shake the container 10 times and allow the bags to soak for 10 minutes. Repeat with fresh acetone. Pour out acetone and place bags on a wire screen to air-dry.

Exception – Roasted soybean: Due to the processing of roasted soy a modification to the extraction is required. Place roasted soy samples into a container with a lid. Pour enough acetone into the container to cover the bags and secure the top. Shake the container 10 times and pour off the acetone. Add fresh acetone and allow samples to soak for twelve hours. After the soak time, pour out the acetone and place the bags on a wire screen to air-dry.

G. To eliminate sample clumping, spread the sample uniformly inside the filter bags by shaking and flicking the bags.

H. Place up to 3 bags on each of eight Bag Suspender Trays (maximum of 24 bags). Stack the trays on the center post of the Bag Suspender with each level rotated 120 degrees in relation to the tray below it. Place the empty 9^{th} tray on top.
NOTE: All nine trays must be used regardless of the number of bags being processed.

I. Verify that the Exhaust Hose is connected to the instrument and securely positioned in the drain.

J. Turn the instrument Power Switch to the ON position.

K. Before inserting the Bag Suspender into the Vessel, read the Temperature Controller on the instrument. If the temperature is higher than room temperature, fill the Vessel with cold tap water. The temperature on the Controller will decrease. When the value on the Controller reaches its lowest number and starts to increase, open the Exhaust Valve and exhaust the water. Repeat this process until the number on the Temperature Controller equilibrates to room temperature.

L. Open the Vessel Lid and insert the Bag Suspender with bags into the Vessel and place the Bag Suspender Weight on top of the empty 9th tray to keep the Bag Suspender submerged.

M. When processing 24 sample bags, add 1900-2000 mL of ambient temperature ADF solution to the fiber analyzer vessel. If processing less than 20 bags, add 100 mL/bag of ADF solution (use minimum of 1500 mL to ensure Bag Suspender is covered).

N. Turn Agitate and Heat ON and confirm agitation.

O. Set the timer for 70 minutes and close the lid.

P. When the ADF extraction is complete, turn Agitate and Heat OFF.

Q. Open the drain valve (slowly at first) and exhaust the hot solution before opening the Vessel Lid.

Caution: The solution in the Vessel is under pressure. The exhaust valve needs to be opened to release the pressure and solution prior to opening the Vessel Lid.

R. After the solution has been exhausted, close the exhaust valve and open the Vessel Lid. Add 1900-2000 mL of 70-90°C rinse water. Turn Agitate on and rinse for 5 minutes. If the Heat is ON, the Vessel Lid should be closed. If the Heat is OFF, the Vessel Lid can be open. Repeat 5 minute hot water rinses 2 more times. Just before draining the 3rd rinse, test the water with pH paper. If acid is present repeat rinses until neutral.

S. After the rinsing procedures are complete, open the Vessel Lid and remove the filter bags. Gently press out excess water from the bags. Place bags in a 250 mL beaker and add 250 mL acetone to cover the bags and soak for 8 minutes.

T. Remove the filter bags from the acetone and place them on a wire screen to air-dry. Completely dry in an oven at 104 ± 2°C. (In most ovens the filter bags will be completely dry within 2-4 hours.) Do not place bags in the oven until the acetone in the bags has completely evaporated.

NOTE: When running a lignin procedure or a sequential (NDF/ADF or NDF/ADF/Lignin) with the F57 Filter Bag it is important **not** to dry the bags overnight after the NDF or ADF procedure. A drying time frame of 2-4 hours at 100°C to 105°C is sufficient to thoroughly dry the bags after each procedure. Extended drying times or too high a temperature can compromise the bag's filtration media. In addition be sure to check the water of the fourth rinse during the ADF procedure to ensure all the sulfuric acid has been removed from the bags. If litmus paper shows the presence of acid during the fourth hot water rinse, repeat until neutral.

U. Remove the filter bags from the oven and immediately place them directly into a collapsible desiccant pouch and flatten to remove any air. Cool to ambient temperature and weigh the filter bags (**W3**).

NOTE: Do not use a conventional desiccator container.

XI. PROCEDURE – ACID DETERGENT LINGIN (ADL) IN BEAKER

A. After performing ADF determinations, place dried sample bags into 3 L beaker and add sufficient quantity (approximately 250 mL) of 72% Sulfuric acid (H_2SO_4) to cover the bags.

Note: Bags must be completely dry and at ambient temperature before adding concentrate acid. If moisture is present in the bags, heat generated by sulfuric acid and de-ionized water will affect the results (sample inside bag will char).

B. Place 2 L beaker inside 3 L beaker to keep bags submerged. Agitate bags at start and at 30-minute intervals by pushing and lifting 2 L beaker up and down approximately 30 times.

C. After 3 hours pour off sulfuric acid and rinse with warm water to remove all acid. Repeat rinses until pH is neutral. Rinse with approximately 250 mL of acetone for 3 minutes to remove water.

Note: Do not place bags in the oven until acetone is completely evaporated.

D. Complete drying in oven at 104°C for 2 to 4 hours. Remove bags from oven and place directly into MoistureStop weigh pouch and flatten to remove air. Cool to ambient temperature and weigh **(W4).** Calculate blank bag correction using weight loss of a blank bag upon sulfuric acid extraction **(C2).**

E. Place each bag in a pre-weighed crucible and ash at 525°C for 3 hours. Cool the contents of the crucible with post-ashing and record weight. Calculate the ash weight **(W5)** by subtracting the crucible weight from the ash and crucible weight. Calculate Blank bag ash correction using weight loss upon ignition of a Blank bag sequentially run through ADF and sulfuric acid extraction **(C3).**

XII. DATA ANALYSIS AND CALCULATIONS

Calculations: All weights recorded in grams (g)

Note: If acceptable, Batch Blank Bag CB1, CB2, and CB3 will be included in the running average for C1, C2, and C3.

A. **% ADF** (as-received basis) = $\dfrac{100 \times (W3 - (W1 \times C1))}{W2 - W1}$

Where: W1 = Weight of the bag

W2 = Sample + bag weight

W3 = Dry weight of bag + fiber after ADF extraction process

C1 = Blank bag correction (running average of final oven-dried weight / original blank bag weight)

B. ADL (after 72% Sulfuric acid (H_2SO_4))

% ADL (as-received basis – or if the sample is dried and being reported on a dry-weight basis)

$$= \frac{100 \times (W4 - (W1 \times C2))}{W2 - W1}$$

% Lignin (LOI)

$$= \frac{100 \times (W4 - W5 - (W1 \times C3)}{W2 - W1}$$

C. **% Cellulose** = % ADF - %ADL

Where: W1 = Weight of the bag

W2 = Sample + bag weight

W4 = Dry weight of bag + fiber (after 72% Sulfuric acid (H_2SO_4) extraction process)

W5 = Sample and bag ash weight after ashing process

C2 = Blank bag correction: (Running average of final dry weight (H_2SO_4) / original Blank bag weight)

C3 = Ash corrected Blank bag (Running average of LOI of bag / original Blank bag)

XIII. METHOD PERFORMANCE

A. N/A

XIV. POLLUTION PREVENTION AND WASTE MANAGEMENT

A. Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The USEPA has established a preferred hierarchy of environmental techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the USEPA recommends recycling as the next best option.

B. The quantity of chemicals purchased should be based on expected usage during their shelf life and disposal cost of unused material. Actual reagent preparation volumes should reflect anticipated usage and reagent stability.

C. It is the laboratory's responsibility to comply with all federal, state and local regulations governing waste management, particularly the hazardous waste identification rules and land disposal restrictions, and to protect the air, water and land by minimizing and controlling all releases from fume hoods and bench operation. Compliance with all sewage discharge permits is also required.

XV. DATA ASSESSMENT AND ACCEPTANCE CRITERIA FOR QUALITY CONTROL MEASURES

A. Quality Control materials is within 15% of known values.

B. Batch Blank bag Correction **(CB1, CB2, or CB3)** has an acceptable range between 0.990 and 1.010, which is +/- 1% error tolerance. If Blank Bag Correction is outside acceptable range, it will not be added to running average blank bag correction factor and corrective actions will be taken as noted below.

XVI. CORRECTIVE ACTIONS FOR OUT-OF-CONTROL DATA

A. If Quality Control materials data is out of accepted criteria, then perform maintenance and/or recalibration, then re-analyze samples in that run or batch.

B. If Batch Blank bag Correction data **(CB1, CB2, or CB3)** is out of acceptable criteria, then re-analyze samples in that run or batch. Additionally, all NEON project samples in that run or batch should be reported to NEON Science and the Collections and Laboratory Analysis group (CLA) as soon as possible about potential of fiber particle loss from the filter bags. The grinding method of the samples in that run or batch needs to be evaluated.

C. If a set of NEON Samples are re-analyzed and the issues above persist and/or there is insufficient sample for re-analysis, data will be reported using quality flag entries as follows:

1. accuracyQF = '**run QA criteria not met'** if QC materials are out of acceptance range, flag all samples in the batch

2. measurementQF = "**possible sample leakage - increased uncertainty'** when blank bag correction indicates potential fiber particle loss, flag all samples in the batch.

3. measurementQF = '**other**' if there is some other issue with an individual sample measurement or set of samples, provide more detail in remarks

D. If there are no data quality issues to report, both quality flag fields will report "**OK**."

XVII. CONTINGENCIES FOR HANDLING OUT-OF-CONTROL OR UNACCEPTABLE DATA

A. Reference A&L Great Lakes Laboratories, Inc. 'Laboratory Quality Assurance Manual'.

XVIII. REFERENCES

A. ANKOM Technology. Acid Detergent Fiber in Feeds – Filter Bag Technique (for A200 and A2001). ADF Method, Method 5. 2017.

B. ANKOM Technology. Method 8 – Determining Acid Detergent Lignin in Beakers. 2016.

XIX. TABLES, DIAGRAMS, FLOWCHARTS AND VALIDATION DATA

A. Form A – Lignin Bench sheet

Form A

ADF		**Balance Used:**					**Date:**			**Weighed By (Initials):**				
Lignin		**Technician:**					**Date:**							
		W1	**W2**	**W3**			**W4**		**W5**					
Sample Number	Bag #	Bag Wt.	Bag Wt + Sample(g)	ADF Final Bag Wt.	Sample Weight (g)	ADF Fiber %	ADF-C Final Bag Wt.	ADF-C %	OM LOI	Ash Weight	Crucible Weight	Crucible Wt. After Ashing	Lignin % LOI	Cellulose %
	1				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	2				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	3				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	4				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	5				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	6				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	7				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	8				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	9				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	10				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	11				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	12				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	13				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	14				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	15				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	16				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	17				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	18				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	19				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	20				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	21				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	22				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
	23				0	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
BLANK	24				0.0000	#DIV/0!		#DIV/0!	0.0000	0.0000			#DIV/0!	#DIV/0!
						C1B		C2B					C3B	
			Running Blank Average			1.0001		0.9988					0.9965	
						C1		C2					C3	