Extraction of polar compounds, lipids, and proteins from CH_3CN quenched samples (mammalian cells, suspended cells, ground tissue) by Teresa Fan, University of Kentucky

FRACTIONATION

Note: This procedure follows [Fan_Quench_cell_tissue].

PREPARATION

Note: The following types and quantities (in parenthesis) of tubes must be labeled on the lid (sequential numbering to match metadata file) and side (number and abbreviated sample ID) for EACH sample extracted. Only those listed in #1 must be weighed before sample is added. Transparent tape over sample labels wrapped completely around each tube ensures ID remains through freeze/thaw, sonication, MeOH rinsing, etc. and is highly recommended:

1. Pre-Tared tubes: Must be weighed pre and post on the same day.
    a. (1) 5ml snap-cap Eppendorf tube (cat #: 0030119401). May be weighed on 4 place balance. For total polar fraction. Will be discarded at the end of the procedure.
    b. (1) 1.5 ml microfuge tube snap cap (USA Scientific 1615-5500). Must be weighed on a 5 place balance. For protein fraction.
2. (1) 2 ml glass screw-thread Target vial (Fisher C4010-1W) and matching white caps with septa (Fisher 00175245). For lipid fraction.
3. (2) 1.5 ml screw top microfuge tube (USA Scientific 1415-4700). For ICMS aliquots.
4. (2) 0.5 ml reduced volume screw top microfuge tubes (USA Scientific 1405-9300) and (1) 1.5 ml microfuge tube snap cap (USA Scientific 1615-5500). For FT-MS aliquots.
5. (2) 2 ml screw top microfuge tube (USA Scientific 1420-8700). For NMR aliquots.

Additional supplies needed for extraction:

6. Fine tip transfer pipets (e.g. SAMCO 232 or VWR, 16001-192)
7. Gel loading tips (e.g. USA Scientific, 1022-000)
8. Fan folded paper to hold pipet tips and transfer pipets
9. Ice bucket, put following items in the bucket
    a. Cold chloroform
    b. chloroform:methanol:BHT (2:1:1mM)
    c. cold methanol

PROCEDURE

1. Add 1 ml of chloroform into the 15 ml tube (Sarstedt, 62.554.002) containing the cells/tissue in CH3CN:water at 2:1.5.

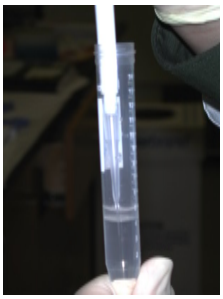
Note: To ensure volume accuracy, wet tip with chloroform before drawing 1 mL.

2. Shake the tube vigorously for > 60 times and vortex occasionally (the mixture will have milky consistency).
3. Centrifuge the conical tube at 3500xg (or 5000 rpm) for 20 min at 4°C using the swinging bucket rotor (see phase separations in Fig. 6)
4. Carefully transfer the majority of the top layer (CH_3CN:water, polar fraction) into a weighed (polar tare) 5ml snap-cap Eppendorf tube using a fine tip transfer pipette (gel loading tip can be substituted, but will require multiple transfers). Save the transfer pipette in a paper fan for step 9 below.
5. Transfer the majority of the lower layer (chloroform, lipids fraction) into a 2 ml glass screw-thread vial using a 200 µl pipet with a gel loading polypropylene tip. Avoid pipetting the aqueous layer (see Fig. 7). Stop removing lipid layer as the protein layer starts to break up or when the vial is full to the shoulder. Save the tip for step 9.

Note: In order to minimize loss of precipitated protein (for normalizing metabolite content), pay special attention in step e and f, **not to pipet any protein**. If protein precipitates contaminate the upper layer, re-centrifuge.

Fig. 6

Fig. 7



6. Reduce the volume of the lipid phase, placing the 2 ml glass vials in a vacuum centrifuge (Eppendorf Vacufuge) for 20-30 min. If you do not have a vacuum centrifuge, you can collect the lipid phase in two 2 ml glass vials to be reduced, pooled, dried, and reconstituted by RCSIRM prior to analysis.

Note: Vacuum centrifuge must be located in a chemical fume hood. Ensure lipids don't dry out completely, otherwise lipids will warm up due to friction in vacufuge and become oxidized.

7. Centrifuge the 15 ml tube at 3500xg (or 5000 rpm) for 20 min at 4°C using the swinging bucket rotor. Remove the vials with the partially reduced lipid phase from the vacuum centrifuge and transfer a little more of the lower layer from the 15 ml tube to the glass vial. Do not try to take more polar (top) layer.
8. Transfer the remaining protein precipitate along with a small amount of both layers into a 1.5 ml microfuge tube (tare weight recorded with a 5-place, 0.1 mg resolution balance, e.g. Mettler Toledo AX 105) Protein tare
    a. Mix the protein residue by pipetting it with a 1 ml pipettor (if precipitates are chunky, cut 1 ml tips with razor blade to make a wider opening), set pipette to 0.5ml, so tip doesn't have protein all over and it's easier to recover as much protein as possible

b. Aspire the mixed middle protein fraction into a pretared 1.5 ml microfuge tube
c. Wash the 15 ml tube with 200-500 µl (depending on how much precipitate is left) of chloroform:methanol:BHT (2:1:1mM)
d. Pool wash with the middle fraction (1.5 ml tube) and shake rigorously again to extract remaining lipids from the precipitate

Note: CH_3CN-chloroform mixture is not as effective in extracting lipids as chloroform-methanol mixture based on FT-MS analysis. BHT is antioxidant in order to preserve poly unsaturated FA. Leaving some polar layer is essential to facilitate extraction, as the wash does not contain water.

9. Centrifuge the middle part in the 1.5 ml tube again to separate layers
    a. Centrifuge the 1.5 ml tube using the Eppendorf centrifuge in room 335 at the maximum speed (14000 rpm) for 20 min at 4°C.
    b. Transfer the upper layer into the same 5 ml vial (polar fraction from step 4) (5ml Eppendorf tube) using the transfer pipet from step 4.
    c. Transfer the lower layer into the 2 ml glass vial from step 5 using the same gel loading tip.
    d. The pellet and remaining liquid left in the tube should be less than 50 µl.

Note: When removing the lower layer, the upper layer moves down and becomes more accessible due to conical shape of the tube. First, take a little of the upper phase (to 5 ml tube), then take as much as possible of the lower layer (to glass vial). Finally, take off as much as possible of the upper phase and add it to the 5ml Eppendorf tube from step 4.
Note: Here can be a stop point. The fractions can be stored in -80°C until the next day.

DRYING AND STORAGE

10. Upper layer (CH_3CN-Water polar fraction, in 5 ml Eppendorf tube, from step 4):
    a. Record the extract weight. (Polar + tare) Calculate the total extract weight (difference) and compute 1/8 and 1/16 of the total (for standard 3.5 ml extraction this will be approximately 200 and 100 µl).
    b. Centrifuge the 5 ml centrifuge tubes with pulse by pressing 'pulse' button and holding it until the rate reaches ~2,000 – 2,400 rpm in order to let any droplets on the tube wall and cap go down.
    c. On a 4-place balance, weigh two aliquots (g polar ICMS A and B) of approximately $1/8^{th}$ the total volume of the polar extract into each of (2) 1.5 ml microcentrifuge tubes with screw caps.
    d. On a 4-place balance, weigh three aliquots (g polar FTMS A, B and C) of $1/16^{th}$ of the total volume of the polar extract into one 1.5 ml snap cap vial and each of two in small volume screw top microfuge tubes (USA Scientific 1405-9300) for FT-ICR-MS.
    e. On a 4-place balance, weigh the remaining extract aliquoted into 2 equal parts to 2.0 ml microfuge tubes for NMR.
    f. Lyophilize all aliquots with a liquid N_2 pretrap.

   g. The dry aliquots should be stored in -80°C.
2. **Note:** a 4-place balance weighing is more accurate than volumetric pipetting; aliquot weight can be converted to volume based on the water density of 1 g/ml.
3. **Note:** two aliquots are prepared in case of loss or need for reanalysis. The second aliquot for IC-MS is optional. If the total volume of the polar fraction is less than 1.0 ml, transfer entire remaining polar fraction, after removal of aliquots for FT-MS and ICMS, to one (1) 2.0 ml tube for NMR.

11. Lower layer (chloroform, lipids fraction, in 2 ml glass tube, from step 5):
    a. Dry in vacufuge at room temperature (it takes ~ 10-30 min).
    b. Immediately reconstitute in 200-500 µl of chloroform-MeOH:1 mM BHT (2:1:1 mM), depending on the residue weight. Weigh this sample as it is reconstituted on a 4 place balance. (dry lipid +300ul chloroform/methanol/BHT)
    c. Store in -80°C.
12. The middle part (protein, in 1.5 ml tube, from step 8):
    a. Spin (table-top microfuge) and/or tap down the protein containing residue to the bottom of the microfuge tube (off the wall).
    b. Add 500µl methanol, vortex, and centrifuge at maximum speed at 4C for 20min. Discard supernatant and dry residual in speedvac at room temperature (generally takes ~ 20 to 30 minutes) or lyophilized with liquid N_2 pretrap.
    c. Weigh the pellet on the 5-place balance. (Protein + tare)
    b. Store in -80°C until protein extraction (see Fan_Protein_Quant_SOP).