DIBANET workshop for Biomass Sampling and Analysis
University of Limerick, Ireland
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Dry Extracted Samples Through Hydrolysis
E→H Rev: 1.00 (1-2-10)

This procedure is for dry sieved samples that have already been extracted and are ready for the acid hydrolysis stage.

FIRST DECIDE ON THE NUMBER OF SAMPLES (REFERRED TO HERE AS "X") THAT WILL BE ANALYSED.

Equipment Needed:

- An autoclave capable of reaching 121C and staying there for 1 hour.
- Analytical balance, accurate to 0.1 mg and weighing scale up to 300g
- Convection drying oven, with temperature control of 105 ± 3^{o}C
- Water bath, set at 30 + 3 °C
- Filtration setup, equipped with a vacuum source and vacuum adaptors for crucibles
- Desiccator containing desiccant
- Chromatogrpahy system (HPLC or IC)
- UV-Visible spectrophotometer, diode array or single wavelength, with high purity quartz cuvettes of pathlength 1 cm
- A deionised water maker.

Chemicals and Materials Needed:

- Sulfuric acid, 72% w/w (specific gravity 1.6338 at 20^{o}C)
- Sugar standards : L(-) Fucose, D(+)glucose, D(+)xylose, D(+)galactose, L(+)arabinose, and D(+)mannose
- 12 Pressure tubes, minimum 90 mL capacity, glass, with screw on Teflon caps and o-ring seals (Ace glass # 8648-30 tube with #5845-47 plug, or equivalent)
- 9 Stir rods sized to fit in pressure tubes and approximately 5 cm longer than pressure tubes.
- 9 Filtration flasks, 250 mL
- 10 porcelain crucibles
- 10 Filtering crucibles, 25 mL, porcelain, medium porosity, e.g. Coors #60531
- Disposable syringes, 3 mL, fitted with polypropylene syringe filters
- Autosampler vials with crimp top seals to fit

DAY BEFORE:

1. Take 2X small empty crucibles and put them in the oven overnight at 105C (MC of dried).
2. 2X clean and labeled filter crucibles should be put in the oven at 105C.
3. 2X Buchner flash adapters should be cleaned with deionised water.
4. 2X Buchner flasks should be cleaned thoroughly, first with NaOH then thoroughly cleaned, to ensure that no NaOH is left, with water and then deionised water. These should then be put in the oven at 105C.also be ready for tomorrow.
5. At least (2X+3) pressure tubes (Ace Glass) (a few extra should be washed incase, on inspection the next day, some are not clean) should be cleaned thoroughly, first with NaOH then thoroughly cleaned, to ensure that no NaOH is left, with water and then deionised water. These should then be put in the oven at 105C.
6. The tops to the pressure tubes should also be cleaned with deionised water.
7. 2X glass rods should be clean and dry today for their use tomorrow.
8. Make sure that we have the appropriate SRS's sugar solutions ready in the freezer for tomorrow. IT IS IMPROTANT THAT THE APPROPRIATE SUG SOLUTION IS USED FOR THE SAMPLE TYPES BEING ANALYSED, i.e. the SUG should have similar concentrations to that of the samples.
9. Make sure that a clean circular NIR cell is ready for tomorrow if access to the NIR is possible.
10. Get the samples (X) for tomorrow and put them by the analytical balance. The samples will be in red test tubes and should be labeled UNIVERSALCODE-E-BATCH NUMBER.
11. Get X of the "shaking boxes" and make sure they are clean. Put them by the balance.
12. Get X clean weighing boats and put by the balance.
13. The pressure tube holder and funnel need to be clean and by the balance.
14. Make sure that there is enough 72% acid in the automatic titrator reservoir for the hydrolysis next day. If there is not add more to the reservoir and "prime" according to the instruction booklet. Check that the density of the acid is correct – 3.00ml of 72% H_2SO_4 should equal 4.92g. If it does not there is either an error with the titrator and it needs to be sorted or the density of the acid itself is wrong in which case a new bottle needs to be used/ordered. If you do not have an automatic titrator then a transfer pipette can be used.

DAY 1 – ACID HYDROLYSIS

1. Take 2X small (empty) crucibles from the oven and place in the dessicator. These will be the crucibles used for determining the moisture content of the samples. It is important that they are left to cool for approx 20 minutes in the dessicator before weighing.
2. Take 3 SUG test tubes (accurately weighed sugar standard solutions) from the freezer (these should already have been pre-weihed) and allow to defrost (but make sure they are standing up and not lying plat). IT IS IMPROTANT THAT THE APPROPRIATE SRS IS USED FOR THE SAMPLE TYPES BEING

ANALYSED, i.e. the SRS should have similar concentrations to that of the samples.

3. **Check the water level in the waterbath**. There must be enough water in the water bath so that the water level is above that of the acid in the pressure tubes.
4. Set the water bath at 30C and check that this is the actual temperature by using a thermometer. **It is crucial that a constant 30C is maintained.**
5. Take a new data recording sheet (E→H) and fill in the samples data (universal code, nir code, old code, date sampled etc.). Also fill these data in the Batch Database Description file.
6. Make sure that a very clean sample bottle (500ml) is ready for filling with deionised water at a later point.
7. Make sure that the deionised water "squeezy bottle" is clean and ready for use after the primary hydrolysis.
8. Hydrolysis of Sample 1 (should take approx. 10 minutes for two pressure tubes):
    a. Take the red test tube containing the extracted material and empty the contents into the "shaking box".
    b. Shake the box for 2-3 seconds.
    c. Take the clean NIR circular (non-moving) cell and, using the weighing boat, fill the cell with the sample.
    d. Carefully place the cell over the correct scanning position in the NIR and then close the lid.
    e. Press scan.
    f. While this is scanning you can take a small crucible from the dessicator and record its number and weight (accuracy 0.1mg).
    g. Add approximately 300mg of the sample to this crucible and record the weight again (accuracy 0.1mg).
    h. Repeat f-g for the other small crucible.
    i. **NOTE FOR SAMPLES WHERE WE HAVE VERY LITTLE MATERIAL WE WILL NOT DO THE MOISTURE CONTENT WITH CRUCIBLES BUT WILL INSTEAD ONLY DO THE NIR SCAN**.
    j. The NIR scan will now have completed. Save the spectra in product group (E-H) with the filename being NIRCODE-BATCHNUMBER.
    k. Put the sample that was in the NIR cell back in the mixing box.
    l. Take one of the pressure tubes and record the number.
    m. Take the pressure tube holder (the drinks bottle) and insert the pressure tube into this so that it stands up vertical and straight. Put this in the centre of the weighing scales (from the top, so the top lid will need to be closed) and pull the cardboard box over the scales. Wait for weight to be steady then press TARE.
    n. Take the funnel and place it in the pressure tube ensuring that it points straight down to the centre base of the pressure tube and that you do not move the pressure tube and bottle when this funnel is inserted.
    o. Make a mental note of the weight shown on the scales. This is the weight of the funnel. The target will be that we will add approx 300mg (+ or – 10 mg) of sample to by using the weighing boat. I**t is important that the**

sample gets to the bottom of the pressure tube and does not get stuck on the sides. If it does get stuck on the sides then you will need to push it down to the bottom with the glass rod.

p. Once this level (300mg) is achieved carefully remove the funnel without shaking the pressure tube and bottle. Note that the cardboard box shield should still be over the scales.
q. Wait for the weight on the weighing scales to steady and then record this weight to the nearest 0.1 mg.
r. Push the cardboard box shield back in order for the titrator to be inserted. Take the automatic titrator and lower it down so that its nozzle points to the bottom centre of the pressure tube but ensure that the pressure tube or bottle are not touched by the nozzle or its supports.
s. Press Tare.
t. Add 3.00 ml (4.92 g) of sulphuric acid to the pressure tube (with the automatic pipette) making sure that the acid only touches the bottom of the tube. Record the volume dispensed (from the reading on the titrator) and the weight on the scales (to the nearest 0.1mg). If you do not have an automatic titrator then a transfer pipette can be used.
u. Then take the pressure tube and bottle support out of the scales. Slowly (so that there is not a jerk at the end) take the pressure tube out of the bottle support. **Always keep the pressure tube vertical and ensure that the sample and acid do not go up the sides**.
v. Take a clean glass rode and mix the acid and sample for about 30 seconds. It is crucial that the sample is mixed with the acid and that both stay at the base of the pressure tube and do not go up the sides.
w. Then put the pressure tube (with rod) in the water bath.
x. Record the time when this is done. Also record this time in the "Acid Stirring Column".
y. Repeat steps (l)-(x) for a second pressure tube using the same sample from the shaking box.
z. Take the red test tube and place it inside the shaking box so that (at a later point) the remaining material can be transferred back into the test tube.

9. Repeat Step 8 for the remaining (X-1) samples.
   a. IMPORTANT – The acid and samples in the pressure tubes in the bath need to be mixed at regular intervals. This must be done very carefully, do not take the pressure tube out of the bath when you do it. Use careful circular motions at the base of the pressure tube with the glass rod to stir. This is done to ensure that the sample and acid do not go up the side of the pressure tube. If some does go up the side attempt to push it back down again by using the glass rod. Because the way the water in the water bath distorts the vision it is only possible to see one side of the pressure tube at a time. Hence, after stirring for a few seconds (and ensuring no material is on the sides) rotate the pressure tube 180 degrees and repeat (this will allow you to inspect the other side of the pressure tube). There is an ACID STIRRING TABLE on page 2 of the Data Recording Sheet. On this record the time the first test tube is put in the bath and then record a note

of the time at every session when the existing pressure tubes in the bath are stirred. The way I used to do it was to stir all tubes in the bath after every 2 pressure tubes have been added or taken away. However this only works if the tubes are added/withdrawn at a similar rate. **In effect, the tubes should be stirred about every 10 minutes they are in the bath, so at least 5 times per tube.**

10. Just before you are ready to take the first pressure tube out of the water bath go and fill the deionised water bottle (500ml) with deionsed water and then transfer some to the empty and clean deionised "squeezy" bottle.
11. It should now be 1 hour since the first pressure tube was put in the water bath. When it is exactly one hour, take the tube out of the water bath and put on the 600g scales (using the tube holder as support). Record the time when this was done.
12. Lift the stirring rod up (but not out of) the pressure tube so that it is not adding to the weight, try and get all of the acid and biomass off the rod by rubbing it against the inside of the glass pressure tube. Tare the weight when a steady value is reached. Then use the squeezy bottle to spray the deionsied water around the edges of the rod to remove all of the sample and acid from the rod. Then take the rod from the tube and put to the side. Then add more mixing water until 84.00g (+ or – 0.04 g) is added. Record the exact weight of the water added. Then put the top tightly on the pressure tube and invert several times to mix the water and acid. Then put the pressure tube in the autoclave rack.
13. Repeat 11-12 for the other pressure tubes when their hour is up. Remember to keep stirring the pressure tubes in the water bath at regular intervals while doing this.
14. **SRS Standards**:
    a. Before adding acid to the SRS test tubes ensure that the micro-pipette is functioning properly by calibrating the right volume.
        i. We need to dispense 348 υl of 72% sulphuric acid, which is equivalent to 0.571g of acid. Take a red test tube and, using the automatic titrator, dispense some of the 72% sulphuric acid into this test tube. This will be used for obtaining the acid to add to the SRSs.
        ii. Take a new test tube, place on the scale and tare the weight. Find the right setting on the micro-pipette so that 0.571g (or close to) is consistently dispensed (do a few times to check with the same test tube, taring each time, for consistency).
    b. Then take a defrosted SRS test tube and slowly open the top making sure that no liquid is in the top or lost. Place the SRS test tube in a holder so that it is pointing straight up in the scales and tare the weight. Add 348 υl of 72% sulphuric acid by using the micro-pipette ensuring that the test-tube is not moved. Record the weight of the acid added. Then put the lid back on the test tube and shake about, invert and Vortex the mixture and then transfer to a pressure tube. Make a note of the number of the pressure tube and the amount of SRS solution in the test tube (from the SRS label) and the amount of acid added. This will correspond to SRS-1.
    c. Repeat (b) for another test-tube/pressure-tube and SRS-2. Add the pressure tubes to the autoclave rack.

15. Autoclaving:

a. Check the water level of the autoclave, it should be just below the bolts.
b. Put the autoclave rack in.
c. Put the top on the autoclave and close the bolts – do opposite bolts at the same time but not too tightly. Once all bolts have been closed, go back and tighten (with two hands) each pair of bolts.
d. MAKE SURE THAT THE PRESSURE RELEASE VALVE IS CLOSED!!!#
e. MAKE SURE AGAIN!!!!!!
f. Turn on the autoclave and press "Variable Cycle", make sure the settings are at 60 minutes and 121C.
g. Press "Start".

16. It is now likely to be lunch time!!!

17. While the pressure tubes are in the autoclave:
    a. Make sure to have 2X dry, cool and clean filter Buchner flasks and crucible holders.
    b. Also take 2X filter crucibles out of the oven (105C) and put in a dessicator and approx 20 minutes later record the weight of the filter crucibles in the appropriate box (weigh to 0.1mg), **make sure to record which scales were used**. These filter crucibles are now ready for the filtration stage of the hydrolysis. REMEMBER TO MATCH THE APPROPRIATE PRESSURE TUBE WITH THE APPROPRIATE FILTER CRUCIBLE.
    c. Make sure 2X clean and dry Buchner flask adaptors (the cut black ones) are ready.
    d. Make sure the surface used for the filtration is clean.
    e. Prepare test tubes for putting the hydrolysis samples in. The coding system used is BATCHNUMBER-UNIVERSALCODE-H-PRESSURETUBENUMBER. Also record the date on the label. For the SRS tubes the coding system is BATCHNUMBER-DATE-SRS(1or2)-PRESSURETTUBENUMBER.
18. When the autoclave has finished remove the rack and allow the hydrolyzates to slowly cool to room temperature before opening the lids.
19. Just before doing the filtration take the appropriate numbered filter crucibles for the pressure tubes and place them in buchner flasks (securing it with the Buchner flask adaptors) and attach the suction device. Put the pressure tube in a flower pot that is directly infront of the Buchner flask (with appropriate filter crucible).
20. Turn on the pumps.
21. Filtration of hydrolysate:
    a. Take a pressure tube and invert it and shake it vigorously. Pour the contents onto the appropriate filter crucible making sure not to overfill the filter crucible.
    b. Repeat for the other pressure tubes and their corresponding filter crucibles.
    c. The filtration step is made much quicker if the solids are brought into suspension by carefully vortexing (with your hand) the pressure tube prior to pouring the solution into the filter crucible.
    d. If any solids or liquids are spilt from the filter crucible or pressure tube make a note of this.

e. When all of the hydrolysate has been removed from the pressure tube and has passed through the filter crucible turn off the pump and carefully remove the filter crucible and Buchner flask adaptor from the Buchner flask. Then shake the Buchner flask with your hand. Transfer some of the hydrolysate to the appropriate test-tube for that pressure tube. The test tube should have the following label: BATCHNUMBER-UNIVERSALCODE-H-PRESSURETUBENUMBER. The tube should be filled to approx 85% capacity. It should be then put to the side to use for UV-Vis analysis later.
f. Then put the Buchner flask adaptor and filter crucible back on the Buchner flask and turn on the pump again.
g. Repeat e-f until all the hydrolysates have been put in test tubes.

22. Transfer of remaining acid insoluble residue:
    a. Using deionised water transfer the remaining solid residue from the pressure tubes to the appropriate filter crucibles.
    b. The best way to do this is to use the squeezy bottle to dislodge all of the material from the sides of the tube so that they fall to the bottom. Then, by producing a vortex as before, get the solids in suspension and pour them into the filter crucible. There will be some solids still stuck on the sides but these should be in a straight line (representing the path of the the water to the filter crucible).
    c. If the pressure tube is kept at an angle of about 50% with the top pointing into the filter crucible and the “solids line” at the lowest point of the tube (i.e. on the path that water flowing down the tube would follow), a squeezy bottle that is full of deionised water should, if squeezed strongly and angled correctly, produce a stream that goes to the back of the pressure tube and then runs down so dislodging the stuck biomass and running it into the filter crucible.
    d. Ensure all the sample is transferred to the filter crucible.
    e. Repeat for all pressure tubes and their appropriate filter crucibles and then turn off the pumps.
23. When 22 is complete put the filter crucibles on a tray and put it in the 105C oven to dry them overnight.
24. ACID SOLUBLE LIGNIN:
    a. Firstly the UV-Vis device needs to be turned on at least an hour before it is to be used.
    b. A blank will need to be prepared. This will be a solution of 4% sulphuric acid in deionised water. This blank can also be used to dilute the hydrolysates if necessary. Take 200ml of deionsed water and add 11.714g of 72% sulphuric acid. Mix thoroughly.
    c. On the spectrophotometer, fill the sample cell with the blank and run this as a background/blank. The sample cell is cleaned by using the pipette to suck out the sample that is in the cell.
    d. Using the hydrolysis liquor aliquot obtained earlier measure the absorbance of the sample at an appropriate wavelength on a UV-Visible spectrophotometer.

e. Dilute the sample as necessary, using the blank, to bring the absorbance into the range of 0.7 – 1.0, recording the mls of hydrolysate and mls of blank factor.

f. Record the absorbance to three decimal places. Reproducibility should be $\pm$ 0.05 absorbance units.

g. Analyze each sample (i.e. each test tube) in duplicate, at minimum. (This step must be done within six hours of hydrolysis.)

h. Repeat d-g for all test tubes.

25. PUTTING TEST TUBES IN THE FREEZER:
    a. Unless the hydrolysates are to be diluted on the same day (in which case refer to the dilution procedure) they will need to be stored as frozen. It is important that the samples freeze when they are standing up so that there are no leaks. Therefore use a test tube rack to get all of the test tubes vertical and then place them in the freezer.
    b. The next day the samples will be frozen, take them out of the test tube rack and transfer to a blue sample bag (Ziploc). Label the bag with the batch number and the date.

DAY 2 – WEIGHING FOR ACID INSOLUBLE RESIDUE CONTENT

1. Take the 2X crucibles that were used for determining the moisture content of the samples and place in the dessicator. 20 minutes later weigh these crucibles to the nearest 0.1mg. Then transfer them to the ashing muffle furnace, making a note of what position each filter crucible is in. Put a porcelain lid on each of the crucibles.
2. Take the 2X filter crucibles out of the oven and place in the dessicator (the green rack can be used to help the crucibles stand up straight). 20 minutes later weigh these filter crucibles to the nearest 0.1mg. Then transfer them to the ashing muffle furnace, making a note of what position each filter crucible is in. Put a porcelain lid on each of the filter crucibles.
3. When it is a suitable time (e.g. when people are going home) plug in the furnace and start Program 2. That follows this heating regime:

    Ramp from room temperature to 105 °C
    Hold at 105°C for 12 minutes
    Ramp to 250 °C at 10°C / minute
    Hold at 250 °C for 30 minutes
    Ramp to 575 °C at 20 °C / minute
    Hold at 575 °C for 180 minutes
    Allow temperature to drop to 105 °C
    Hold at 105 °C until samples are removed

DAY 3 – WEIGHING FOR ASH CONTENT OF ACID INSOLUBLE RESIDUE

1. Check the temperature of the ashing furnace. If it is 105C then it is OK to take the filter crucibles out. If it is more than 105 then slowly open the furnace door and allow the furnace to cool until the temperature is 105C at which point the filter crucibles can be taken out.
2. The filter crucibles can be put in the green rack so that they stand up better in the dessicator. However make sure that they have their lids on when they are put in the dessicator because otherwise, when the dessicator is opened for weighing the crucibles, the ash will be blown away.
3. 20 minutes after the filter crucibles have been put in the dessicator weigh them to the nearest 0.1mg.
4. After the filter crucible has been weighed, empty the ash out and blow to remove the ash and weigh it again to the nearest 0.1mg.
5. Repeat steps 1-4 for the crucibles with the original biomass samples in.