Methanol Chloroform Extraction of Serum Protocol

Biomaterials Required:
~0.5 mL serum (per sample) collected with Heparin***
Samples:
List sample ID, human serum, collection date, one-freeze thaw cycle and approx estimated volume)
Bovine Serum (BS): non-sterile, Rockland, Cat #D200-00-0100, Lot #26415, one freeze-thaw cycle (1/0.5ml)

Other reagents and solutions:

- Methanol and chloroform (reagent or HPLC grade; Fisher brand) mix 1:1 (volume) fresh in a tightly sealed (Corning screw top bottle) that has been pre-cooled (-20°C); store mixture (-20°C) so it is ice cold when ready for use
- Ice cold DI water

Other needed materials/equipment:

A. Parafilm
B. Glucose Meter (Optimum EZ glucose meter and test strips)
C. Pasteur pipets (9" borosilicate glass; Fisher brand; 22-183-632 or 13-678-20D)
D. Borosilicate glass serological filter-plugged 1mL pipets (VWR 93000-692)
E. Refrigerated centrifuge (Eppendorf 5810R)
F. Lyophilizer tubes (Fisher cat# 14-962-26D) – **Use for all areas where "culture tube" is indicated as well. Centrifuge rotor can only fit these tubes.**
G. Lyophilizer/freeze-dryer- Stringer Lab (LabConco 1L benchtop)
H. Speedvac (LabConco Centrivap Micro IR)- Stringer lab
I. "Industrial" Sharpie pen for labeling tubes
J. Disposable glass vials (Fisher brand; 03-339-25C borosilicate glass) for speedvac
K. Generic round bottom 15mL flasks (bulbs)

** Blood must be stored on ice immediately after collection and frozen at -80°C as soon as possible (<1 hour).
***The only acceptable anti-coagulant for use with sample collection or storage for this protocol is heparin. EDTA will render samples unusable. Sodium or lithium heparin work equally well.

Use glass graduated cylinders and Pasteur pipets and the glass serological pipets for Chloroform/Methanol measurements.

All samples and solutions must be kept on ice or in the refrigerator at all times!
Only use glass pasteur pipettes for transferring!

Prechill all tubes/reagents in ice or in -20 before use!

1. Thaw samples in the fridge (4°C) and mix needed reagents/solutions.
2. Label two culture tubes for each sample; put the second set of tubes in the fridge (4°C).
3. Gently invert sample tubes (DO NOT VORTEX) and remove two 20 µL aliquots (one if total volume is less than 300) and add to labeled microcentrifuge tubes for post-extraction analysis. Freeze these aliquots at -80C.
4. Measure and record the glucose concentration of the bovine serum, and any samples that have not undergone glucose testing by spotting serum (2.5 uL by micropipetter is enough) onto a fresh glucose test strip for each sample.
5. Add total volume of sample (up to 1mL) to each culture tube labeled with "A." Record the actual volume that goes into each tube. This can vary if a clot has formed in the sample. Keep samples on ice.
6. For samples less than 750 uL add 1mL of cold methanol:chloroform to each tube, cap and vortex thoroughly. Add 2mL for samples of more than 750 uL and 0.5 mL for samples of less than 250 uL. Be careful not to let the methanol:chloroform come in contact with the tube cap, the caps are not solvent-tight.
7. Centrifuge tubes at 1300 x *g*, 4°C for 20 min.
8. Following centrifugation, transfer the supernatant and lower fluid layer to the second labeled culture tube ("L")(keep on ice). The samples may form a pellet and supernatant, or a supernatant, solid layer and lower layer of fluid. Either is acceptable, just be sure to remove all fluids leaving only the pellet (it doesn't matter of you get some of the pellet). Reserve this fluid layer in the refrigerator (4°C) or on ice (figure 1). Pellets from whole blood samples will be larger and harder than those of serum or plasma.

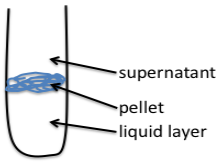

Figure 1: Representative depiction of blood or serum sample following centrifugation in step 5.

9. Resuspend the pellet of each sample in cold methanol:chloroform (0.5mL). Vigorously vortex to break up as much of the pellet as possible. Centrifuge (1300 x *g*, 4°C for 20 min).
10. Collect the supernatant (at this point, there should only be a supernatant and pellet but there may be some "floaters") and add it to the fluid layer that was reserved in step 6. Reserve pellet and any floaters.
11. Add 0.5mL of ice-cold DI water to each of the fluid tubes. Vortex well.

12. Cool the fluid for 17 minutes at -20°C. Do not freeze samples for more than 20 minutes! If you should run over, remove tubes from the freezer re-vortex and repeat this freezing step (step 10). If the aqueous phase freezes, ice crystals will form and precipitate in the organic phase, and the extraction will not be effective. If the sample freezes, thaw it (4°C) and repeat step 10.
13. While the lipid tubes chill, add 0.5mL of ice-cold DI water to the aqueous tubes (with the pellet). Suspend the pellet by vigorously vortexing or use a spatula as needed.
14. After the 20min freezer incubation, the fluid samples will have separated into a supernatant (the aqueous top layer) and a protein layer (see figure 2). Add the top layer to the resuspended pellet (step 13). Do not disrupt or penetrate the white protein layer. This remaining protein layer and lower liquid layer is the lipid fraction which can be capped and placed in the fridge (4°C) or left on ice until it is dried (step 15).

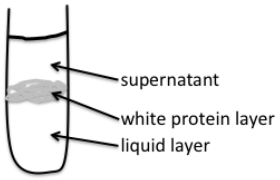

Figure 2: Representative depiction of blood or serum sample following freezer incubation in step 10.

15. Transfer the lipid fractions to the 3.7mL borosilicate vials (measure and record volumes using serological glass pipets) and dry the lipid fractions in a speedvac at 50°C on heat + IR setting for 1h or until dry – record drying time for each sample. Cap tubes[1], wrap in parafilm and store at -20°C (can be stored for months).
16. Vortex the tubes containing the pellet and the newly added aqueous top layer (these are the water soluble samples) and centrifuge (1300 x *g*, 20 min, 4°C).
17. Transfer the supernatant to a lyophilizer bulb/flask. Cover with lid or parafilm.
18. Add 0.5mL ice cold DI water to pellet and resuspend as in step 10 and centrifuge (1300 x *g*, 20 min at 4°C).
19. Transfer the supernatant to the existing samples in the lyophilizer bulbs and discard the pellet.
20. Allow the water soluble fractions to sit in the lyophilizer bulbs for 5 minutes. Carefully observe each glass. If oil droplets have formed at the bottom of the glass, use a Pasteur pipet to remove them and add to the lipid fraction.
    Measure and record the volume in the glasses before freezing with glass serological pipet! NOTE: Add 1mL of distilled water to each bulb to dilute out remaining methanol before freezing.
21. Freeze the lyophilizer glasses overnight in the -80°C freezer. Tilt the bulbs after covering each with a kimwipe.
    Lyophilize for at least 24 h to ensure that methanol is removed. Do not exceed 48 h.
22. When the aqueous fractions are dry, they can be sealed with parafilm and stored (4°C) for up to a week.
    Resuspend each sample in 0.500 mL D_2O at the time of assay or before long-term storage (record the volume transferred out of the bulb after resuspension).

[1] solvent vapors can cause degradation and failure of plastic caps

Serum samples are filtered before being run on NMR. For long-term storage (years) transfer samples to microcentrifuge tubes, seal with parafilm, and store at -80°C.

23. To assay lipid samples, resuspend samples in 0.600 mL[2] of a 1:2 deuterium methanol:deuterium chloroform solution (volume to volume), cap with a new cap and centrifuge (1300 x *g*, 10 min, 4°C). Transfer supernatants to NMR tubes for assay.

Sample Name	Initial volume	Pre speed-vac lipid volume	speed vac time	sample aqueous volume (w/out 2mL DI H2O)	total aqueous volume

[2] for a smaller original sample (i.e., ≤ 200µL) use 500µL

Serum Extract Filtration

Filtration needs to be done on all samples that display the "protein bumps" upon NMR acquisition. Currently, this has only been seen in plasma and serum samples in all species.

Materials:

1. Pall Nanosep 3K Omega Centrifugal Devices (Cat# OD003C34)
2. Deuterium oxide (Acros Organics Cat# AC426931000) – 2.5mL per sample total
3. Microcentrifuge with rotor capable of 14,000 x g force
4. Long Glass Pasteur Pipets (Wilmad LabGlass Cat# 803A)
5. 5mm 7-inch long NMR sample tubes (Wilmad LabGlass Cat# 528-PP-7)

Samples:

Sample	Pre-filter Volume (uL)	Post-filter Volume (uL)	Volume of D_2O added to filtrate (uL)	Vol. Formate IS added

Procedure:

1. Prepare filters by rinsing with about 500uL ultrapure H_2O in each centrifugal device (do not add to top of tube, leave about 1cm of space at top) and spinning at 14,000xg for 4 minutes. Discard filtrate, which contains glycine trapped on the filters
2. Rinse once more with new ultrapure H_2O. Then rinse each filter 3 times with D_2O. Discard all filtrate and remove excess water off the top of the filter.
   If rinsed filters are to be stored, rinse twice with D_2O, and remove excess water from the top and bottom of the filter before storing at -20 ^oC (for up to a month). Before using stored filters rinse once more with D_2O.
3. Resuspend lyophilized extracts in 500 uL D_2O.
   Add the entire volume of the extracted sample to the washed centrifugal device. Do not exceed 500uL. Record this volume.
4. Spin each sample at 14,000xg at 4^oC for 20 minutes.
5. After the spin, take the liquid off of the top of the filter without disturbing the material on/in the filter. Gently scrape the filter with the same pipet tip. Then add the reserved liquid back on top of the original filter.
6. Spin at 14,000xg at 4^oC for 25 minutes.
7. Discard filters and close centrifuge tubes.
8. Measure filtrate volume. Add D2O to end volume of 450uL, or volume of largest sample.
9. To this, add 50uL of 5mM formate internal standard. Record exact volume of formate AND CONCENTRATION of formate used.

If not running NMR on same day:

12. Store samples with parafilm covering each cap at 4C if expecting to run NMR within 5 days. If longer, store at -20C.

PREPARATION OF FATTY ACID METHYL ESTERS OF TOTAL LIPIDS WITH BF_3-METHANOL

Reference: Modified method of Morrison, W. R. and Smith, L. M. (1964). Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron trifluoride—methanol. J. Lipid Res. 5: 600-608.

1. Dry chloroform completely under nitrogen
2. Add 1 ml of BF_3-methanol (Sigma Aldrich)
3. Close the tube under nitrogen
4. Mix well and incubate at 60^O C for 3-3.5 h
5. Add 1 ml of hexane, mix
6. Add 0.5 ml of water
7. Vortex well
8. Centrifuge at ~3000 rpm (table top centrifuge) for 5 min
9. Transfer hexane layer into another tube
10. Re-extract the aqueous layer with another 1 ml of hexane
11. Combine hexane layer
12. Dry under nitrogen
13. Re-dissolve in small volume (~50 µl) of chloroform
14. Purify methyl ester by TLC as described below

TLC PURIFICATION OF FATTY ACID METHYL ESTERS

Mangold, H. K. (1969) in Thin-Layer Chromatography (Springer, New York), E. Stahl (Ed), pp. 363-421).

1. Apply the product in chloroform on a TLC plate (5 x 20 – 20 x20 cm plate depending upon the number of spots) applying authentic methyl ester sample side by side
2. Run the plate up to 12 cm with hexane – ether - acetic acid (80 : 20 : 1.5, v/v) as the developing solvent in a TLC chamber
3. After the run is complete, dry the plate and spray with 0.1 % Rhodamine 6G.
4. Identify the methyl ester spots/bands with respect to the retention time Of the authentic standard and mark
5. Scrape the spots/bands from the TLC plate and put the powder in to a tube
6. Extract the contents with 1.5 ml of chloroform – methanol (1 : 1)
7. Re-extract the powder with 1 ml of the same solvents
8. Dry the solvents under nitrogen
9. Re-dissolve in 100 µl of hexane for GC analysis