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Measuring leaf carbon fractions with the ANKOM2000 Fiber Analyzer

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ABSTRACT

Here we describe the standardized protocol used by the [Canadian Airborne Biodiversity Observatory](#) (CABO) to measure carbon fractions in leaf samples, using the [ANKOM2000 Fiber Analyzer](#). Prior to the analysis, leaf samples are oven-dried, and then ground with a cyclone mill (2-mm screen). Then, carbon fractions are measured using a sequential digestion procedure to measure neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL). Finally, the residues are ashed in a muffle furnace to determine inorganic recalcitrant materials. This protocol is based on the [manufacturer's protocols](#).

EXTERNAL LINK

www.caboscience.org

MATERIALS

NAME	CATALOG #	VENDOR
Fiber Filter Bags F57	CA-11022-996	VWR international Ltd
Neutral detergent solution	CA11023-022	VWR international Ltd
Acid detergent solution	CA11023-012	VWR international Ltd
Sulfuric acid	A300S-212	Thermo Fisher Scientific
Sodium sulfite	S430-500	Thermo Fisher Scientific
Alpha-amylase	CA11023-004	VWR international Ltd

STEPS MATERIALS

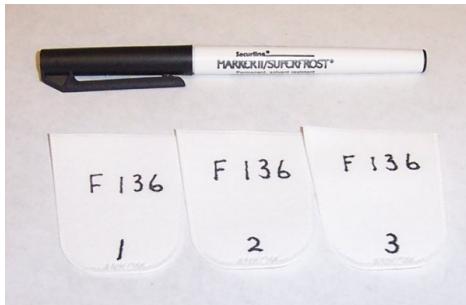
NAME	CATALOG #	VENDOR
Sodium sulfite	S430-500	Thermo Fisher Scientific
Alpha-amylase	CA11023-004	VWR international Ltd
Paper pH Strips	13-640-508	Thermo Fisher Scientific
Alpha-amylase	CA11023-004	VWR international Ltd

BEFORE STARTING

Leaf samples need to have been dried for 72 h at 65°C and then ground with a cyclone mill (2-mm screen).

Sample preparation

- 1 Use a solvent resistant marker to label the filter bags to be used in the analysis.



- 2 Weigh and record the weight of each empty filter bag to the nearest 0.0001 g (in Fulcrum). Zero the balance.



NOTE: Do not pre-dry filter bags. Any moisture will be accounted for by the blank bag correction.



Sartorius Practum

Analytical balance

Sartorius 224-1S



- 3 Place 0.45–0.50 g of prepared sample in up to 23 of the bags and record the weight to the nearest 0.0001 g (in Fulcrum) of each. Avoid placing the sample in the upper 4 mm of the bag.
- 4 Include at least one empty bag in the run to determine the blank bag correction (C1).



NOTE: A running average blank bag correction factor (C1) 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 C1 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.

- 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 s) before removing each bag from the heat sealer.



Impulse Heat Sealer

Heat Sealer

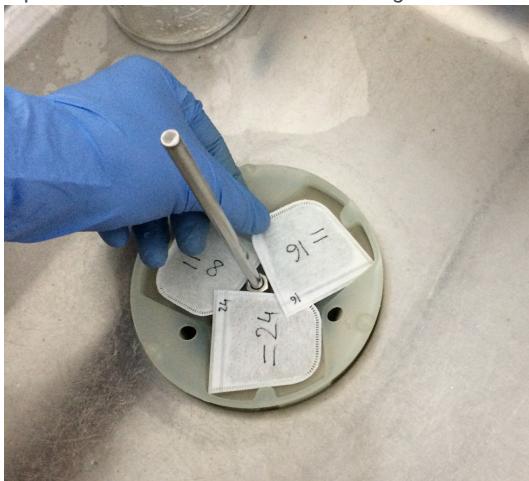
Uline H-163 [🔗](#)



- Spread the sample uniformly inside the filter bags by gently shaking the bags to eliminate clumping.

Neutral Detergent Fiber

- 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 9th tray on top.



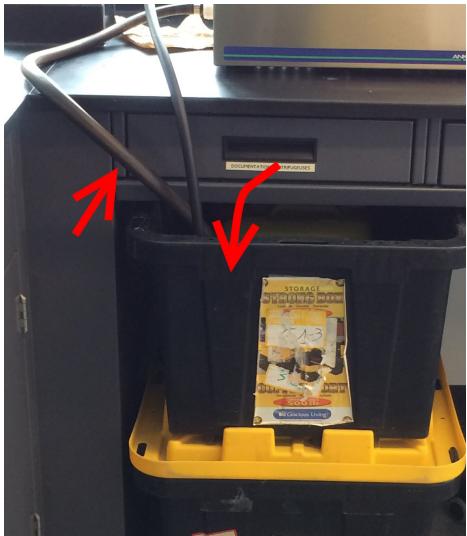


NOTE: All nine trays must be used regardless of the number of bags being processed.

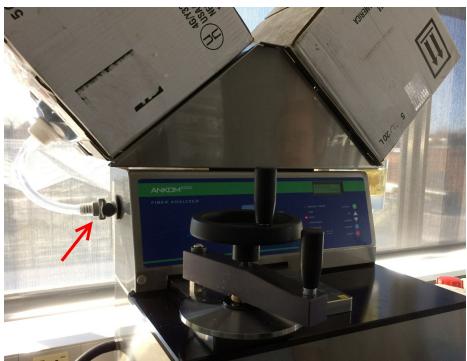
- 8 Verify that the hot water supply is on (room B-236).



- 9 Verify that the drain hose is securely positioned in an empty **yellow** waste bin.



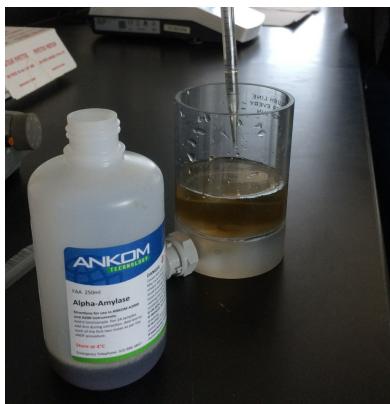
- 10 Attach the Neutral Detergent (ND) solution hose to the Cubetainer and then to Port A on the instrument.



- 11 Add 50 ml of tap water in the alpha-amylase dispenser. Add **8 ml** of alpha-amylase and enough tap water to fill the dispenser (until the line). Attach the Amylase Dispenser Assembly to Port B on the instrument.

Keep the pipette and alpha-amylase bottle next to the instrument for step 16.

Weigh **20 g** of Sodium sulfite and keep it for step 16.



The ANKOM2000 will automatically add the amylase solution to the first and second rinse.

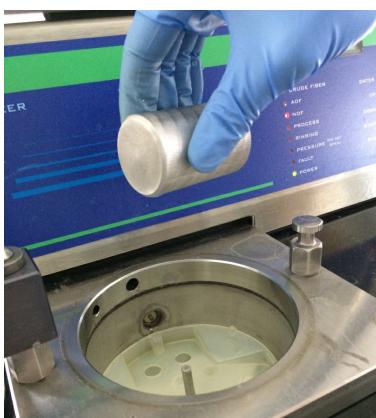


Alpha-amylase

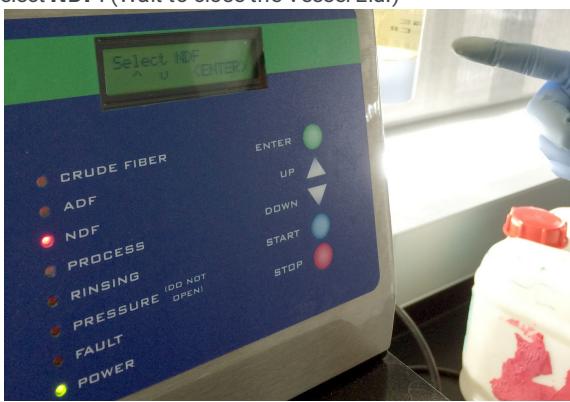
by VWR international Ltd

Catalog #: CA11023-004

- 12 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.



- 13 Follow the instructions on the ANKOM2000 display:
Select **NDF**. (Wait to close the Vessel Lid.)



14 The screen will show: Insert samples. Press **Enter**.

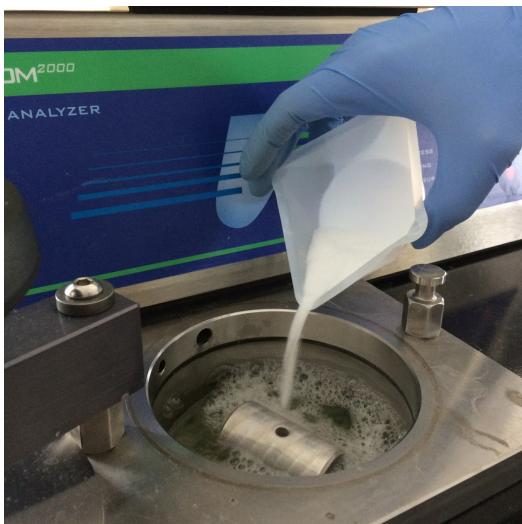


15 Confirm hot water is on (>70 °C) and press **START**.



16 After the ND solution has been automatically inserted and agitation begins, manually add 20g of Na₂SO₃ and 4.0 ml of alpha-amylase.

20 g Na₂SO₃ 4 ml alpha-amylase



 **Sodium sulfite**
by Thermo Fisher Scientific
Catalog #: S430-500

 **Alpha-amylase**
by VWR international Ltd
Catalog #: CA11023-004



Fiber analyzer

Ankom A2000



- 17 Close the Vessel Lid.



Fiber analyzer

Ankom A2000



This step takes about 01:30:00 .

- 18 When the NDF extraction and rinsing procedures are complete, open the Vessel Lid and remove the filter bags. Gently press out excess water from the bags.



HOT! Be careful!



Fiber analyzer

Ankom A2000



- 19 Completely dry the filter bags in an oven at $102 \pm 2^\circ\text{C}$ for 3 h.

03:00:00

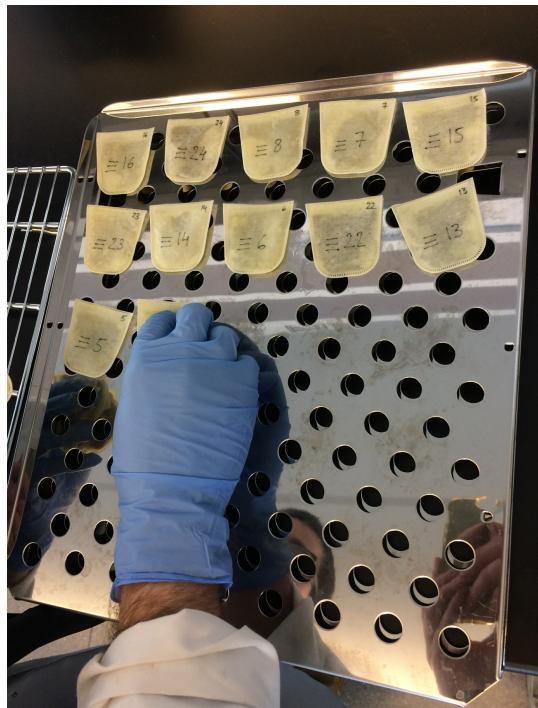
During that time, go to Step 20 and Flush Procedure (Step 21).



Oven

Oven forced-air convection

Fisher Isotemp 15-103-0510



- 20 During that time, run a flush procedure if necessary.

Detach both cubitainers from ports A and B.



The Flush procedure allows you to clean the system with water. This procedure should be used when changing from one procedure to another (e.g., when preparing to do an ADF analysis after completing an NDF analysis) or when doing an NDF procedure after a previous NDF procedure with more than 2 h between procedures, or before storing the instrument.



Amylase tends to be sticky. You should always clean the Amylase Dispenser Assembly and flush Port B after every NDF procedure unless the next procedure you are running is another NDF, and you run it within 2 h of the previous procedure.

- 21 Flush procedure:

Verify that the drain hose is securely inserted into a waste bin.



- 22 Flush procedure:

Press the arrow keys on the Keypad until you see "Select Flush" on the Display.

Select Flush
^ v <Enter>

- 23 Flush procedure:

Press **ENTER** on the Keypad.

- 24 Flush procedure:

Attach the Amylase Dispenser Assembly to port B.

- 25 Flush procedure:
Fill the dispenser with 100 ml of hot water.



- 26 Flush procedure:
Attach the Amylase Dispenser Assembly to port A.

- 27 Flush procedure:
Fill the dispenser with about 100 ml of hot water.



If you press and hold the START key on the Keypad during the Flush operation, water will flow into the Vessel. This will rinse the bottom of the Vessel, but it will not rinse all the way to the top. If you need to rinse anything from the top part of the inside of the Vessel, pour hot water into the Vessel as needed during the Flush operation.

- 28 When dry, remove the filter bags from the oven and immediately place them directly into a collapsible desiccant pouch and flatten it to remove any air. Cool to ambient temperature for **00:30:00**.



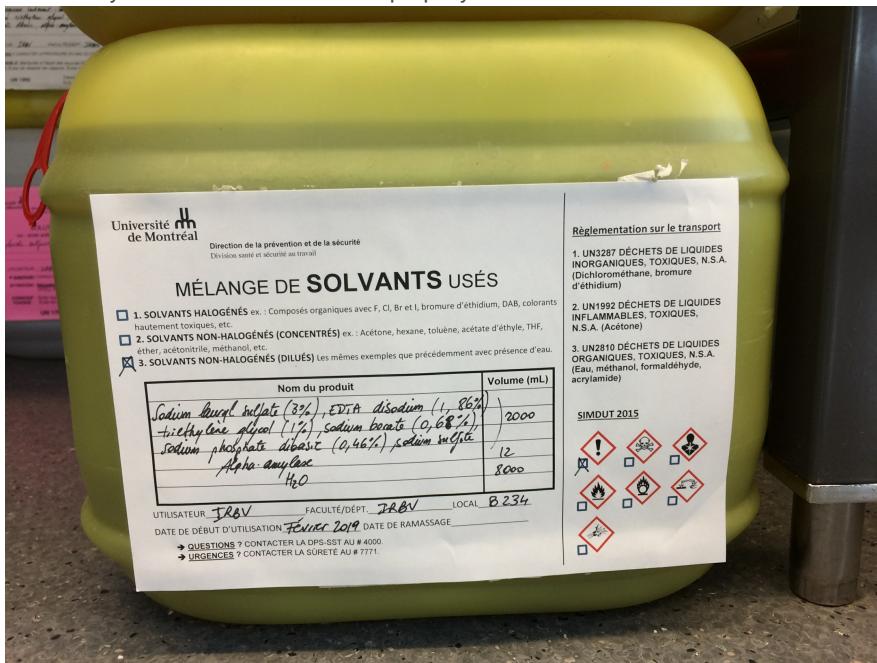
NOTE: Do not use a conventional countertop or cabinet desiccator.

- 29 Weigh the filter bags to the nearest 0.0001 g and add record the weight (in Fulcrum).



If not processing immediately with the Acid Detergent Fiber (ADF) steps, put the filter bags in the dessicator until the next step.

- 30 Remove the yellow waste bin and label it properly.



Wait few hours to let the waste cool down before adding the label (it does not stick when it is hot).

- 31 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 9th tray on top.



NOTE: All nine trays must be used regardless of the number of bags being processed.

- 32 Insert the drain hose securely into an empty **white** waste bin.



33 Read the Temperature Controller on the right side of the instrument.



If the temperature is higher than 20 °C, cool the Vessel as follows:

- a. Fill the Vessel with cold water.
- b. When the Temperature Controller reads 20 °C, run the Flush Procedure to drain the water.
- c. Repeat steps a and b if necessary.

34 Attach the acid detergent (AD) solution hose to the Cubetainer and then to Port B on the instrument.

35 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.

36 Follow the instructions on the ANKOM2000 display:

- a. Select ADF.
- b. Close the Vessel Lid.
- c. Confirm hot water is on (>70°C).
- d. Press **START**.

⌚ 01:00:00

37 When the ADF extraction and rinsing procedures are complete, open the Vessel Lid and remove the filter bags. Gently press out excess water from the bags.



HOT! Be careful!

38 Completely dry the filter bags in an oven at 102 ± 2°C for 3 h.

⌚ 03:00:00



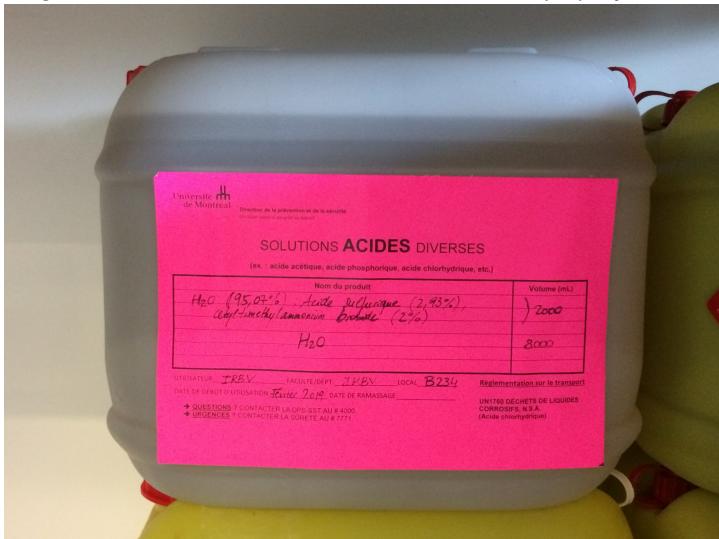
Oven

Oven forced-air convection

Fisher Isotemp 15-103-0510



- 39 During that time, remove the white waste bin and label it properly.



The label will not stick if the content is hot. Wait a few hours to cool down before sticking the label.

- 40 During that time, run a flush procedure if necessary.

Detach both cubitainers from ports A and B.



The Flush procedure allows you to clean the system with water. This procedure should be used when changing from one procedure to another (e.g., when preparing to do an ADF analysis after completing an NDF analysis) or when doing an NDF procedure after a previous NDF procedure with more than 2 h between procedures, or before storing the instrument.

- 41 Flush procedure:

Verify that the drain hose is securely inserted into a waste bin.



- 42 Flush procedure:
Press the arrow keys on the Keypad until you see "Select Flush" on the Display.



- 43 Flush procedure:
Press **ENTER** on the Keypad.
- 44 Flush procedure:
Attach the Amylase Dispenser Assembly to port B.

- 45 Flush procedure:
Fill the dispenser with 100 ml of hot water.



- 46 Flush procedure:
Attach the Amylase Dispenser Assembly to port A.

- 47 Flush procedure:
Fill the dispenser with 100 ml of hot water.



If you press and hold the START key on the Keypad during the Flush operation, water will flow into the Vessel. This will rinse the bottom of the Vessel, but it will not rinse all the way to the top. If you need to rinse anything from the top part of the inside of the Vessel, pour hot water into the Vessel as needed during the Flush operation.

- 48 Remove the filter bags from the oven, immediately place them into a collapsible desiccant pouch, and flatten it to remove any air. Cool to ambient temperature.



NOTE: Do not use a conventional desiccator container.

- 49 Weigh the filter bags to the nearest 0.0001 g and record the weight (in Fulcrum).



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Analytical balance

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If not proceeding immediately with the acid lignin method, place the filter bags in the dessicator until the next step.

50 Prepare a 72% H₂SO₄ solution or take a 250 ml bottle of 72% H₂SO₄.



Danger, highly corrosive.

Exothermic reaction when mixing H₂SO₄ and water. **ALWAYS ADD ACID TO WATER (slowly) AND NOT THE OPPOSITE!**

Wear gloves, labcoat, safety glasses.

Work under the chemical hood.



Under the chemical hood, place a stirring plate, 2 L glass beaker and a magnetic stir bar.

Use a cylinder to measure 242 ml of dH₂O and pour in the beaker.

Start stirring (slow speed).

Use a glass cylinder to measure 758 mL of H₂SO₄ and **SLOWLY** pour into the beaker.

Wait at least 1 h to for the solution to cool down.

In a 1 L glass cylinder, adjust the final volume to 1 L with dH₂O.

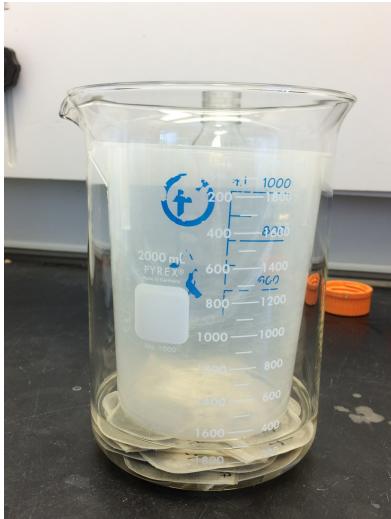
When completely cooled, aliquot in 250 ml bottles.

51 Place dried filter bags with samples into a 2 L glass beaker and completely cover the bags with 72% H₂SO₄ (approximately 250 ml).

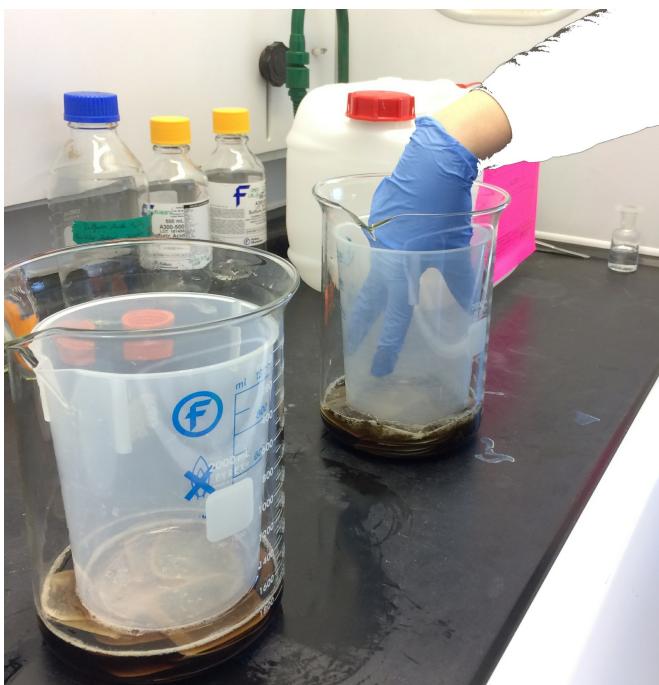


Filter bags **must be completely dry** and at ambient temperature before adding concentrated acid. If moisture (even ambient moisture) is present in the bags, heat generated by the H₂SO₄ and H₂O reaction will adversely affect the results.

- 52 Place a 1 L beaker inside the 2 L beaker to keep the filter bags submerged.



- 53 Agitate the bags at the start and at 30-minute intervals by gently pushing and lifting the 1 L beaker up and down approximately 30 times.



54 After 3 h, pour off the H₂SO₄ and rinse with tap water to remove all of the acid.



If acid remains in the filter bags when they go into the drying oven, the samples will burn, leading to erroneous results.



H₂SO₄ and rinse water must be poured in a **white** waste bin, with a pink label.

55 Repeat rinses until the pH paper shows neutral color when touching the bags. Handle the bags gently during rinsing. Fine lignin particles can exit the filter bag if not handled carefully.



Paper pH Strips

by Thermo Fisher Scientific

Catalog #: 13-640-508

56 Dry the bags in an oven at 100 °C for 3 h.

⌚ 03:00:00



Oven

Oven forced-air convection

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- 57 Remove the filter bags from the oven and place them directly into a Desiccant/Moisture Stop pouch. Flatten the pouch to remove air. Cool to ambient temperature and weigh the bags to the nearest 0.0001 g. Record the weight (in Fulcrum).



Ashing

- 58 Rinse 24 crucibles and covers with dH₂O.
- 59 Dry the crucibles and covers in an oven at 150 °C for 1 h.

 Oven
Oven forced-air convection
Fisher Isotemp 15-103-0510 



- 60 Cool down crucibles (with lids on) in a metal dessicator for 1h30min.

⌚ 01:30:00



Fisherbrand™ Stainless Steel Desiccator
with Stainless Steel Shelves
Metal Dessicator

Fisher S02123



- 61 Weigh empty crucibles, with lids, to the nearest 0.0001 g. Record the weight (in Fulcrum).



Do not wear lab gloves when weighing in this section. Gloves cause static.



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Analytical balance

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- 62 Fold filters bags into four and insert each bag into its crucible, with the corresponding sample number. Cover with the lid.

- 63 Put the crucibles (with lids on) in the muffle furnace at 500 °C for 5 h.

⌚ 05:00:00



Thermo Scientific™ Thermolyne™ Largest
Tabletop Muffle Furnaces
Muffle furnace

Thermolyne 10-505-13



64 Change the temperature at 150 °C, O/N.

⌚ 14:00:00



Thermo Scientific™ Thermolyne™ Largest
Tabletop Muffle Furnaces
Muffle furnace

Thermolyne 10-505-13 [🔗](#)



65 Turn off the muffle furnace and transfer the crucibles (with lids on) in a metal dessicator for 1h30min.

⌚ 01:30:00



Fisherbrand™ Stainless Steel Desiccator
with Stainless Steel Shelves
Metal Dessicator

Fisher S02123 [🔗](#)



66 Weigh crucibles with lids on to the nearest 0.0001 g. Record the weight (in Fulcrum).



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Analytical balance

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67 Wash crucibles and lids with tap water.



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