**Cell Wall Crude Composition Assays**

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**References:**

* Foster CE, Martin TM, Pauly M. Comprehensive compositional analysis of plant cell walls (lignocellulosic biomass) part II: carbohydrates. J Vis Exp. 2010 Mar 12;(37):1837. doi: 10.3791/1837. PMID: 20228730; PMCID: PMC3145335.
* Roig-Oliver M., Bresta P., Nadal M., Liakopoulos G., Nikolopoulos D., Karabourniotis G., Bota J., Flexas J., Cell wall composition and thickness affect mesophyll conductance to CO2 diffusion in Helianthus annuus under water deprivation, Journal of Experimental Botany, Volume 71, Issue 22, 31 December 2020, Pages 7198–7209, <https://doi.org/10.1093/jxb/eraa413>

**Background:**

The following protocol for crude cell wall component analysis was developed by modification and combination of the above references and those listed under the individual subheadings. The equipment and supplies to conduct this protocol in full as written are available in the IGB GEGC theme at UIUC. This protocol was developed and successfully used on wild type and transgenic Nicotiana tabacum.

**Expected Performance:**

For a guideline of general success in completing this protocol, please see the below table of published and our currently unpublished expected standard errors for each item reported.

|  |  |  |
| --- | --- | --- |
| **Cell Wall Component** | **Roig-Oliver et al 2020 standard error** | **Long Lab unpublished standard error** |
| Moisture (%) | NA | 0.33 - 0.47 |
| mg AIR/mg leaf tissue (dry matter basis) | 0.08 – 0.14 | 0.007 – 0.012 |
| ug Starch (as Glucose)/mg leaf tissue (dry matter basis) | NA | 2.19 - 7.18 |
| ug Total Soluble Sugar (as glucose)/ mg leaf tissue (dry matter basis) | NA | 1.83 - 3.66 |
| ug Hemicellulose (excluding Cellulose bound such as Glycans, as glucose)/mg AIR | 5.41 – 30.73 | 4.61 – 13.56 |
| ug Cellulose (including Cellulose bound such as Glycans, as glucose)/mg AIR | 5.26 – 29.81 | 2.30 – 3.21 |
| ug Pectin (as Galacturonic Acid)/mg AIR | 1.02 – 8.02 | 1.96 – 11.30 |

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# Tissue Sampling, Moisture Analysis, and Grinding:

**References**

**Reagents**

* Liquid nitrogen
* Dry ice

**Materials**

* Aluminum foil
* Sharpies
* 15 mL centrifuge tubes (Fisher [05-539-4](https://www.fishersci.ca/shop/products/fisherbrand-easy-reader-polypropylene-centrifuge-tubes-9/055394))
* Grinding beads, 4 mm SPEX stainless steel grinding bead (SPEX; [2150](https://www.fishersci.ca/shop/products/stainless-steel-grinding-balls-4-11-mm-diameter/2150?searchHijack=true&searchTerm=SPEX%3B+2150&searchType=RAPID&matchedCatNo=SPEX%3B+2150))
* 2 mL, screw cap tubes (Fisher, [02681375](https://www.fishersci.com/shop/products/fisherbrand-sterile-microcentrifuge-tubes-screw-caps-6/02681375))

**Equipment**

* Scissors
* Tweezers, Large
* Cryo-gloves
* Liquid nitrogen dewar, 1 L
* Styrofoam cooler
* -80C Freezer
* Spatula
* Funnel
* Analytical balance
* Freeze-dryer
* Tissue grinder, SPEX Sample Prep Genogrinder 2010
* Microtube rack

**Protocol**

1. Sample whole leaves with the midrib cut out (~6-11 g) into labeled aluminum foil envelopes at mid-day.
   1. **Optional:** If starch quantification is not desired, dark adapting the leaves prior to sampling would increase quantity of AIR extracted and simplify AIR cleanup protocol.
2. Flash freeze in liquid nitrogen for ~10-20 seconds. Holding the envelope completely submerged with a large pair of tweezers until bubbling slows or ceases.
   1. Envelope must be firmly held with large tweezers while submerged in liquid nitrogen or the envelope will poof up and float on top of the liquid nitrogen.
3. Quickly place envelope between two blocks of dry ice until it can be transferred to the -80C freezer for long term storage.
4. Weigh and record the weight of empty, labeled 15 mL centrifuge tubes.
5. Place pre-weighed tubes on dry ice and allow to cool for 15-20 minutes.
6. Transfer sample envelopes from -80C storage to dry ice. Use a funnel, tweezers, and spatula transfer as much plant tissue from envelope to pre-weighed labeled tube as possible (3-6 g) while keeping all items on dry ice.
   1. Any unused tissue can be stored long term at -80C in the foil envelopes.
7. Weigh tubes with sample and record.
   1. Weigh one at a time and leave tubes on dry ice between weighing.
8. Remove caps, cover tubes with parafilm and poke several holes in the parafilm using a needle or 10 ul pipette tip.
   1. Although tube manufacture is fairly consistent, for most accurate measurements label caps and return the exact pre-weighed cap to the exact pre-weighed tube for downstream measurements.
9. Prepare freeze-dryer by pre-cooling and vacuuming to equilibration based on manufacturer’s instructions.
10. Transfer tubes to freeze dryer and lyophilize at >-40C and <0.2 bar for approximately 5 days until a steady state weight is reached.
    1. Drying time may vary depending on moisture content, tissue size, number of samples, species, etc. Samples are dry when weights do not change across multiple days.
11. Recap and weigh tube and dry sample, recording weight.
    1. Difference in initial tissue weight and final tissue weight divided by initial sample weight times 100 is percent moisture.

( Wtinitial – Wtfinal) /Wtinitial X 100 = % Moisture

1. Add two 4 mm SPEX stainless steel grinding bead (SPEX; 2150) to each tube and recap.
2. Grind samples on SPEX Sample Prep Genogrinder 2010 for 10 minutes at 1200 rpm, remove from grinder, inverted tube several times to remove beads and unground portions of leaf from conical tip of tube, and grind an additional 5 minutes at 1200 rpm
   1. Ensure the Genogrinder is properly balanced before grinding.
   2. Follow all manufacturer instructions for Genogrinder.
   3. Capped, ground tissue samples can be stored at room temperature for several months.
3. Weigh and record the weights of pre-labeled 2 mL screw cap tubes.
   1. Although tube manufacture is pretty consistent, for most accurate measurements label caps and return the exact pre-weighed cap to the exact pre-weighed tube for downstream measurements.
4. Weigh 100-200 mg ground leaf tissue into each tube, record weight.
   1. Capped and weighed tissue aliquots can be stored at room temperature for several months.

# Fractionation of Sugars and Starch/Alcohol Insoluble Residue (AIR):

**References**

* Foster CE, Martin TM, Pauly M. Comprehensive compositional analysis of plant cell walls (lignocellulosic biomass) part II: carbohydrates. J Vis Exp. 2010 Mar 12;(37):1837. doi: 10.3791/1837. PMID: 20228730; PMCID: PMC3145335.
* Marília Gaspar, Paula Moreira Felix Costa, Marcos Pereira Marinho Aidar, A new rapid and sensitive enzymatic method for extraction and quantification of starch in plant material, Hoehnea Volume 34, Issue 4, December 2006, Pages 425-431,   <https://doi.org/10.1590/S2236-89062007000400001>.
* Pak S. Chow, Simon M. Landhäusser, A method for routine measurements of total sugar and starch content in woody plant tissues, Tree Physiology, Volume 24, Issue 10, October 2004, Pages 1129–1136, <https://doi.org/10.1093/treephys/24.10.1129>

**Reagents**

* Ethanol, 100%, 200 proof, 1 gallon (Decon Labs, [2716](https://deconlabs.com/products/200-proof/))
* Water, distilled
* Chloroform, ACS grade, 99.8%, 1 L (Fisher, [AA32614K2](https://www.fishersci.com/shop/products/chloroform-acs-99-8-thermo-scientific/AA32614K2))
* Methanol, ACS grade, 1 L (Fisher, [A412-1](https://www.fishersci.com/shop/products/methanol-certified-acs-fisher-chemical-10/A4121#?keyword=METHANOL%20ACS%204L%2099.8%%20(67-56-1)))
* Acetone, ACS grade, 1 L (Fisher, [A18-1](https://www.fishersci.com/shop/products/acetone-certified-acs-fisher-chemical-12/A181#?keyword=ACETONE%20REAG%204L%20(67-64-1)))

**Prepared Reagents**

* 80% Ethanol, ~100 mL
  + 80 mL of ethanol, add 20 mL of distilled water mix.
    - Ethanol is miscible in water. Measure separately before mixing.
* 1:1 v/v Chloroform:Methanol wash, 50 mL
  + In a chemical fume hood, mix 25 mL chloroform with 25 mL methanol.
  + Wear proper PPE including lab coat, goggles, and gloves.

**Materials**

* Tips, Repeat pipettor, 25 mL (Fisher, [13-683-707](https://www.fishersci.com/shop/products/eppendorf-combitips-advanced-pipetter-tips-standard-eppendorf-quality-tips-9/p-4909476#?keyword=combitip%20repeat%20pipette%20tip))
* 2 mL, screw cap tubes, (Fisher, [02681375](https://www.fishersci.com/shop/products/fisherbrand-sterile-microcentrifuge-tubes-screw-caps-6/02681375))
* 8 mL, 13 X 16 mm glass tubes (Fisher, [14-930AA](https://www.fishersci.com/shop/products/kimax-reusable-tubes-rubber-lined-screw-cap-5/14930AA))
* Glass Pasteur pipet, (Fisher, [13-678-8C](https://www.fishersci.com/shop/products/wheaton-disposable-pasteur-pipets/136788C?crossRef=136786B#?keyword=136786B))

**Equipment**

* Repeat pipettor (Fisher, [13-683-552](https://www.fishersci.com/shop/products/repeater-e3-bundle-including-charger-stand/13683552#?keyword=combitip%20repeat%20pipetter) or equivalent)
  + Alternatively, a standard 100-1000 ul micropipette can be used but is not as efficient.
* Graduated cylinder, 100 mL
* Vortex
* Water bath
* Floating foam tube racks ([Fisherbrand™ HS2165C](https://www.fishersci.ca/shop/products/hdpe-floating-hdr-foam-tube-rack/360992328))
* Centrifuge, microtube
* Speed-vac
* -20C Freezer
* Chemical Fume hood
* Oven, 35oC
* Microtube rack

**Protocol**

1. Add 1 mL of 80% ethanol to each sample.
   1. A repeat pipettor is useful for large sample numbers.
2. Vortex to mix the samples until all powder dissolved.
3. Incubate the samples at 80 °C in a water bath for 20 minutes.
4. Centrifuge the tubes at max speed (>15,000 g) for 3 minutes to precipitate solids.
5. Decant the supernatant into an 8 mL tubes, glass round bottom 13 x 100 mm.
   1. 8 mL tubes are used because they fit in the GEGC speed-vac. Use tubes appropriate for the drying apparatus in the lab.
   2. **Optional:** If sugar quantification is not desired, ethanolic supernatant can be discarded in the hazardous waste (UIUC DRS UI # 80,501 Ethanol/Acetone/Water).
6. Repeat addition of 80% ethanol, heating at 80 °C for 20 min, centrifuging, and decanting the supernatant 6 times, until supernatant is clear. All the supernatants from the same sample should be combined into the correctly labeled 8 mL tube after each centrifugation.
   1. Store 8 mL supernatant tubes on ice or at 4 °C while tissue is incubating at 80 °C.
   2. After final 80% ethanol extraction, the ethanolic supernatant can either be stored at -20oC for several months or continue with the protocol below at “Sugar Fraction – Ethanolic Supernatant, step 7”.
   3. After final 80% ethanol extraction, the remaining pellet should be washed and dried the same day; continue with the protocol below at “Starch/AIR Fraction – Pellet, step 7”.

## Sugar Fraction – Ethanolic Supernatant

1. After combining the last ethanolic supernatant, remove the ethanol using a Speed Vac Concencentrator, following manufacturers recommendations.
   1. Thermo-Fisher SPD12 Speed-Vac at 0.1 HPr, 35 °C, and Thermo-Fisher RVT404 refrigerated vapor trap -108 °C. Ensure Speed-Vac is properly balanced.
      1. There are three on switches: refrigerated vapor trap, speed-vac, and vacuum pump on floor. All three must be on for operation.
   2. Ethanolic sugar supernatants and desiccated sugar from ethanolic sugar supernatants can be stored at -20C for several months.
2. Add 1 mL of distilled or ultrapure water to the dried sugars in the tube.
3. Vortex to resuspend all sugar.
   1. If polystyrene 8 mL tubes were used, the 1 mL of resuspended sugar must be transferred to a polypropylene microcentrifuge tube. If glass or polypropylene 8 mL tubes were used, continue to the next step.
4. In a chemical fume hood, add 500uL of chloroform.
   1. Prime the pipette tip by filling it with 500 ul chloroform, ejecting the chloroform back into the reagent container, and filling it again. This will prevent the chloroform from leaking out of the pipette and is only necessary when a fresh pipette tip is used.
   2. Chloroform is an organic solvent. Wear proper PPE including lab coat, gloves, and googles. Read safety data sheet before handling.
5. Vortex.
6. Centrifuge the tubes at >4000 g for 5 minutes to separate the solvent phases.
7. Transfer only the aqueous sugar phase (upper phase) to a new labeled 2 mL tube.
   1. If glass Pasteur pipet is used for transfer, discard in sharps container.
   2. Cleaned aqueous sugar samples can be stored for several months at -20C.
   3. Discard chloroform in hazardous waste (UIUC DRS UI # 80,050 Chloroform/Methanol only).
8. Proceed with “Optional: Charcoal Cleanup of Sugar Samples” or directly to “Glucose Quantification for Analysis of Total Sugar, Digested Hemicellulose, and Digested Cellulose.”

## Starch/AIR Fraction – Pellet

1. In a chemical fume hood, to remaining starch pellet add 1 mL 1:1 v/v chloroform:methanol wash.
   1. Wear appropriate PPE including gloves, goggles, and lab coat.
2. Vortex thoroughly.
3. Centrifuge the tubes at 25,000 g for 3 minutes to precipitate solids.
4. Decant the supernatant into a hazardous waste container (UIUC DRS UI # 80,050 Chloroform/Methanol only).
5. Add 1 mL acetone.
6. Vortex thoroughly.
7. Centrifuge the tubes at >15,000 g for 3 minutes to precipitate solids.
8. Decant the supernatant into a hazardous waste container (UIUC DRS UI # 80,501 Ethanol/Acetone/Water).
9. Remove caps and dry the starch/AIR pellet at 35C overnight.
   1. Dried starch/AIR pellets can be stored at room temperature long term until further analysis.
10. Proceed to “Enzymatic Starch Digestion of Starch/AIR Pellet” protocol described below.

# Enzymatic Starch Digestion of Starch/AIR Pellet

**NOTE:** If lignin or cell wall-bound phenolic determination or quantification is desired, aliquot 100-200 mg of AIR into a new screw cap tube, record the weight, and proceed with starch digestion on the aliquot only. Use the recorded weight in calculations. Lignin and phenolic speciation is outside the scope of this protocol as written. Reference Foster et al. 2010 or Roig-Oliver et al. 2020 for additional protocols.

**References**

* Foster CE, Martin TM, Pauly M. Comprehensive compositional analysis of plant cell walls (lignocellulosic biomass) part II: carbohydrates. J Vis Exp. 2010 Mar 12;(37):1837. doi: 10.3791/1837. PMID: 20228730; PMCID: PMC3145335.
* Marília Gaspar, Paula Moreira Felix Costa, Marcos Pereira Marinho Aidar, A new rapid and sensitive enzymatic method for extraction and quantification of starch in plant material, Hoehnea Volume 34, Issue 4, December 2006, Pages 425-431,   <https://doi.org/10.1590/S2236-89062007000400001>.

**Reagents**

* Water, distilled
* α-amylase (Bacillus licheniformis) enzyme, 3000 U/mL (Neogen, [E-BLAAM-40mL](https://www.megazyme.com/alpha-amylase-bacillus-licheniformis?number=E-BLAAM-40ML))
* Amyloglucosidase (Aspergillus niger) enzyme, 3260 U/mL (Neogen, [E-AMGDF-10ML](https://www.megazyme.com/amyloglucosidase-aspergillus-niger?number=E-AMGDF-10ML))
* 3-(4-Morpholino)propane sulfonic acid (MOPS), 99%, 25 g (Fisher, [AAA12+1414](https://www.fishersci.com/shop/products/mops-99-thermo-scientific/AAA1291414#?keyword=1132-61-2))
* Sodium hydroxide, 100 g (Fisher, [S318-100)](https://www.fishersci.com/shop/products/sodium-hydroxide-pellets-certified-acs-fisher-chemical-7/S318100#?keyword=NaOH)
* Acetic acid, ACS grade (Fisher, [A38-500](https://www.fishersci.com/shop/products/acetic-acid-glacial-certified-acs-fisher-chemical-9/A38500#?keyword=glacial%20acetic%20acid))
* Acetone, ACS grade, 1 L (Fisher, [A18-1](https://www.fishersci.com/shop/products/acetone-certified-acs-fisher-chemical-12/A181#?keyword=ACETONE%20REAG%204L%20(67-64-1)))
* Ice

**Prepared Reagents**

* Sodium hydroxide, 1M, 5 mL
  + 0.2 g sodium hydroxide bring to 5 mL volume with distilled water.
  + For pH adjustment of buffers.
* MOPS, 10 mM, pH6.5, 50 mL
  + 0.1045 g MOPS into ~40 mL distilled water. pH to 6.5 with NaOH. Bring to 50 mL volume with distilled water. Refrigerate.
  + Store refrigerated for up to 2 months.
* Acetate Buffer 100 mM , pH 4.5, 50 mL
  + 300 ul acetic acid into 40 mL water, pH to 4.5 with NaOH. Bring to 50 mL volume with distilled water. Refrigerate.
  + Store refrigerated for up to 2 months.
* 120 U/mL α-amylase (Bacillus licheniformis) in MOPS buffer, 50 mL.
  + 2 mL thermostabile α-amylase into 48 mL of 10 mM pH 6.5 MOPS.
  + Prepare fresh daily.
* 30 U/mL amyloglucosidase (Aspergillus niger) in acetate buffer, 50 mL.
  + 460 ul amyloglucosidase into 49.54 mL of 100 mM pH 4.5 acetate buffer.

**Materials**

* Transfer pipet (Fisher, [137119bm](https://www.fishersci.com/shop/products/fisherbrand-disposable-graduated-transfer-pipettes-5/137119BM#?keyword=137119bm))
* Pipette tips, 100-1000 ul
* Tips, Repeat pipettor, 25 mL (Fisher, [13-683-707](https://www.fishersci.com/shop/products/eppendorf-combitips-advanced-pipetter-tips-standard-eppendorf-quality-tips-9/p-4909476#?keyword=combitip%20repeat%20pipette%20tip))
* Microcentrifuge tube, 2 mL (Fisher, [05-408-138](https://www.fishersci.com/shop/products/fisherbrand-premium-microcentrifuge-tubes-2-0ml-9/05408138#05-408-138))

**Equipment**

* Micropipette, 100-1000 ul
* Repeat pipettor (Fisher, [13-683-552](https://www.fishersci.com/shop/products/repeater-e3-bundle-including-charger-stand/13683552#?keyword=combitip%20repeat%20pipetter) or equivalent)
  + Alternatively, a standard 100-1000 ul micropipette can be used but is not as efficient.
* Analytical balance
* Spatula
* Graduated cylinder, 50 mL
* pH Meter
* Refrigerator
* Water bath
* Heating block
* Ice bucket
* Vortex
* Floating foam tube racks ([Fisherbrand™ HS2165C](https://www.fishersci.ca/shop/products/hdpe-floating-hdr-foam-tube-rack/360992328))
* Centrifuge, microtube
* Speed-vac
* Microtube rack

**Protocol**

1. Add 500 µL of 120 U/mL α-amylase in MOPS buffer to each starch/AIR fraction pellet sample tube from “Fractionation of Sugars and Starch/AIR” protocol.
2. Vortex to suspend all solids. Flicking the tube may help dislodge the dried pellet.
3. Incubate for 30 minutes at 75 °C in the water bath.
4. Add an additional 500 µLof α-amylase in MOPS buffer (120 U/mL) to each sample tube.
5. Vortex to suspend all solids. Flicking the tube may help dislodge the dried pellet.
6. Incubate for 30 minutes at 75 °C in the water bath.
7. Add an additional 500 µLof α-amylase in MOPS buffer (120 U/mL) to each sample tube.
   1. Sample tubes should contain a total of 1500 ul of α-amylase in MOPS buffer (120 U/mL).
8. Vortex to suspend all solids. Flicking the tube may help dislodge the dried pellet.
9. Incubate for 30 minutes at 75 °C in the water bath.
10. Heat in heating block at 99 °C for 10 minutes to deactivate enzyme.
11. Centrifuge at >13,000 g for 10 minutes.
12. Quantitatively transfer 800 ul to a new, labelled 2 mL microcentrifuge tube.
    1. **Optional:** If starch quantification is not desired, α -amylase in MOPS supernatant can be discarded down the sink and skip to step 13 under “De-starched AIR – Pellet”.
    2. After final MOPS digestion, the MOPS supernatant should continue digestion the same day; continue with the protocol below at “Starch Fraction for Quantification– MOPS Supernatant, step 13”.
    3. After final MOPS digestion, the remaining pellet should be washed and dried the same day; continue with the protocol below at “De-starched AIR - Pellet – Pellet, step 13”.

## Starch Fraction for Quantification– MOPS Supernatant

1. Cool down the water bath to 50 °C, supernatant starch tubes can be stored at room temp on the counter while water bath cools.
   1. Leaving the lid off, especially if it is a shaking water bath, will help cool. For faster cooling, remove some water from the water bath and refill with cool distilled water. Do not use tap water as mineral buildup or heated chlorine could damage the water bath.
2. Add 500 µL 30 U/mL amyloglucosidase in acetate buffer to each sample tube.
3. Vortex.
4. Incubate for 30 minutes at 50 °C in the water bath.
5. Add another 500 µL 30 U/mL amyloglucosidase in acetate buffer to each sample tube.
6. Vortex.
7. Incubate for 30 minutes at 50 °C in the water bath.
8. Place the tubes in ice until cool to the touch to stop the reaction.
9. Proceed to [“Total Starch (As Glucose) Quantification by NZYtech GOD-POD Method”](https://www.protocols.io/view/total-starch-as-glucose-quantification-by-nzytech-kxygxzm8kv8j/v1) or store the samples at -20°C up to one month.

## De-starched AIR - Pellet

1. Add 1 mL distilled water to remaining de-starched AIR pellet with 1 mL water.
2. Vortex to resuspend pellet.
3. Centrifuge at >13,000 g for 10 minutes.
4. Discard supernatant.
5. Repeat 1 mL water wash, vortex, centrifuge, and supernatant discard twice more.
6. Add 500 ul acetone to the pellet.
7. Vortex to resuspend pellet.
8. Centrifuge at >13,000 g for 10 minutes.
9. Use a micropipette, remove 300 ul of acetone supernatant and discard in the hazardous waste (UIUC DRS UI # 80,501 Ethanol/Acetone/Water).
   1. The pellet is not very stable even after centrifugation at max speed. Do not remove all the acetone or AIR loss will occur. If AIR weight (mg AIR/g dry tissue) is a critical experimental measure, consider leaving all acetone to be removed by the Speed-vac for most accurate measurement. At 300 ul acetone, Speed-vac took ~8 hours to reach dry. If no acetone is removed, dry time could approach 15 hours.
10. Remove the remaining acetone using a Speed Vac Concencentrator, following manufacturers recommendations until a steady state weight is obtained (~6-10 hours)
    1. Thermo-Fisher SPD12 Speed-Vac at 0.1 HPr, 35 °C, and Thermo-Fisher RVT404 refrigerated vapor trap -108 °C. Ensure Speed-Vac is properly balanced.
       1. There are three on switches: refrigerated vapor trap, speed-vac, and vacuum pump on floor. All three must be on for operation.
    2. **Some protocols recommend air drying on the bench, in our experience this resulted in incomplete drying. Some protocols recommended drying in an oven at +35°C, in our experience this resulted in rock hard pellets that were not possible to grind or weigh for AIR aliquots.**
    3. If Speed-Vac unattended overnight is not possible, we found that Speed-Vac for 1.5 hours to remove all pooled acetone, drying on the bench overnight, and returning to Speed-vac the following day until a steady state weight was reached worked well.



Wet

Dry

1. After steady-state weight is obtained, recap the samples with their corresponding caps and weigh, recording weight.
2. Subtract the original 2 mL tube weight (recorded in “Tissue Sampling” step 14) from the steady-state weight to get the final de-starched dried AIR weight. Divide the de-starched dried AIR weight by the dried leaf tissue weight (recorded in “Tissue Sampling” step 15) to obtain mg AIR/mg Leaf (dry matter basis).
3. Proceed to either “Test AIR Pellet for Starch Residue” protocol described below or “Separation of Matrix Polysaccharide (Hemicellulose/Pectin) from Cellulose/Glycan Fractions”.

# Optional: Test AIR Pellet for Starch Residue

**Background**

Residual starch will break down into glucose under succeeding acid digestions and cause cell wall components to be artificially high. Highly recommend this optional step on at least a representative subset of samples to verify no residual starch.

**Reagents**

* Water, distilled
* Lugol Iodine Solution ([Sigma 32922-250 mL](https://www.sigmaaldrich.com/US/en/product/sigma/32922))
* Corn starch (Fisher, [S25580](https://www.fishersci.com/shop/products/corn-starch-2/S25580#corn%20starch) or equivalent from grocery)

**Materials**

* Pipette tips, 20-200ul
* Microcentrifuge tube, 2 mL (Fisher, [05-408-138](https://www.fishersci.com/shop/products/fisherbrand-premium-microcentrifuge-tubes-2-0ml-9/05408138#05-408-138))

**Equipment**

* Pipette, 20-200 ul
* Vortex
* Microtube rack

**Protocol**

1. Aliquot approximately 10-30 mg portion of de-starched AIR into a new, labelled tube.
   1. Exact weight is not important.
2. Aliquot approximately 10-30 mg of corn starch int a new, labelled tube as a positive control.
3. Label an empty tube as a negative control.
4. Add 200 ul of distilled water to each tube.
5. Vortex.
6. Add 20 µl Lugol’s Iodine solution to each tube.
7. Vortex.
8. Incubate at room temperature for 5 minutes.
9. Observe and record color of AIR.

A group of test tubes

Description automatically generated

**neg**

**neg**

**neg**

**pos**

* + 1. If starch is present, the AIR will appear dark in color and the “Enzymatic Starch Digestion of Starch/AIR Pellet” protocol should be repeated on the main AIR sample before pulling another aliquot for another iodine test.
    2. If starch is not present, discard iodine test aliquot and continue the “Separation of Matrix Polysaccharide (Hemicellulose/Pectin) from Cellulose/Glycan Fractions” protocol using the main starch-free AIR sample.

# Separation of Matrix Polysaccharide (Hemicellulose/Pectin) from Cellulose/Glycan Fractions

**References**

* Foster CE, Martin TM, Pauly M. Comprehensive compositional analysis of plant cell walls (lignocellulosic biomass) part II: carbohydrates. J Vis Exp. 2010 Mar 12;(37):1837. doi: 10.3791/1837. PMID: 20228730; PMCID: PMC3145335.

**Reagents**

* Water, distilled
* Trifluoroacetic acid (TFA), CAS 76-05-1, ReagentPlus grade, 99% ([Sigma-Aldrich T6508-25ML](https://www.sigmaaldrich.com/US/en/product/sigald/t6508))
  + **Note:** Molarity of trifluoroacetic acid is often not provided on the label, instead density is given. To determine molarity (mol/L = M), convert density to g/L and then divide by molecular weight of reagent.
    - For the recommended TFA above, density is 1.489 g/mL at 20 °C and molecular weight is 114.02 g/mol
* Ice
* Acetone, ACS grade, 1 L (Fisher, [A18-1](https://www.fishersci.com/shop/products/acetone-certified-acs-fisher-chemical-12/A181#?keyword=ACETONE%20REAG%204L%20(67-64-1)))

**Prepared Reagents**

* Trifluoroacetic acid (TFA), 2 M, 100 mL
  + In a chemical fume hood, slowly add 15.32 mLs of 13.059 M TFA to 86.68 mLs of distilled water.
  + Wear proper PPE including lab coat, goggles, and gloves.

**Materials**

* 2 mL, screw cap tubes, (Fisher, [02681375](https://www.fishersci.com/shop/products/fisherbrand-sterile-microcentrifuge-tubes-screw-caps-6/02681375))
* Pipette tips, 100-1000 ul
* Gel-Loading Tips, 1-200μL (Fisher, [02-707-138](https://www.fishersci.com/shop/products/fisherbrand-gel-loading-tips-1-200-l-4/02707138))

**Equipment**

* Chemical fume hood
* Graduated cylinder, 100 mL
* Microbalance, Sartorius MCE3.6 P-2500-M or equivalent
* Microspatula with V-shaped spoon (Fisher, [21-401-25B](https://www.fishersci.com/shop/products/fisherbrand-hayman-style-microspatulas-v-shaped-spoon/2140125B#micro%20spatula))
* Vortex
* Centrifuge, microtube
* Pipette, 100-1000 ul
* Pipette, 20-200 ul
* Microcentrifuge
* Heating block, standard tube, dry bath, capable of reaching 130°C
* Ice bucket
* Microtube rack

**Protocol**

1. Tare a new, labelled 2 mL screw cap tube on a microbalance.
2. Weigh 2-3 mg of AIR residue obtained from “Enzymatic Starch Digestion of Starch/AIR Pellet: De-starched AIR - Pellet “ into the tube.
3. Record weight of sample.
4. Repeat twice more for each sample. AIR aliquots of samples should be weighed in triplicate.
5. Centrifuge at >13,000 g for 10 minutes to pull AIR aliquot to bottom of tube.
6. Pre-heat a heating block to 121 °C in a chemical fume hood.
7. In a chemical fume hood, add 375 ul of 2 M trifluoroacetic acid to each AIR aliquot and cap tightly.
   1. TFA is a strong acid. Wear proper PPE including lab coat, gloves, and googles. Read safety data sheet before handling.
8. Microcentrifuge briefly to pull all material to the bottom of the tube.
   1. If cell wall material is on the side of the tube, it will bake on to tube instead of digesting.
9. In a chemical fume hood, heat in dry bath heating block at 121°C for 90 minutes.
10. After 90 minutes, transfer the tubes to ice and let cool completely.
    1. **CAUTION:** The boiling point of TFA is 72.4°C. Tubes must cool below this temperature before opening the lids to avoid both sample loss and injury.
11. Centrifuge the tubes at >13,000g for 15 minutes.
12. Using a 200 ul pipette with a gel loading tip, very slowly transfer two sets of 150 ul supernatant to a new, labelled tube.
    1. Although 375 ul of TFA was added to the tube, **DO NOT TRY TO REMOVE ALL SUPERNATANT**. The pellet is very fragile and will be disturbed if more than 300 ul is removed. If there is not a high level of confidence in pipetting skill, only remove 200-250 ul of supernatant.
    2. The use of the gel loading tip helps minimize any floating pellet pieces that might get carried over into supernatant and effect hemicellulose/pectin measurements.
    3. The supernatant contains the matrix polysaccharides including pectin and digested hemicellulose. Supernatant can be stored at -20C until further analysis or proceed to measure total hemicellulose, as glucose, by sulfuric phenol assay at “Glucose Quantification for Analysis of Total Sugar, Digested Hemicellulose, and Digested Cellulose” and total pectin by “Quantitative determination of uronic acid micro plate assay”
    4. The pellet contains the cellulose/glycan fraction should be washed and dried the same day; continue with the protocol below at “Cellulose/Glycan Pellet” step 13.

## Cellulose/Glycan Pellet

1. Add 1 mL of distilled water to the remaining pellet and vortex.
2. Centrifuge the tubes at >13,000g for 10 min.
3. Using a 1 mL pipette, very slowly and carefully remove about 800 ul of the supernatant being careful not to disturb the pellet.
   1. Leaving behind residual supernatant is acceptable because any residual hemicellulose or pectin will be diluted past the level of interference in the next several washes.
   2. Supernatant could still contain residual TFA. Discard the supernatant into a hazardous waste container (UIUC DRS UI # 224289 TFA, sulfuric acid, phenol, sodium tetraborate, sulfamic acid, 3-phenylphenol, DMSO, water).
4. Add 1 mL acetone to the tube and vortex.
5. Centrifuge the tubes at >13,000g for 10 min.
6. Using a 1 mL pipette, very slowly and carefully remove about 800 ul of the supernatant being careful not to disturb the pellet.
   1. Discard acetone wash in the hazardous waste (UIUC DRS UI # 80,501 Ethanol/Acetone/Water).
7. Repeat acetone wash in steps 16 -18 twice more.
8. Air dry overnight with the lids off at room temperature in chemical fume hood.
   1. Leaving the fume hood open at a working distance increases airflow across the tubes and encourages safe evaporation of the ~200 ul of remaining acetone.
9. Store dried cellulose/glycan pellet at room temperature or proceed to “Optional: Glycan Removal From Cellulose Fraction” or directly to “Digestion of Cellulose/Glycan Fraction”.

# Optional: Glycan Removal From Cellulose Fraction

**Note:** This protocol was tested but it did not change the final results significantly between the treatments of our specific experiment, so we opted to not use this portion of the protocol for publication results.

**References**

* Foster CE, Martin TM, Pauly M. Comprehensive compositional analysis of plant cell walls (lignocellulosic biomass) part II: carbohydrates. J Vis Exp. 2010 Mar 12;(37):1837. doi: 10.3791/1837. PMID: 20228730; PMCID: PMC3145335.

**Reagents**

* Water, distilled
* Acetic Acid, Glacial, ACS Grade ([Fisher A38-212](https://www.fishersci.com/shop/products/acetic-acid-glacial-certified-acs-fisher-chemical-9/A38212#glacial%20acetic%20acid))
* Nitric Acid, ACS grade ([Fisher A200-500](https://www.fishersci.com/shop/products/nitric-acid-certified-acs-plus-fisher-chemical-8/A200500))
* Ice
* Acetone, ACS grade, 1 L (Fisher, [A18-1](https://www.fishersci.com/shop/products/acetone-certified-acs-fisher-chemical-12/A181#?keyword=ACETONE%20REAG%204L%20(67-64-1)))

**Prepared Reagents**

* Updegraff reagent (acetic acid: nitric acid: water, 8:1:2 v/v), 110 mL
  + In a chemical fume hood, slowly add 80 mL of concentrated glacial acetic acid to 20 mL of distilled water.
  + Swirl gently to mix and let cool.
  + Add 10 mL concentrated nitric acid (~70%), swirl to mix.
  + Wear proper PPE including lab coat, goggles, and gloves.

**Materials**

* Pipette tips, 100-1000 ul

**Equipment**

* Chemical fume hood
* Graduated cylinder, 200 mL
* Pipette, 100-1000 ul
* Heating block, standard tube, dry bath, capable of reaching 130°C
* Ice bucket
* Microtube rack
* Vortex
* Centrifuge, microtube

**Protocol**

1. In a chemical fume hood, to the tube containing the cellulose/glycan pellet from either “Separation of Matrix Polysaccharide (Hemicellulose/Pectin) from Cellulose/Glycan Fractions” add 1 mL of Updegraff reagent to the tube.
   1. WEAR PPE: COAT, GLOVES, GOGGLES. Extremely corrosive.
2. Cap tube, vortex.
3. In a chemical fume hood, heat in dry bath heating block at 100°C for 30 minutes.
4. After 30 minutes, transfer the tubes to ice and let cool completely.
5. Centrifuge the tubes at >10,000g for 15 min.
6. Using a 1 mL pipette, very slowly and carefully remove about 850 ul of the supernatant being careful not to disturb the pellet.
   1. Leaving behind residual supernatant is acceptable because any residual glycan will be diluted past the level of interference in the next several washes.
   2. Discard the supernatant into a hazardous waste container (UIUC DRS UI # 100259 Acetic acid/Nitric acid/Water).
7. Add 1.5 mL water to the tube and vortex.
8. Centrifuge the tubes at >13,000g for 10 min.
9. Using a 1 mL pipette, very slowly and carefully remove about 850 ul of the supernatant being careful not to disturb the pellet.
   1. The water wash could still contain residual acid. Discard the supernatant into a hazardous waste container (UIUC DRS UI # 100259 Acetic acid/Nitric acid/Water).
10. Add 1.5 mL acetone to the tube and vortex.
11. Centrifuge the tubes at >13,000g for 10 min.
12. Using a 1 mL pipette, very slowly and carefully remove about 850 ul of the supernatant being careful not to disturb the pellet.
    1. Discard acetone wash in the hazardous waste (UIUC DRS UI # 80,501 Ethanol/Acetone/Water).
13. Repeat acetone wash in steps 10 -12 twice more.
14. Air dry overnight with the lids off at room temperature in chemical fume hood.
    1. Leaving the fume hood open at a working distance increases airflow across the tubes and encourages safe evaporation of the ~200 ul of remaining acetone.
15. Store dried cellulose/glycan pellet at room temperature or proceed to directly to “Digestion of Cellulose/Glycan Fraction”.

# Digestion of Cellulose/Glycan Fraction

**References**

* Foster CE, Martin TM, Pauly M. Comprehensive compositional analysis of plant cell walls (lignocellulosic biomass) part II: carbohydrates. J Vis Exp. 2010 Mar 12;(37):1837. doi: 10.3791/1837. PMID: 20228730; PMCID: PMC3145335.

**Reagents**

* Water, distilled
* Sulfuric Acid, ACS, 95 to 98% (Fisher, [23-749-903](https://www.fishersci.com/shop/products/sulfuric-acid-acs-95-98-milliporesigma/23749903))
* Ice

**Prepared Reagents**

* Sulfuric Acid, 72% (w/v), 25 mL
  + In a chemical fume hood, slowly add 16.31 mL of 98% sulfuric acid to 8.69 mL of distilled water.
    - To calculate a weight by volume dilution of sulfuric acid from another weight by volume concentration, add density to the dilution equation.

* + - If a different percent concentration by w/v is desired, density can be found in [online calculators or tables.](https://www.handymath.com/cgi-bin/sulfurictble11.cgi?submit=Entry)
  + Sulfuric acid is extremely corrosive. Wear proper PPE including lab coat, goggles, and gloves.

**Materials**

* Pipette tips, 20-200 ul
* Pipette tips, 100-1000 ul

**Equipment**

* Chemical fume hood
* Graduated cylinder, 20 mL
* Pipette, 20-200 ul
* Pipette, 100-1000 ul
* Vortex
* Centrifuge, microtube

**Protocol**

1. In a chemical fume hood, to the tube containing the cellulose/glycan pellet from either “Separation of Matrix Polysaccharide (Hemicellulose/Pectin) from Cellulose/Glycan Fractions” or “Optional: Glycan Removal From Cellulose Fraction”, add 175 ul of 72% sulfuric acid to the tube.
   1. WEAR PPE: COAT, GLOVES, GOGGLES. Extremely corrosive.
2. Vortex.
3. Centrifuge 2000 rcf for 1 in to pull solids off tube walls and into sulfuric acid.
4. Incubate in a chemical fume hood for 30 minutes at room temperature.
5. Vortex.
6. Incubate in a chemical fume hood for an additional 15 minutes at room temperature.
7. Add 825 ul water to each tube.
8. Vortex.
9. Centrifuge 10000 rcf for 5 minutes.
10. Store digested cellulose samples at -20oC for several months or directly proceed to measuring total glucose by “Glucose Quantification for Analysis of Total Sugar, Digested Hemicellulose, and Digested Cellulose”.
    1. **NOTE:** Some protocols recommend heating the sample in 72% sulfuric acid or more dilute. We do not recommend this practice. The sulfuric acid is sufficient to digest the cellulose down into its glucose components. Heat treatment will degrade the released glucose and cause an increase in variability or may cause artificially low cellulose results.
    2. **NOTE:** A brown pellet or residue after sulfuric acid digestion at room temperature is residual lignin. Do not try to dissolve this pellet by more aggressive sample treatments.

# Total Starch (As Glucose) Quantification by NZYtech GOD-POD Method

**Protocol**

1. Use the “Starch Fraction for Quantification– MOPS Supernatant” obtained from “Enzymatic Starch Digestion of Starch/AIR Pellet” in the publicly available published method at Lynn Doran, Amanda P. De Souza 2021. Total Starch (as Glucose) Quantification by NZYtech GOD-POD Method. protocols.io [https://dx.doi.org/10.17504/protocols.io.b27yqhpw.](https://www.protocols.io/view/total-starch-as-glucose-quantification-by-nzytech-kxygxzm8kv8j/v1)

# Optional: Charcoal Cleanup of Sugar Samples

**Note:** Secondary metabolites and pigments can interfere with the absorption or the sulfuric-phenol reaction in the “Glucose Quantification for Analysis of Total Sugar, Digested Hemicellulose, and Digested Cellulose” assay leading to high variability and inflated total soluble sugar results. In a direct comparison on the same samples, charcoal cleanup of sugar extracts, we saw a trend toward lower standard deviation between biological replicates for sugar (as glucose) per mg dried leaf tissue.

**References**

* Kondo, M, Mulianda, R, Matamura, M, et al. Validation of a phenol-sulfuric acid method in a microplate format for the quantification of soluble sugars in ruminant feeds. *Anim Sci J*. 2021; 92:e13530. <https://doi.org/10.1111/asj.13530>

**Reagents**

* Water, distilled
* Charcoal, Activated ([Sigma C9157-500G](https://www.sigmaaldrich.com/US/en/product/sial/c9157))

**Materials**

* Microcentrifuge tube, 2 mL
* Pipette tips, 100-1000 ul

**Equipment**

* Microspatula with V-shaped spoon (Fisher, [21-401-25B](https://www.fishersci.com/shop/products/fisherbrand-hayman-style-microspatulas-v-shaped-spoon/2140125B#micro%20spatula))
* Microbalance, Sartorius MCE3.6 P-2500-M or equivalent
* Vortex
* Centrifuge, microtube
* Pipette, 100-1000 ul

**Protocol**

1. Label a new tube for each sample.
2. Weigh 2 to 3 mg of activated charcoal into each tube, recording the weight.
3. Multiply the weight of charcoal by 100 to obtain the volume of sugar ethanolic extract to add from “Fractionation of Sugars and Starch/AIR: Sugar Fraction – Ethanolic Supernatant”.
   1. If sugar ethanolic extract will need to be diluted for “Glucose Quantification for Analysis of Total Sugar, Digested Hemicellulose, and Digested Cellulose”, dilute the sugar extract prior to adding to the activated charcoal to maximize the amount of contaminants the charcoal is able to remove.
4. Add the appropriate volume of sugar ethanolic extract to the charcoal.
5. Vortex.
6. Incubate at room temperature for 5 minutes.
7. Vortex.
8. Centrifuge 10000 rcf for 10 minutes.
9. Transfer supernatant to a new, labelled tube.

# Glucose Quantification for Analysis of Total Sugar, Digested Hemicellulose, and Digested Cellulose

**Note:** This protocol quantifies total soluble sugars as glucose. It can be used for total soluble sugar analysis on the ethanolic extract of the dried and ground leaf tissue from “Fractionation of Sugars and Starch/AIR: Sugar Fraction – Ethanolic Supernatant” or from “Optional: Charcoal Cleanup of Sugar Samples”. Trifluoroacetic acid digestion converts hemicellulose to its component simple sugars which can be quantified using this protocol. For hemicellulose quantification, use the TFA digest from “Separation of Matrix Polysaccharide (Hemicellulose/Pectin) from Cellulose/Glycan Fractions: Supernatant”. 72% Sulfuric acid digestion converts cellulose/glycan to its component simple sugars which can be quantified using this protocol. For cellulose quantification, use the sulfuric digest from “Digestion of Cellulose/Glycan Fraction”.

The only modifications to the protocol when being used for total sugar, hemicellulose, or cellulose is in the standard preparation and quantity of sample added to each well. Sample quantity will vary between experiments for all components based on species and environmental factors.

**References**

* Kondo, M, Mulianda, R, Matamura, M, et al. Validation of a phenol-sulfuric acid method in a microplate format for the quantification of soluble sugars in ruminant feeds. *Anim Sci J*. 2021; 92:e13530. <https://doi.org/10.1111/asj.13530>
* Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S, Lee YC. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal Biochem. 2005 Apr 1;339(1):69-72. doi: 10.1016/j.ab.2004.12.001. PMID: 15766712. https://pubmed.ncbi.nlm.nih.gov/15766712/
* Pak S. Chow, Simon M. Landhäusser, A method for routine measurements of total sugar and starch content in woody plant tissues, Tree Physiology, Volume 24, Issue 10, October 2004, Pages 1129–1136, <https://doi.org/10.1093/treephys/24.10.1129>

**Reagents**

* Sulfuric acid, Merck/Millipore brand, 95-98% ([Fisher 23-749-903](https://www.fishersci.com/shop/products/sulfuric-acid-acs-95-98-milliporesigma/23749903))
  + Quality of the sulfuric acid can affect the development of assay color. Old sulfuric acid (>10 years) did not generate linear standard curves.
* Phenol, ACS Grade ([Fisher, AA33213A3](https://www.fishersci.com/shop/products/phenol-acs-99-stab-thermo-scientific/AA33213A3#?keyword=Phenol))
* Glucose solution, 1mg/mL ([Sigma G6918-100ML](https://www.sigmaaldrich.com/US/en/product/sigma/g6918))
* MilliQ or distilled water
* **Optional:** Trifluoroacetic acid (TFA), CAS 76-05-1, ReagentPlus grade, 99% ([Sigma-Aldrich T6508-25ML](https://www.sigmaaldrich.com/US/en/product/sigald/t6508))
  + **Note:** Molarity of trifluoroacetic acid is often not provided on the label, instead density is given. To determine molarity (mol/L = M), convert density to g/L and then divide by molecular weight of reagent.
    - For the recommended TFA above, density is 1.489 g/mL at 20 °C and molecular weight is 114.02 g/mol

**Prepared Reagents**

* Phenol 5% (w/v), 100 mL
  + 5 g phenol up to 100 mL with milliQ water.
  + Use within a week.
  + WEAR PPE: COAT, GLOVES, GOGGLES. Weigh and prepare in a chemical fume hood.
  + Alternatively, Phenol, 5% (w/v) can be purchased. ([Fisher 18-612-218](https://www.fishersci.com/shop/products/phenol-5-w-v-solution-4-9-5-1-spectrum-chemical/18612218))
* **Optional:** Trifluoroacetic acid (TFA), 2 M, 100 mL
  + In a chemical fume hood, slowly add 15.32 mLs of 13.059 M TFA to 86.68 mLs of distilled water.
  + Wear proper PPE including lab coat, goggles, and gloves.

**Materials**

* Pipette tips, 1-10 ul
* Pipette tips, 20-200 ul
* Combitips advanced™ Standard Pipettor Tips, 0.5 mL (5 ul to 100 ul) ([Fisher 13-683-702](https://www.fishersci.com/shop/products/eppendorf-combitips-advanced-pipetter-tips-standard-eppendorf-quality-tips-9/13683702?keyword=true))
* Combitips advanced™ Standard Pipettor Tips, 5 mL (50 ul – 1000 ul) ([Fisher 13-683-705](https://www.fishersci.com/shop/products/eppendorf-combitips-advanced-pipetter-tips-standard-eppendorf-quality-tips-9/13683705?keyword=true))
* 96-well plates, 330 ul, polypropylene or PTFE only. ([Biotix 63300102](https://biotix.com/products/plates/330-%CE%BCl-assay-plate-96-well/))
  + Polystyrene is not chemical resistant to sulfuric acid and phenol. Degradation of the plastic will occur over time. Use at your own risk.
* Microcentrifuge tubes, 2 mL
* Optional: aluminum foil

**Equipment**

* Chemical fume hood
* Pipette, 1-10 ul
* Pipette, 20-200 ul
* Repeat pipettor, Eppendorf Combitips advanced or equivalent
* Graduated cylinder, 100 mL
* Analytical Balance
* Water bath
* Ice bucket
* UV-Vis Plate reader
* Microtube rack

**Protocol**

1. Move a water bath to a chemical fume hood and pre-heat to 90oC.
2. Prepare glucose standards in microcentrifuge by pipetting the appropriate amounts of 1 mg/mL Glucose standard, distilled water, and if appropriate 2M trifluoroacetic acid into each labeled tube. The standard curve will differ for total sugar, hemicellulose, and cellulose. Please refer to the appropriate table below.
   1. The following standard curves were appropriate for *Nicotiana tabacum* when used with the recommended sample volumes. Depending on species, treatment, and environment, expanded curves or modifications to initial sample volume may need to be used.
      1. The linear curve for the sulfuric-phenol assay seems to be robust and has successfully been extended beyond the ranges given here.
   2. 2M TFA that is present in the hemicellulose extracts seems to interact with the sulfuric-phenol assay, especially at higher sugar concentrations.
      1. There was no significant difference in the slope of the standard curve with and without 2 M TFA from 0-20 ug glucose per well but there was a significant increase in the slope of the curve with 2 M TFA from 0-25 ug glucose per well.
         1. It is recommended to add 2M TFA to the glucose standards for the hemicellulose quantification if the standard curve used for calculating sample concentration will exceed 20 ug glucose per well.
   3. If 2M trifluoroacetic acid is used in the preparation of the hemicellulose standard curve, wear appropriate PPE including a lab coat, gloves, goggles, and prepare standards in a chemical fume hood.

|  |  |  |
| --- | --- | --- |
| **Standard Curve for Total Sugar Analysis or Hemicellulose (without TFA)** | | |
| **ug Glucose/50 ul-well (ug)** | **Amount 1 mg/mL Glucose (ul)** | **Amount distilled water (ul)** |
| 5 | 20 | 180 |
| 10 | 40 | 160 |
| 15 | 60 | 140 |
| 20 | 80 | 120 |
| 25 | 100 | 100 |

|  |  |  |  |
| --- | --- | --- | --- |
| **Standard Curve for Hemicellulose (with TFA)** | | | |
| **ug Glucose/50 ul-well (ug)** | **Amount 1 mg/mL Glucose (ul)** | **Amount distilled water (ul)** | **Amount 2M TFA (ul)** |
| 5 | 20 | 140 | 40 |
| 10 | 40 | 120 | 40 |
| 15 | 60 | 100 | 40 |
| 20 | 80 | 80 | 40 |
| 25 | 100 | 60 | 40 |

|  |  |  |
| --- | --- | --- |
| **Standard Curve for Cellulose** | | |
| **ug Glucose/50 ul-well (ug)** | **Amount 1 mg/mL Glucose (ul)** | **Amount distilled water (ul)** |
| 2 | 8 | 192 |
| 4 | 16 | 184 |
| 6 | 24 | 176 |
| 8 | 32 | 168 |
| 10 | 40 | 160 |
| 12 | 48 | 152 |
| 14 | 56 | 144 |

1. Design a plate layout with all samples, standards, and blanks in triplicate.
   1. A triplicate of blank wells must be included on every plate.
   2. Standard curve only needs to be included on one plate as long as the analysis is completed in a single day and the spectrophotometer lamp is not shut off between plates.
2. Pipette 50 ul of each prepared glucose standard in triplicate into the assigned wells.
3. Pipette 1-50 ul of sample into each of the assigned wells.
   1. Sample volume may need to be determined empirically to find the sample volume that returns an absorbance that fits into the standard curve based on the analyte of interest, species, and environmental conditions sampled in.
   2. If suggested sample volume falls below 5 ul, it is recommended to make the sample dilution in a separate tube for all triplicates and then pipette 50 ul of the pre-diluted sample into the well to improve precision of the triplicate.
   3. Historical values are provided in the table for reference:

|  |  |  |  |
| --- | --- | --- | --- |
| **Species** | **Analyte** | **Suggested volume (ul)** | **Notes** |
| Tobacco | Total Sugar | 1 | Sampled midday |
| Tobacco | Hemicellulose | 10 |  |
| Tobacco | Cellulose | 50 |  |

1. Add distilled water to bring the total volume of each well to 50 ul. For example, if 10 ul of sample was used add 40 ul of distilled water.
2. If the microplate isn’t already in a chemical fume hood, move to a chemical fume hood for the remaining steps. Wear PPE including coat, gloves, and googles for all remaining steps.
3. Inside the hood using a repeat pipettor, add 150uL of sulfuric acid to each well. Try to minimize the time between addition to first well and final well.
   1. A multi-channel pipette or repeat pipettor will allow the fastest addition of sulfuric acid to all samples. The sulfuric acid will degrade the o-rings on the multi-channel pipette and reduce precision over repeated runs. If using a multi-channel, keep extra channel o-rings in stock.Filter tips may help prolong the life of multi-channel pipette parts but they also decreased precision. Repeat pipettor is the best equipment for this protocol as the sulfuric is contained in disposable parts.
4. Immediately after the addition of sulfuric acid using a repeat pipettor, add 30uL of phenol 5% in each well. Try to minimize the time between addition to first well and final well.
5. Incubate the plate by floating in a 90 °C water bath for 5 minutes.
   1. A small boat can be made of aluminum foil to keep the plate above the boiling water and make it easier to remove from the water bath.
   2. Leave the lid off the water bath to avoid condensation dripping in the open plate top and altering the concentration of the glucose in the wells.
6. Place plate on ice bath to cool until cool to the touch.
7. Once the plate is cool, read the absorbance at 490 nm on a UV-VIS spectrophotometer.
   1. Do not leave the plate in the spectrophotometer for longer than is necessary. Sulfuric acid and phenol fumes outside of the hood are bad for health and may damage the spectrophotometer if exposure is extended.
8. Do not shutoff the spectrophotometer lamp between plates. If the lamp remains on continuously, only one glucose standard curve is needed. If the lamp is shut off, a standard curve will need to be included.
9. Average the three technical replicates for each sample and standard.
10. For each plate, subtract the average triplicate blank well absorbance from all average triplicate standards or samples.
    1. Each plate should be zeroed to itself.
11. Generate a linear model for the averaged, zeroed standard curve concentrations against their absorbance.
12. Calculate the desired final concentration.
    1. For total soluble sugar, calculate ug sugar (as glucose) per mg dried leaf tissue.

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Description automatically generated

* 1. For hemicellulose, calculate TFA soluble hemicellulose (as glucose) per mg AIR.

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* 1. For cellulose and bound glycans, calculate cellulose (as glucose) per mg AIR.

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Description automatically generated

1. Properly dispose of all liquid hazardous waste (UIUC DRS UI #224290 - Trifluoroacetic acid, sulfuric acid, phenol, sodium tetraborate, sulfamic acid, 3-phenylphenol, DMSO, water) and solid, contaminated waste (UIUC DRS UI #224289 - Contaminated Debris – No liquid: Trifluoroacetic acid, sulfuric acid, phenol, sodium tetraborate, sulfamic acid, 3-phenylphenol, DMSO.)

# Pectin Quantification by Uronic Acid Microplate Assay

**References**

* [Alba, K., Laws, Andrew P. and Kontogiorgos, Vassilis (2015) Isolation and characterization of acetylated LM-pectins extracted from okra pods. Food Hydrocolloids, 43. pp. 726-735. ISSN 0268005X](http://eprints.hud.ac.uk/id/eprint/23494/1/Accepted_Final.pdf)
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* Blumenkrantz N, Asboe-Hansen G. New method for quantitative determination of uronic acids. Anal Biochem. 1973 Aug;54(2):484-9. doi: 10.1016/0003-2697(73)90377-1. PMID: 4269305.
* Filisetti-Cozzi, T. M. C. C., & Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. *Analytical Biochemistry*, *197*(1), 157–162. <https://doi.org/10.1016/0003-2697(91)90372-Z>
* van den Hoogen BM, van Weeren PR, Lopes-Cardozo M, van Golde LM, Barneveld A, van de Lest CH. A microtiter plate assay for the determination of uronic acids. Anal Biochem. 1998 Mar 15;257(2):107-11. doi: 10.1006/abio.1997.2538. PMID: 9514779.

**Reagents**

* Water, distilled
* D-(+)-Galacturonic acid monohydrate, MW 212.15 g/mol, 97% (Fisher [AAJ6628214](https://www.fishersci.com/shop/products/d-galacturonic-acid-monohydrate-97-thermo-scientific/AAJ6628214?searchHijack=true&searchTerm=Alfa+Aesar+J66282))
  + Monohydrate is crystallisation water and cannot be removed.
  + Pectins GalA is anhydroform due to formation of the glycosidic bonds. If Pectin as GalA wt/wt AIR is desired, multiply mM results by MW 176 g/mol for anhydrous galacturonic acids.
* Sulfamic acid, >99% ([Sigma, 242772-5G](https://www.sigmaaldrich.com/US/en/product/sigald/242772))
* Potassium hydroxide, pellets, Certified ACS ([Fisher P250-3](https://www.fishersci.com/shop/products/potassium-hydroxide-pellets-certified-acs-fisher-chemical-5/P2503#?keyword=))
* Dimethyl sulfoxide (DMSO), Certified ACS ([Fisher D128-500](https://www.fishersci.com/shop/products/dimethyl-sulfoxide-certified-acs-fisher-chemical-4/D128500#?keyword=67-68-5))
* m-Hydroxdiphenyl, 97% ([Fisher P07685G](https://www.fishersci.com/shop/products/3-phenylphenol-tci-america-2/P07685G#M-hydroxydiphenyl))
* Sodium tetraborate decahydrate, crystalline, Certified ACS ([Fisher S248-500](https://www.fishersci.com/shop/products/sodium-tetraborate-decahydrate-crystalline-certified-acs-fisher-chemical-3/S248500#?keyword=sodium%20tetraborate%20decahydrate))
* Sulfuric acid, Merck/Millipore brand, 95-98% ([Fisher 23-749-903](https://www.fishersci.com/shop/products/sulfuric-acid-acs-95-98-milliporesigma/23749903))

**Prepared Reagents**

* Potassium hydroxide, 11M, 5 mL
  + Dissolve 3.085 g into 5 mL water.
  + Strong base. Make in chemical fume hood. Wear appropriate PPE including gloves, coat, and goggles.
* Sulfamic acid, 4M, 5 mL
  + Add 1.94 g to 2.5 ml of 11M KOH, add 11 M KOH until sulfamic acid dissolves. Adjust pH to 1.6, bring to 5 mL volume with water.
  + Took all the way up to 5 mLs (45 drops ~2.5 mLs of 11 M KOH) to dissolve sulfamic acid. pH 1. Not possible to get it to pH 1.6 without making more dilute than 4M sulfamic acid.
  + Strong acid. Make in chemical fume hood. Wear appropriate PPE including gloves, coat, and goggles.
  + Store at room temperature for several weeks.
* m-Hydroxydiphenyl in dimethyl sulfoxide, 100 mg/mL, 5 mL
  + Dissolve 500 mg in 5 mL DMSO, store at room temperature in dark for several weeks.
* Sodium tetraborate decahydrate, 120 mM in sulfuric acid, 100 mL
  + Add 4.5765 g into 100 mL concentrated sulfuric acid. Stir overnight in fume hood.
  + Prepare and store in glass container.
  + Use within one week.
* D-(+)-galacturonic acid monohydrate stock, 50 mmol/L, 1 mL
  + Add 10.61 mg to 1 mL water.
  + Store in the dark, at 4oC for several weeks.
* Sulfuric acid, 80% (v/v), 50 mL
  + Slowly add 10 mL of concentrated sulfuric acid to 40 mL of water.

**Materials**

* Pipette tips, 20-200 ul
* Pipette tips, 100-1000 ul
* Pasteur pipets
* Combitips advanced™ Standard Pipettor Tips, 0.2 mL (2 ul to 40 ul) ([Fisher 13-683-701](https://www.fishersci.com/shop/products/eppendorf-combitips-advanced-pipetter-tips-standard-eppendorf-quality-tips-9/13683701))
* Combitips advanced™ Standard Pipettor Tips, 5 mL (50 ul – 1000 ul) ([Fisher 13-683-705](https://www.fishersci.com/shop/products/eppendorf-combitips-advanced-pipetter-tips-standard-eppendorf-quality-tips-9/13683705?keyword=true))
* 96-well plates, 330 ul, polypropylene or PTFE only. ([Biotix 63300102](https://biotix.com/products/plates/330-%CE%BCl-assay-plate-96-well/))
  + Polystyrene is not chemical resistant to sulfuric acid and phenol. Degradation of the plastic will occur over time. Use at your own risk.
* Microseal 'B' PCR Plate Sealing Film, adhesive, optical ([Bio-Rad MSB1001](https://www.bio-rad.com/en-us/sku/MSB1001-microseal-b-pcr-plate-sealing-film-adhesive-optical?ID=MSB1001))
* Microcentrifuge tubes, 2 mL
* Optional: aluminum foil

**Equipment**

* Chemical fume hood
* Analytical balance
* Microbalance, Sartorius MCE3.6 P-2500-M or equivalent
* Microspatula with V-shaped spoon (Fisher, [21-401-25B](https://www.fishersci.com/shop/products/fisherbrand-hayman-style-microspatulas-v-shaped-spoon/2140125B#micro%20spatula))
* Graduated cylinder, 10 mL
* Graduated cylinder, 100 mL
* pH Meter
* Stir bar
* Stir plate
* Water bath
* Ice bucket
* UV-Vis Plate reader
* Microtube rack
* Repeat pipettor, Eppendorf Combitips advanced or equivalent
* Pipette, 20-200 ul
* Pipette, 100-1000 ul
* Vortex

**Protocol**

1. Move a water bath to a chemical fume hood and pre-heat to 90oC.
2. Prepare a galacturonic acid standard series from 200 nmol to 3.125 nmol in microcentrifuge tubes.
   1. Label 8 microcentrifuge tubes 200, 100, 50, 25, 12.5, 6.25, 3.125, 0.
      1. In our experience, the standard curve was not linear above 200 nmol galacturonic acid per well in repeated attempts.
   2. Prepare the 200 nmol/well standard by pipetting 32 ul of 50 mmol/L D-(+)-galacturonic acid monohydrate stock into 256 ul of milliQ water.
   3. Vortex.
   4. Add 128 ul of water to the standard tubes 100, 50, 25, 12.5, 6.25, 3.125, 0.
   5. Prepare a serial dilution by pipetting 128 ul of the 200 nmol/well standard into the 128 ul of water in the 100 tube.
   6. Vortex.
   7. For best linearity, switch pipette tips between each standard.
   8. Prepare a serial dilution by pipetting 128 ul of the 100 nmol/well standard into the 128 ul of water in the 50 tube.
   9. Vortex and repeat down to the 3.125 tube.
   10. Leave only water in the 0 tube.
3. Move all supplies to chemical fume hood for remainder of the protocol. Wear proper PPE including gloves, goggles, and a lab coat.
4. Design a plate layout with all samples, standards, and blanks in triplicate.
   1. A triplicate of blank wells must be included on every plate.
   2. Standard curve only needs to be included on one plate as long as the analysis is completed in a single day and the spectrophotometer lamp is not shut off between plates.
5. Pipette 36 ul of each prepared galacturonic acid standard in triplicate into the assigned wells.
6. Pipette 36 ul of water into the blank assigned wells.
7. Pipette 16 ul of sample into each of the assigned wells.
   1. Sample volume may need to be determined empirically to find the sample volume that returns an absorbance that fits into the standard curve based on the analyte of interest, species, and environmental conditions sampled in. Above volume was appropriate for greenhouse grown tobacco.
8. Add 20 ul water to each of the sample wells using a repeat pipetter.
   1. If a different sample volume is used, modify amount of water to make total well volume equal to the 36 ul used for prepared standards.
9. Using a repeat pipetter, add 4 ul 4 M sulfamic acid to every well.
   1. A 4 ul volume will not automatically fall from the repeat pipette tip due to the small volume and surface tension. Dot the 4 ul droplet on the side of the well without touching the well. The droplet will be washed into the well with the following volume additions.
   2. Sulfamic acid helps prevent browning due to residual interfering sugars. Some protocols omit 4 M sulfamic acid.
10. Using a repeat pipetter, add 200 ul of 120 mM sodium tetraborate in sulfuric acid to every well.
11. Seal each plate with a microseal B film to prevent evaporation.
12. Incubate the plate by floating in a 80 °C water bath for 60 minutes.
    1. A small boat can be made of aluminum foil to keep the plate above the boiling water and make it easier to remove from the water bath.
    2. Leave the lid on the water bath to avoid dramatic temperature changes during the hour incubation time. The seal on the plates will prevent any contamination condensation that may come off the lid during incubation.
13. Place plate on ice bath to cool until cool to the touch.
14. Carefully and slowly peel off the microseal films and discard in hazardous waste (UIUC DRS UI #224289 - Contaminated Debris – No liquid: Trifluoroacetic acid, sulfuric acid, phenol, sodium tetraborate, sulfamic acid, 3-phenylphenol, DMSO).
15. Once the plate is cool, read the absorbance at 525 nm on a UV-VIS spectrophotometer.
    1. Do not leave the plate in the spectrophotometer for longer than is necessary. Sulfuric acid and phenol fumes outside of the hood are bad for health and may damage the spectrophotometer if exposure is extended.
16. Do not shutoff the spectrophotometer lamp between consecutive readings and/or plates. If the lamp remains on continuously, only one galacturonic acid standard curve is needed. If the lamp is shut off, a standard curve will need to be included.
17. Immediately before use in a separate beaker, add 100 ul of 100 mg/mL m-hydroxydiphenyl in dimethyl sulfoxide to 4.9 mL of 80% sulfuric acid.
18. Using a repeat pipetter, add 10 ul of the above diluted m-hydroxydiphenyl in dimethyl sulfoxide to every well.
    1. **Note**: The recommended volume of diluted m-hydroxydiphenyl in dimethyl sulfoxide added to each well is ¼ the volume recommended in Bethke 2019. Linearity of the standard curve was improved at the lower volume of 10 ul per well of diluted m-hydroxydiphenyl in dimethyl sulfoxide.
19. Incubate at room temperature in a chemical fume hood for 15 minutes.
20. Read the absorbance at 525 nm on a UV-VIS spectrophotometer.
    1. Do not leave the plate in the spectrophotometer for longer than is necessary. Sulfuric acid and phenol fumes outside of the hood are bad for health and may damage the spectrophotometer if exposure is extended.
21. Do not shutoff the spectrophotometer lamp between consecutive readings and/or plates. If the lamp remains on continuously, only one galacturonic acid standard curve is needed. If the lamp is shut off, a standard curve will need to be included.
22. Subtract the absorbance from the second 525 nm reading from the absorbance from the first 525 nm reading for each well.
23. Average the three technical replicates for each sample and standard.
24. For each plate, subtract the average triplicate blank well absorbance from all average triplicate standards or samples.
    1. Each plate should be zeroed to itself.
25. Generate a linear model for the averaged, zeroed standard curve concentrations against their absorbance.
26. Calculate the final concentration of ug pectin (as D-(+)-galacturonic acid anhydrous) per mg AIR
    1. Note the 176 in the final calculation for ug GalacA per mg AIR is the molecular weight for anhydrous Galacturonic Acid which is the form found in pectin. If a direct comparison back to the standard curve is desired, used the molecular weight of the form found in the standard (typically monohydrate).

A screenshot of a computer

Description automatically generated

1. Properly dispose of all liquid hazardous waste (UIUC DRS UI #224290 - Trifluoroacetic acid, sulfuric acid, phenol, sodium tetraborate, sulfamic acid, 3-phenylphenol, DMSO, water) and solid, contaminated waste (UIUC DRS UI #224289 - Contaminated Debris – No liquid: Trifluoroacetic acid, sulfuric acid, phenol, sodium tetraborate, sulfamic acid, 3-phenylphenol, DMSO.)