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

1. Pre-Tared tubes: Must be weighed pre and post on the same day.
    a. 5ml snap-cap Eppendorf tube (cat #: 0030119401)
    b. 1.5 ml microfuge tube
2. 2 ml glass screw-thread vial (Target vial with ID patch and PTFE/Silicone/PTFE Septa; Blue Fisher 03-377B, 03-377-2A)
3. Fan folded paper to hold pipet tips and transfer pipets
4. Ice bucket, put following items in the bucket
    a. Cold chloroform
    b. chloroform:methanol:BHT (2:1:1mM)
    c. cold methanol

1. **PROCEDURE** 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 maximal speed) 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 (cat #: 0030119401) using a fine tip transfer pipette (e.g. VWR, 16001-192). Save the transfer pipette in a paper fan for step 8 below.
5. Transfer the majority of the lower layer (chloroform, lipids fraction) into a 2 ml glass screw-thread vial (Target vial with ID patch and PTFE/Silicone/PTFE Septa; Blue Fisher 03-377B, 03-377-2A or Clear Glass with I-D; Red PTFE/White Silicone/Red PTFE septum, 03-377-7B/03-377-8B) using a 200 µl pipettor with a gel loading polypropylene tip (e.g. USA Scientific, 1022-000). Avoid pipetting the aqueous layer (see Fig. 7). Save the tip in a 1.5 ml microfuge tube for step 8.

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.

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. 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 4-place, 0.01 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.

8. 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 easier accessible due to conical shape of the tube, 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, STORAGE, AND RECONSTITUTION

9. Upper layer (CH_3CN-Water polar fraction, in 5 ml Eppendorf tube, from step 4):
    a. Record the extract weight. (Polar + tare)
    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 particulate on the tube wall and cap go down.
    c. Split aliquots for FT-MS (2x 1/10 of total weight, should be around 2x 100µl) and GC-MS (2x 1/8 of total weight should be around 2x200µl) analysis (see Prep_Polar_Lipid_SOP), and NMR (2x ½ of remaining).
    d. Lyophilize the aliquots with a liquid N_2 pretrap.
    e. The dry sample should be stored in -80°C.
    f. Before NMR analysis, reconstitute the sample in H_2O:D_2O, centrifuge at ≥ 14Kxg for 5 min to remove particulates and transfer supernatant into an NMR tube (see Prep_Polar_Lipid_SOP).
10. 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. (dry lipid +300ul chloroform/methanol/BHT)
    c. Store in -80°C.
    d. Before FT-ICR MS analysis, dilute in the nanospray matrix, and centrifuge to remove particulates.
11. The middle part (protein, in 1.5 ml tube, from step 8):
    a. Add 500µl methanol, centrifuge at maximum speed at 4C for 20min. Discard supernatant and dry residual in speedvac at room temperature (it may take ~ 1h) or lyophilized with liquid N_2 pretrap.
    b. Weigh the pellet on the 4-place balance. (Protein + tare)
    b. Store in -80°C until protein extraction (see Fan_Protein_Quant_SOP).