SOP: PP029.4
Updated 1/4/22

Purification of TDM and SL

Materials and Reagents

1. Methanol, chloroform, HPLC and ACS-grade
2. Acetone, ACS grade
3. Graduated cylinder, glass (100, 500 ml)
4. Chemical fume hood.
5. Magnetic stir plate
6. Round-bottom flasks, 250, 500 ml
7. Rotary evaporator
8. Metal spatula
9. Teflon Oakridge centrifuge tubes
10. Pasteur pipets
11. PIM standard
12. TLC equipment (note 1)
13. Glass tubes, 13 x 100 mm + Teflon caps
14. Glass vials, 2 ml + Teflon caps
15. Preparative TLC plates (Merck 1.05715.0001, via Burgoon)
16. Analytical TLC sheets (Merck 1.05554.0001)
17. Glass pipets (1, 5, 10 ml)
18. Rubber pipet bulb
19. Vortex
20. Reagents and equipment for alditol-acetate derivation (note 2)
21. 25 mm PTFE syringe filters, 0.2 µm
22. Light C18 SepPak reverse phase columns (Waters)
23. Glass syringe, 10 ml

Protocol

1._____ Obtain 5% and 6% MeOH in $CHCl_3$ fractions from column enrichment of TL (note 3).

2._____ Apply these extract to separate preparative TLC plate sets in the hood and run in solvent system 65/25/4 $CHCl_3/CH_3OH/H_2O$ (note 4).

3._____ Transfer TDM-specific and SL-specific silica to Teflon tubes for extraction (note 5).

4._____ Add at least 10 ml 2:1 $CHCl_3/CH_3OH$ to each tube and, ensuring that caps are tight, briefly vortex.

5._____ Centrifuge at 3,000 rpm at 4°C for 5 minutes.

6._____ Transfer the organic supernatant to round bottom flasks, filtering through small filter cones fitted into a glass funnel (note 6).

7._____ Repeat steps 4 to 6 for a total of 2 extractions.

8._____ Dry TDM or SL extract for this set of plates via rotary evaporation.

9._____ Resuspend in small volume of 2:1 and transfer to glass tube (note 7).

10._____ Evaluate crude extracts by analytical TLC (note 8).

11._____ Run several preparative TLC plates before combining appropriate crude TDM or SL extracts for final polishing.

12._____ Evaluate the total amount of crude TDM and SL extract by drying in a tared 2 ml glass vial.

13._____ Combine appropriate extracts of TDM or SL for polishing by C18 SepPak columns (note 9).

14._____ Divide the crude extracts among several glass tubes, in 500 ul aliquots. Make a 10-fold dilution with CH_3OH for each, using Pasteur pipets to mix well (note 10).

15._____ Prep each column with 4 ml 95% acetonitrile, 5 ml CH_3OH, then 2 ml 80% CH_3OH, using a glass syringe (note 11).

16._____ Load each column slowly with the MeOH-diluted crude TDM or SL.

17._____ Wash each column with 4 ml CH_3OH, then slowly elute SL with 2 x 3 ml 25% $CHCl_3$ in CH_3OH, capturing in separate tubes which will be dried down.

18._____ Wash each column with 4 ml 40% $CHCl_3$ in CH_3OH, then elute TDM with 2 x 3 ml 60% $CHCl_3$ in CH_3OH, again capturing in separate tubes.

19._____ Dry all fractions on the N_2 bath and resuspend in 0.5 – 1.0 ml 2:1 $CHCl_3/CH_3OH$ (note 12).

20._____ Evaluate the 25% and 60% fractions, as well as the flowthrough, with analytical TLC.

21._____ If pure, run 50 ug of each on 2D TLC, using 2D TLC, and staining with $CuSO_4$ and (note 13).

22._____ Evaluate each with MALDI-TOF (note 14).

Notes

1. See Thin Layer Chromatography, SOP SP032 and SP033, for a complete list of equipment and reagents.

2. See Preparation of Alditol-Acetate Derivatives, SP022.

3. See Enrichment of Total Lipid SP058.

4. Start with 0.5 ml per plate. One plate in the first set should have 25-50 µg TDM or SL loaded near the edge to discern between bands. The quantity of enriched lipid applied to each plate, and total overall, can be determined by taring a 2.0 ml glass vial and drying down 100-200 ul. This will help calculate the amount of enriched TL applied per plate, for consistency, as well as to monitor how much can be loaded before resolution begins to break down, leading to less pure extracts. Often the 5% MeOH fraction will be mostly TDM, while the 6% will likely have both. Purified TDM from the 5% MeOH fraction via prep plates may not need any further purification, and could be set aside to combine with TDM isolated from other plates.

5. Stain edges of TLC plates with α-naphthol or $CuSO_4$ and charring to discern the total lipid bands and the TDM-specific band. Use the UV light box to view the TDM band, in line with the control. Trace the putative band with a pencil, using bands seen with short-wave UV as a guide. You can trace the SL and TDM bands separately to extract separately. This may not lead to sufficient purity, and follow-up SepPak purification may be necessary.

6. Select a round-bottom flask sufficient such that the total volume of three rounds of extraction will not exceed ½ the volume capacity of the flask. This will make drying the lipid via rotary evaporation more efficient.

7. Use 5-6 ml 2:1 to resuspend crude TDM or SL in each flask, transfer to 13x100 mm tube. Dry down in nitrogen bath, then resuspend in 250 µl 2:1 per plate extracted. For example, if 4 plates, then final resuspension volume would be 1 ml. After the extracted silica has dried overnight, transfer to a small bottle to extract a 3rd and 4th time later, which can be used as crude reference material. Keep collecting all the twice-extracted silica for it to be extracted at the end. It could also serve as an additional amount to be purified further if required.

8. Run 10 µl on analytical TLC (10x10 cm) along with SL standard and TDM, developing with 100/14/0.8 chloroform/methanol/water, and staining with $CuSO_4$ or α-naphthol. The enriched 5% or 6% MeOH fraction can be ran as well. Each set of plates will be evaluated prior to combining for the final clean-up. Some will be sufficiently clear of bands as to not require further polishing. Upwards of 60% of crude material is lost in the "clean-up" stage.

9. Use a conservative approach of no more than 250-500 ug crude TDM or SL to be applied to each SepPak filter.

10. To avoid columns drying out, work in batches of 4-8 tubes, each dilution of which will go through a separate SepPak column.

11. This prepares the lipid for binding to reverse-phase cartridges. The dried lipid will not solubilize well in methanol alone. Ensure that each aliquot is efficiently resuspended, with no apparent precipitation, to avoid product loss. Take care to not introduce air into the columns.

12. Do not use water bath heat for drying. Sulfolipid is very heat-labile, and will become very non-polar as soon as it loses its sulfate group.

13. Run a 50 ug spot in the 1st dimension as 100/14/0.8 chloroform/methanol/water, allow to dry briefly, turn each 90° clockwise, then run in 90/10 chloroform/methanol.

14. Apply 1-2 µg using DHB matrix, and include crude TDM/SL or control from a previous lot. One should see a distinct peak cluster in reflector negative mode for SL between roughly 2200-2700 m/z. TDM has been more difficult to see via MALDI, but in reflector positive mode generally yields ill-defined peaks at 2600-2900 mz.