SOP: PP026.5
Updated 11/30/21

Purification of $PIM_{1,2}$

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

Protocol

1._____ Obtain 33% MeOH in CHCl3 fraction from column enrichment of TL (note 3).

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

3._____ Transfer PIM-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 PIM 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 at least 12 preparative TLC plates before combining appropriate crude $PIM_{1,2}$ extracts for final polishing.

12._____ Evaluate the total amount of crude $PIM_{1,2}$ extract by drying in a tared 2 ml glass vial.

13._____ Resuspend PIM and apply to final prep plates, developing as before (note 9).

14._____ Evaluate by analytical TLC in triplicate (note 10).

15._____ Once $PIM_{1,2}$ has been confirmed with TLC, make alditol acetate derivatives of the sample in triplicate, using 50, 100, and 200 µg material. Include neutral sugar standards in triplicate (note 2).

16._____ Perform gas chromatography on derivatives to confirm presence of mannose and inositol (note 11).

17._____ Perform MALDI-TOF analysis (note 12).

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 $PIM_{1,2}$ loaded near the edge to confirm which band is actually $PIM_{1,2.}$ 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 ug. 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.

5. Stain edges of TLC plates with α-naphthol or $CuSO_4$ and charring to discern the total lipid bands and the PIM-specific band. Hold the plates up to visible light to see a faint dark band aligning with the PIM control and the corresponding PIM bands at the edge. Working in the hood, scrape away silica below PIM area with a glass slide and discard. Carefully scrape PIM area and distribute silica evenly to Teflon tubes.

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 PIM in the 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 $PIM_{1,2}$ standard, developing with 65/25/4 chloroform/methanol/water, and staining with $CuSO_4$ or α-naphthol. The enriched 33% MeOH fraction can be ran as well. There is often a spurious band just below $PIM_{1,2}$ that will be purified out later. 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 no more than 5 mg per plate. Extract as before, and evaluate cleaned prep by TLC versus control. Passage the final preparation through 25 mm PTFE Acrodiscs fitted to a glass syringe, making certain to rinse the disk with 1-2 ml 2:1. Obtain a final weight in a fresh vial. Weights of crude and polished $PIM_{1,2}$ will enable determination of percent loss.

10. Run 50 µg on 3 10x10 mm HPTLC plates using 65/25/4 solvent system. Develop with α-naphthol, copper sulfate, and Dittmer-Lester. The first two should be charred with the heating gun to activate staining, but that sprayed with Dittmer-Lester will come up on its own after 10 min or so. Do not char this one, but do scan immediately as the color will fade.

11. See Gas Chromatography of Glycolipids, SP045.3.

12. Apply 1-2 µg using DHB matrix, and include crude PIM or $PIM_{1,2,}$ control from previous lot. One should see a peak cluster in reflector positive mode between roughly 1690-1770 m/z.

References

Brennan, P. J. and C. E. Ballou. Journal of Biological Chemistry. 1967. 242:3046.
Khoo, K.-H., *et. al.* Glycobiology. 1995. 5:117.

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