

# BIOMASS SEQUENTIAL EXTRACTION PROTOCOL

(Courtesy from Ana Paula Alonso)

## I. Extraction and methylation of fatty acids

1. Weigh ~ 5 mg of lyophilized fine powder of tissue and add one tungsten bead (5 mm) previously rinsed with hexanes.
2. Warm up **C:17 heptadecanoic acid (TAG) internal standard solution (2 mg/mL)** from freezer that is dissolved (in hexanes: isopropanol [2 : 1]) for 5-10 minutes at 60°C.
3. Add 50 µL of C:17 internal standard with a 100 µL Hamilton syringe to the different samples. Complete to 1 mL with hexanes: isopropanol [2 : 1] using a 1 mL Hamilton syringe.
4. Shake in bead beater 5 minutes (frequency: 30 Hz).
5. Centrifuge samples at 17,000 x g for 15 minutes in a microcentrifuge.
6. Carefully remove supernatant (solvent too fluent) to a 13 x 100 mm glass test tube (**PREVIOUSLY RINSED WITH HEXANE**) using a Pasteur pipet.
7. Repeat steps 3-6 (**DO NOT ADD C17 INTERNAL STANDARD**) twice with respectively 1 mL hexanes: isopropanol [2 : 1] for extraction 2 and 3. Combine supernatant from the different extractions.
8. Check if there is a pellet in the extract and if it is the case centrifuge at 800 x g in a swinging bucket (Legend X1R) for 2 minutes.
9. Carefully remove supernatant to a new 13 x 100 mm glass test tube (**PREVIOUSLY RINSED WITH HEXANE**) using a Pasteur pipet.
10. Add 500 µL of hexanes to the pellet, pipet up and down 3 times and transfer liquid to the original 2 mL Startedt tube containing your soybean extract.
11. Dry down the combined extracts at 60°C under nitrogen. **KEEP THE PELLETT TO DO PROTEIN, STARCH and CELL WALL EXTRACTION!!! DRY IT AND STORE IT AT -80°C.**
12. Add to the extract 150 µL of toluene with a Hamilton syringe and vortex for 5-10 seconds.
13. Add 0.5 mL of a Methanol solution containing 2.5 % (v/v) of sulfuric acid (with graduated glass pipet) freshly prepared. Flush nitrogen for 5-10 seconds.
14. Vortex for 30 seconds.
16. Incubate samples for 120 minutes at 80°C in a dried incubator or oven. Check tubes for leaks after 15-20 minutes and vortex tubes every 30-45 minutes during incubation.
17. Add 250 µL of 5% NaHSO<sub>4</sub> in H<sub>2</sub>O and vigorously mix.
18. Add 1 mL hexanes and vortex vigorously for 1 minute.
19. Spin at 2000 rpm (560 x g) for 5 minutes at room temperature.
20. Take 100 µL of supernatant with a 0.5 mL Hamilton syringe and transfer methylated fatty acids to a 2 ml glass vial. Then, transfer 500 µL of supernatant into a 13 x 100 mm glass test tube using a Hamilton syringe.
21. Add 900 µL of hexane to the 100 µL sample in the vial.
22. Dry down the sample in the 13 x 100 mm glass tube at room temperature under nitrogen and store at 4°C.

23. Samples are ready for GC-MS run.

## **II. Extraction and quantification of proteins**

### 1. Extraction:

1. Dry pellet from previous lipids extraction under a nitrogen flow.
2. Add 0.5 mL of extraction buffer (20 mM Tris-HCl, pH 7.5, 150mM NaCl, and 1% SDS) previously pre-warmed at 42°C.
3. Vortex (on position 7) for 15 minutes at 42°C (push samples half way down into the foam; start at 10 then reduce speed).
4. Centrifuge at 17,000 x g for 10 minutes.
5. Transfer supernatant into a 2 mL tube using a 1000 µL pipet.
6. Add 0.5 mL of pre-warmed extraction buffer.
7. Vortex (on position 7) for 10 minutes at 42°C.
8. Centrifuge at 17,000 x g for 10 minutes.
9. Pipet supernatant into a 2 mL tube.
10. Repeat step 6-9 one more time. **At the end of the protein extraction keep the pellet to do starch and cell wall analysis.**

### 2. Quantification:

#### Note and material needed:

- **DC Protein Assay Kit II from BIO-RAD (Cat# 5000112)**
  - Incubator set up at 42°C (*Used to warm up protein samples and extraction buffer*)
  - 10000 µg/mL BSA solution (*Aliquots are located in the -20 freezer. You take one tube/aliquot each time you need to quantify proteins*).
  - 13 x 100 mm glass tubes
  - Two 50 mL conical tubes (*One to prepare reagent A' and one for reagent B*)
1. Use a BSA solution (10000 µg/ml) to generate a standard curve. The solution is at -20°C. Thaw at room temperature then store on ice.
  2. Prepare the different BSA dilutions as indicated in the table. **Make a master mix for each point meaning that you will have to prepare 3.5 samples for each point** [Example for 250 µg/mL: 3.5 x 2.5 µL (BSA)+ 3.5 x 97.5 µL (Extraction buffer)].
  3. Vortex and use the minicentrifuge to bring down droplets. Distribute 100 µL into the bottom of each glass tube as indicated in the table.
  4. Prepare "Protein" samples as indicated in the table. **Read instructions below the table!**
  5. **Add 500 µL of reagent A'** (add 20 µL of reagent S to each mL of reagent A that will be needed for the run, example: when you have 20 samples you need to prepare 8 mL [14 samples + 2] of reagent A that you mix with

160  $\mu$ L of reagent S in a 50 mL tube) **into each test tube**. (If crystals appearance when adding S + A, warm up at 42°C)

6. Add 4 mL reagent B into each test tube and vortex immediately.

7. Incubate for 15 min and transfer 1 mL into cuvettes.

8. Read the absorbance at 750 nm. The absorbance will be stable at least one hour.

Tube #	Sample	VOL ( $\mu$ L)	Extraction buffer ( $\mu$ L)	Final vol. ( $\mu$ l)	Emission (750 nm)
1	O-BSA-1	0	100	100	N/A
2	250-BSA-1	2.5	97.5	100	
3	250-BSA-2	2.5	97.5	100	
4	250-BSA-3	2.5	97.5	100	
5	500-BSA-1	5	95	100	
6	500-BSA-2	5	95	100	
7	500-BSA-3	5	95	100	
8	1000-BSA-1	10	90	100	
9	1000-BSA-2	10	90	100	
10	1000-BSA-3	10	90	100	

### **III. Extraction and quantification of starch**

#### 1. Extraction:

1. Add 1 mL of ultrapure water to the pellet from previous lipid and protein extractions. Vortex and remove bead.
2. Centrifuge at full speed for 10 minutes.
3. Remove carefully supernatant and add 1.0 mL of ultrapure water to the pellet.
4. Vortex and centrifuge at full speed for 10 minutes.
5. Remove carefully supernatant.
6. Add 0.5 mL of 0.1 M Acetate buffer pH 4.8.
7. Loosely cap with lids, wrap samples in an aluminum foil and put them in a tray containing water.
8. Autoclave samples at 120°C during one hour.
9. Let the samples cool down (tighten caps) for 15 minutes at room temperature.
10. Add 0.5 mL of 0.1 M Acetate buffer pH 4.8 and incubate at 55°C in a dry bath + vortex until pellet is resuspended and then add 10  $\mu$ L of amyloglucosidase to each tube.
11. Mix by inverting and incubate at 55°C for 2 hours, inverting every 30 minutes.
12. Centrifuge samples at full speed for 15 minutes.
13. Carefully pipet 0.5 mL of upper phase into a new 2 mL tube.

13. Proceed to quantification of samples using **Total Starch assay procedure kit from Megazyme (Cat# K-TSTA)**.

2. Quantification:

1. Add the following components to a 1.5 mL tube:

Tube	Sample name (tube #)	Sample ( $\mu$ L)	GOPOD reagent (mL)	OD (510nm)
1	Blank (acetate)	35	1	
2	Glucose-1	35	1	
3	Glucose-2	35	1	
4	Glucose-3	35	1	

2. After adding all the components, vortex gently (vortex set up on 7-8) and centrifuge briefly with a minicentrifuge.

3. Incubate the tubes at 50°C for 20 minutes.

4. Transfer the whole content of the 1.5 mL tube into a plastic cuvette and read the absorbance at 510 nm.

