Perchloric acid extraction

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Variations of PCA extraction protocols are also stored below.

PERCHLORIC ACID STARCH / METABOLITE EXTRACTION FOR ARABIDOPSIS; Retsch Mill

Materials:
Retsch Mill, M300
Microfuge tube holder for Retsch Mill (Qiagen, 24 tube holder)
3 mm Stainless steel balls, or silicon carbide particles #6 grit (BioSpec #11079140sc)
2 Styrofoam boxes, one with ice, other for LN_2
2 ml Microfuge tubes (Sarstadt work best)
1.5 ml Microfuge tubes
LN_2
Cold 3.5% M Perchloric Acid (in fridge)
Neutralizing Buffer (2M KOH, 150 mM Hepes, 10 mM KCL)
80% Ethanol
0.2 M KOH
1 M Acetic Acid
Dry Bath for microfuge tubes, 95°C
200 mM Sodium Acetate pH 4.8
Amyloglucosidase (0.2 U μl^{-1})
α-Amylase (1 U μl^{-1})

Procedure:
1. Label and weigh 2 ml microfuge tubes, it is helpful to weigh them with a silicon carbide particle already in the tube so you don't have to add it later
2. Harvest leaf material and, **push to bottom of tube**, and immediately freeze in LN2
   **** Make sure there is not too much material, ≈ 500 mg FW maximum, or it will not grind well ****
3. After freezing, quickly weigh again, to determine fresh weight
4. Store in -80º C until ready to continue with assay
5. Place Retch mill microfuge tube holder in Styrofoam box and cool with LN_2
6. Get plant samples from step 4 and place a stainless ball in each sample tube
   **** Be sure to push plant material to bottom so that ball can move freely ****
7. Place samples in cooled microfuge tube holder and place and secure holder containing vials into Retsch Mill, you will need cryo gloves
8. Tighten the outer knob first, then move the ratching set pin into the locked position and tighten further (3 clicks)
   Make sure each holder has a microfuge tube holder, however the second microfuge tube holder does not have to be filled with tubes
9. Grind samples for 30 sec at a frequency of 30 (maximum speed). If the sample isn't pulverised enough, carefully open tube while cold and partially break up leaf material with a pair of chilled forceps. Grind again in Retsch mill for 30 seconds.
10. Add perchloric acid to each vial, use aprox 2 µl per mg of tissue (typically 600 µl for 3 large Arabidopsis leaves), record the amount of perchloric acid you add to each tube
    ** **Allow tube to warm slightly before closing so you don't have an explosion** **

11. Place tubes on ice and allow to incubate for 5 min
12. Centrifuge at maximum speed, 4°C for 10 min
13. After centrifugation carefully remove the supernatant without getting any pellet and place into a 1.5 ml microfuge tube save pellet for starch analysis. If you added 600 µl perchloric acid you should be able to recover 500 µl
14. Add ≈ 0.25 µl Neutralizing buffer per µl of recovered perchloric acid (125 µl for 500 µl perchloric acid) to bring the pH to around 7, If unsure check pH using pH sticks, be sure to record the volume of neutralizing buffer you use. Note if the solution turns yellow the pH is > 7, pink pH < 7
15. Freeze and thaw sample to precipitate salts
16. Centrifuge for 2 minutes. Pipette off supernatant and freeze this or assay for carbohydrates

Starch

17. Resuspend pellet in 2X 850 µl water to remove phytoglycogen
18. Centrifuge at maximum speed for 5 min at 4°C, discard supernatant.
19. Add 2X 850 µl 80% EtOH vortex
20. Centrifuge at maximum speed for 5 min at 4°C, discard supernatant,
21. Dry samples in speed vac to remove any remaining ethanol, this can interfere with enzymes later
22. Resuspend pellet in 500 µl 0.2 M KOH, vortex or shake to break up pellet
23. To gelatinize starch incubate microfuge tube at 95° C for 30 minutes, use dry block heater
    **** make sure to use tube locks ****
24. Once it is done incubating allow to cool for 2 - 5 minutes and then add 50 µl of 1 M Acetic Acid, this brings the pH to around 5, if you are unsure check pH using pH paper. We want a pH of around 5 to keep the amyloglucosidase and α-amylase that will break the starch down happy. Make sure to record the volume of Acetic Acid you add so you can determine moles of glucose later on.
25. Add 200 µl 200 mM sodium acetate buffer pH 4.8
26. In a 15 ml tube make up an enzyme mix using the following formula
    + 2 µl amyloglucosidse (0.2 U μl^{-1}) * # of samples
    + 50 µ l α-amylase (1 U μl^{-1}) * # of samples
27. Add 50 µl enzyme cocktail to each sample and incubate on a shaker at room temperature for 1 - 2 days
28. Centrifuge for 20 minutes and place supernatant in a fresh tube to use for glucose assay.
29. Assay 5 µl of starch sample for glucose

Math:

To calculate soluble sugar amounts in mol / g FW use the following formula

Molarity of sample * 500e-6 + 125e-6 (total volume of sample) * 600e-6 (original volume of sample) ÷ 500e-6 (volume sample was before adding neutralizing buffer) ÷ fresh weight of plant sample in grams = moles of sugar per gram FW

To calculate starch amounts in mol glucose / g FW use the following formula

Molarity of sample * 800e-6 L (total volume of sample) ÷ fresh weight of plant sample in grams = moles of starch glucose per gram FW

STARCH EXTRACTION REAGENTS

3.5% Perchloric Acid:

In 400 ml of distilled H_2O

+ 20 ml of 70% perchloric acid
remember acid into water never the reverse

Neutralizing Buffer:
2M KOH, 150 mM Hepes, 10 mM KCl
In 250 ml of distilled H_2O
+ 8.936 g Hepes
+ .186 g KCl
+ 28.055 g KOH

80% Ethanol, 500 ml
In 100 ml distilled H_2O
+ 400 ml 100% ethanol

0.2 M KOH:
In 250 ml distilled H_2O
+ 2.81 g KOH

1 M Acetic Acid:
In 235.625 ml distilled H_2O
+ 14.375 ml glacial acetic acid
remember acid into water never the reverse

200 mM Sodium Acetate pH 4.8, 500 ml
In 500 ml distilled H_2O
+ 13.61 g Sodium Acetate (MW 136.08)
pH to 4.8

Amyloglucosidase Stock
The Amyloglucosidase comes from Megazyme (E-AMGDF) and is sold in a 50% glycerol solution and is ready to use directly from the bottle.

Alpha Amylase Stock 1 U μl^{-1}:
1. In 7 ml 200 mM Sodium Acetate Buffer pH 4.8 add 3 ml Glycerol, 30%
2. Place 2 ml of alpha amylase (1000 U/ml; Megazyme E-ANAAM) into two 1.5 ml microfuge tubes
3. Centrifuge max speed at 4°C for 5 minutes
4. Pipette off and discard supernatant and add 1ml glycerol buffer made in step 1 to each tube this will give you 1 U μl^{-1}.
   Store tubes in -80°C
   **** Remember to check specific activity of enzyme ****

Ziru Li

Perchloric acid extraction for NMR samples

Last modified 07/15/10

5. Measure the leaf area and pre-weigh leaf before gas exchange experiment.
6. Prechill a mortar in liquid nitrogen. Add 1ml of 70% (v/v) perchloric acid with 250 umol

maleate and 5 umol methylphosphonate.

7. At different times during the gas exchange experiment, take out the leaf and flash-freeze / freeze-clamp it. Then grind up the leaf to a fine powder in perchloric acid.
8. Place the frozen powder at -10C and let thaw.
9. Centrifuge at 15,000g for 10 min to remove pellet.
10. Pour the supernatant to a new tube. Neutralize supernatant with 2M $KHCO_3$ to ~pH5, then centrifuge again at 10,000g for 10 mins to remove pellet.
11. Lyophilize the resulting supernatant. The lyophilized powder can be stored in liquid nitrogen at this step.

12. Reconstitute plant extract in 0.5ml water containing 10% D2O, neutralize to pH7.5 and buffer with 50mM HEPES.
13. Chelate divalent cations by adding sufficient amounts of 1,2-cyclohexylenedinitrilotetraacetic acid (50-100umol).

Note: This protocol is adapted from Aubert et al., *JCB* **133**:1251-1263, 2006 and Rivasseau et al., *PCE* **32**:82-92.

Perchloric acid extraction of DXP in poplar leaves

Last modified July 11, 2011

The following protocol is for extracting sugar phosphate from poplar leaves and may be generalized to extracting any polar compound from a leaf sample. The protocol starts with an acclimatized poplar leaf or a leaf in gas exchange experiment.

14. Acclimatize poplar leaf.
15. Pre-cool a mortar and pestle immediately before the next step (freeze-clamping).
16. Dip the clamp end of the freeze clamp in liquid N_2, and then quickly clamp the leaf with the freeze clamp and then place the clamp with the entire leaf in liquid N_2. The leaf will become brittle. Quickly knock off parts of leaf outside of the clamp area, and then drop the freeze-clamped part of the leaf into the mortar. If leaf piece does not want to come off the freeze clamp, peel it off with a pair of tweezers.
17. Add some liquid N_2 into the mortar and start grinding. Start with a slow crush, use up and down motions (avoid circular grinding motions) to avoid loss of leaf tissues from the mortar. Crush the leaf into fine powder as the liquid N_2 evaporates. This should be easy as the leaf at this point is quite brittle.
18. Add 0.88mL of extraction buffer into the mortar, grinding while adding. The extraction buffer will quickly freeze to a chunk of ice. Continue crushing and grinding until the chunk has been ground into a green snow. Grind harder in a circular motion. If the mortar/pestle is too cold to hold onto, insulate yourself with some layers of paper towels. Place a layer of tin foil after grinding so condensations would occur on top of the foil.
19. Let thaw.
20. Transfer the plant extract with a Pasteur pipette (break the end for a bigger opening if needed) into a vial, and centrifuge at max (>20,000g) for 10 mins. 20 mins is desired if time permits. Start vacuuming and cooling down the lyophilizer during this time.
21. Transfer 0.50mL of the supernatant to a new vial (ideally glass), and add enough neutralizing buffer to neutralize plant extract to 7. For ~500uL of supernatant 200uL of neutralizing buffer is a good starting pointing. Pipette up and down to thoroughly mix the solution, be careful as a

lot of bubbles (CO_2) will come out, and then pH it with pH paper.

22. Centrifuge at max for 1 min.
23. Transfer the supernatant to a new tube, then flash freeze in liquid N_2.
24. Let thaw.
25. Centrifuge at max for 10 min.
26. The purpose of steps 10-12 is to get rid of the salts in the solution, if desired this part can be repeated for one or two more times to get rid of more salts, in practice it probably makes little difference.
27. Flash-freeze again in liquid N_2.
28. Lyophilize the frozen samples to get the solutes.
29. Pulse-centrifuge the resulting fluffy solids (~10 seconds) to settle down the powders.
30. Re-constitute the powders in HPLC buffer and load onto LC-MS.

Note:

31. Actual amount of DXP = 2 x the amount determined in the end since only 0.5ml of supernatant was taken in step 8.
32. The clamped leaf area is ~9.6cm^2.

Materials needed:
Ice-cold extraction buffer (perchloric acid + EDTA)
Neutralizing buffer

3.5% Perchloric Acid:
In 380 ml of distilled H2O
+ 20 ml of 70% perchloric acid
remember acid into water never the reverse

Extraction Buffer:
20mM EDTA in perchloric acid
In 100mL of 3.5% Perchloric acid
+ 0.832g of EDTA

Neutralizing Buffer:
2M KHCO3, 20mM EDTA

HPLC Buffer:
50mM CH3COONH4, pH-ed to 11 with NH4OH, 20mM EDTA