Metabolite extraction from tissues using a bead beater

i.e using the TissueLyser II or TissueLyser LT (Qiagen)

Use at a minimum 5 samples/replicates per condition.

This bead beating method will work for softer tissues (not for skin, etc.). The TissueLyser II is more powerful than the TissueLyser LT.

See Materials section below for tube options. With the TissueLyser LT, less samples can be processed at the same time when using 2 ml Eppendorf tubes than when using Screw Cap tubes.

***Important:** When dissecting the tissues, work quickly and on ice! Try to obtain very similar-sized tissue pieces for each sample in the range of 10 – 30 mg.*

1. Prepare 1.5 ml microcentrifuge tubes and place on ice.
2. Dissect the tissue(s) on ice. Rinse the tissue in ice-cold 150 mM NH4OAc pH 7.4[(1)] to remove any blood, if desired, and blot dry.
3. Cut the tissue in smaller pieces (see above) and transfer the pieces to a cold microcentrifuge tube.
4. *Immediately snap-freeze the tissue samples in liquid nitrogen for 5 min*.
5. Store the tissues in a -80 ˚C freezer until ready for further processing.

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6. Measure out - and weigh - the needed amount of tissue per sample.
   a) Precool a microcentrifuge tube for each sample to be extracted on dry ice.
   b) One by one: place the tube on a microscale and zero it. Back on dry ice, add the tissue piece(s) to be extracted, and weigh the tube again. Record the weight of the sample. Place the sample back on dry ice.

   Alternatively, you can skip the weighing of the individual samples, but you need to know approximately how large the needed tissue pieces have to be and try your best to use very similar amounts of tissue per sample.

7. Calculate the amount of the extraction solution (80% MeOH) needed per sample:
   Use i.e., 1 ml per 20 mg tissue. *Adjust the volume of MeOH extraction solution according to the tissue weight*.
8. Add one stainless steel bead per 2 ml Eppendorf tube.
   Include one tube for a sample processing blank containing no tissue.
   Label the top of the Eppendorf tube close to the hinge (not in the middle area) and on the side with a good quality permanent marker.
9. Add the calculated volumes of ice-cold 80% MeOH to the 2 ml tubes. To the blank tube, add the average volume of 80% MeOH used in the experiment.
10. Precool these prepared 2 ml tubes on dry ice.
    Precool the TissueLyser II or LT tube holders for about 5-10 min in a -20 ˚C freezer.
11. Add the frozen tissues to the prepared 2 ml tubes on dry ice.
    Homogenize the tissues as recommended by the manufacturer, i.e.,
    when using the TissueLyser II, try 2-4 rounds of 30 s at 30 oscillations/sec;
    when using the TissueLyser LT, you could try rounds at the max speed of 50 oscillations/s.
    - *In between the rounds, cool the samples for 5 min on dry ice and the tube holders at -20 ˚C.*
    - Continue with rounds of homogenization for each individual sample as needed, until the sample looks completely homogeneous without any visible small chunks.
    - Bigger pieces will take longer to homogenize than smaller pieces.
    - Use the blank sample to balance the number of samples in the tube holder.
12. Place the samples for another 30-60 min at -80°C to aid effective extraction and protein precipitation.
13. Allow the samples to warm up a bit on ice. Vortex the samples for 30 sec.
14. Spin at 16,000 g for 15 min at 4 °C.
15. Transfer the supernatants to glass vials placed on ice, being careful not to disturb the pellets (you can leave some liquid behind).
    Keep the tubes with the pellets to determine protein content, if desired (see below).

Note: Re-centrifuge the supernatants if the samples develop visible 'flecks' or cloudiness.

Store the extracts at -80°C if not continuing right away (but not long-term!).

16. Transfer a volume of extract that is equivalent to 5 mg of tissue to a new glass vial (or microcentrifuge tubes if drying in a speed vac). This volume should be the same for each sample since the MeOH volumes were already adjusted according to mg of tissue sample.

    Alternatively: if not the same volume/mg ratio was used for each sample, then the aliquots to be dried can be determined based on the protein content of each individual sample. Continue with section A below instead.

17. Dry the samples using a Genevac EZ-2 Elite evaporator or a speed vac without applying heat. Do not dry longer than needed and remove the vials promptly after the program ends.

18. Store the dried samples at -80 °C until ready for LC-MS analysis.

19. Optional: determine the protein content of the pellets for potential post LC-MS acquisition data normalization based on protein concentration (see A14 below).

A. If normalization by protein content is desired, do the following:

14. Determine the protein content of the pellet.
    Decant the bead and any remaining MeOH from the tubes, and place the tubes briefly upside down on a Kimwipe tissue to drain. Add about 0.6 ml **0.2 M NaOH** (per 20 mg tissue) to the pellets. Heat for 20 min at 95 °C until the pellets are completely resuspended (flick the tubes to check). Cool the samples to RT. Vortex briefly. Spin for 15 min at 16,000 x g. Determine the protein concentration of the supernatant by **BCA method**, using an i.e., 1:10 sample dilution. Calculate the total protein content for each sample.

15. Transfer a volume of the extracts that is equivalent to a certain amount of protein, i.e., in the 300-500 µg range, into glass vials *(as a loose guideline, use about 5 mg of tissue equivalent if input tissue weight was measured accurately.)*
    i.e., if total protein in a sample is 3200 µg, transfer 500/3200 x 1000 µl (the volume of 80% MeOH used for extraction) = 156 µl.

16. Make all the sample volumes the same by adding fresh 80% methanol to those samples with a smaller sample volume.
17. Dry the samples using a Genevac EZ-2 Elite evaporator or a speed vac without applying heat. Do not dry longer than needed and remove the vials promptly after the program ends.
18. Store the dried samples at -80 °C until ready for LC-MS analysis.

EQUIPMENT AND MATERIALS NEEDED (or similar):

- TissueLyser II or TissueLyser LT (Qiagen), or from other manufacturer
- Liquid nitrogen, if obtaining fresh tissues
- Dry ice
- Ice
- Regular 1.5 ml microcentrifuge tubes
- Tubes for homogenization:
  - 2 mL SafeLock Eppendorf tubes, Cat# 022363352, Eppendorf.com (also available from Fisher Scientific

  or
  - 2 ml Free Standing Screw Cap tubes with cap and O-ring, 02-682-558, Fisher Scientific
- 5 mm Grinding Balls (440C Stainless Steel), OPS DIAGNOSTICS, No. GBSS 196-2500-10.
- HPLC grade methanol
- HPLC grade water

- Glass vials		13-622-351	Fisher Scientific	Chromacol™ GOLD-Grade Inert Glass Vials; Thermo 2SVWGK
- Caps		03-452-327	Fisher Scientific	9 mm Screw Caps, SureSTART™ Level 2 (Silicone/PTFE septum); Thermo Scientific 6ASC9STB1
	or	03-379-123	Fisher Scientific	9 mm autosampler vial screw thread caps (PTFE/silicone septum); Thermo Scientific C500054A

- 0.2 M NaOH in water
- BCA assay kit