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Internal Metabolite Extraction for Targeted and Untargeted Metabolomics Using Ultrahigh Resolution Mass Spectrometry

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Metabolomics Protocols ...



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Protocol status: Working

We use this protocol in our group and it is working.

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Materials

Per sample:

- 4× 8ml amber glass vials (combusted)
- 4x Pasteur pipettes (combusted)
- 2× 2ml LC vials with glass inserts, plus 2 for pooled samples (combusted)
- 2x ~1.5ml centrifuge tube (autoclaved)
- 1x glass test tube (combusted)
- 2x small piece of combusted aluminum foil (plus a couple bigger sheets)
- 2-4× 200ul pipette tips (plus a couple more for reagents)
- 1× 1000ul pipette tips (plus a couple more for reagents)
- 1× 50 mg/1 cc PPL cartridge

Reagents (all organics and water use Optima grade, for acid use trace metal grade):

- 40:40:20 acetonitrile:methanol:water + 0.1M formic acid (1.2ml/sample)
- 6M Ammonium hydroxide (51 µL / sample)
- 5ug/ml deuterated biotin standard (10 µL / sample)
- 0.01M hydrochloric acid (for solid phase extraction)
- Methanol (for solid phase extraction)
- 95:5 water:acetonitrile (750ul/sample)
- deuterated internal standard mix (25ul/sample)

Other:

- VacuFuge
- Centrifuge
- Vacuum manifold and pump
- Tubing, trap, and filter for vacuum filtration setup
- Analytical balance
- Forceps and scissors (rinsed with MeOH)
- Assorted pipettes and tips
- Glass syringe
- Assorted combusted small beakers and graduated cylinders
- Pipette filling bulb for Pasteur pipettes
- Lab tape (more than you think) and sharpies for labeling
- Kimwipes and gloves

Troubleshooting

Extraction of metabolites from filter samples

- 1 Filters should be stored at -80 °C until ready for extracting.
- 2 Use combusted aluminum foil surface for all weighing and cutting. Rinse tweezers and scissors (especially inside edges) with methanol prior to beginning. Step 3 should be carried out as quickly as possible in order to avoid degradation.
- 3 Cut the filter so that the pieces are small enough to stack on the bottom of an 8 mL glass vial.
 - 3.1 Place the pieces of filter in an 8 mL glass vial, preferably amber vials to avoid photodegradation.
 - 3.2 Add 1000 μ L of ice cold 40:40:20 acetonitrile:methanol:water + 0.1M formic acid to each vial (with glass syringe).
 - 3.3 Spike in 25 μ L of a 1 μ g/mL stock solution of deuterated standards*.
 - 3.4 Vortex gently to separate the filter pieces so that the extraction solvent is in contact with both sides of each filter. Make sure filter pieces are completely covered.
 - 3.5 You can extract a portion of a filter, and weigh the filter fragments to track how much of the filter was extracted.
- 4 Sonicate vials with filter and extraction solvent for 10 minutes.
- 5 Pipette extraction solvent using a combusted Pasteur pipette from the vials into 2 mL Eppendorf tubes, leaving behind the filter.
- 6 Rinse the filter by adding 500 μ L of fresh extraction solvent to the glass vial. Repeat this step three times. You will need two Eppendorf vials for each sample because you will have a total of 2.5 mL of extraction solvent from each sample.
- 7 Spin the extracts in the Eppendorf vials at 20,000 $\times g$ for 5 minutes.



- 8 Transfer the supernatant into new, combusted 8 mL glass vials, taking care not to pipette out the particulate matter at the very bottom of the Eppendorf tube. Neutralize the sample with 51.2 μ L of 6 M ammonium hydroxide.
- 9 Spin down the samples in the Vacufuge, but do not let them completely dry out. The length of time required for this step will depend on the volume of fluid.
- 10 Bring up the samples in 500 μ L 0.05 μ g/mL deuterated injection mix (D2 biotin, D6 succinic acid, D4 cholic acid, D7 indole 3 acetic acid, and ^{13}C phenylalanine). Note: sometimes these should be brought up in a different volume depending on the particular samples.

Optional steps to remove excess salt

- 11 Transfer 250 μ L of the solution from Step #10 to a new amber vial, and dry the solution down in the vacufuge (to near dryness only).
- 12 Bring the sample up to about 500 μ L with 0.01 M HCl to lower the pH to 2.
- 13 Use a 50 mg/1 cc PPL cartridge and pass one cartridge volume of 100% methanol through the cartridge. Use an old test tube inside the filtration manifold to collect the waste methanol
- 14 Turn on the vacuum pump. Pre-rinse the cartridge with one cartridge volume of 0.01M HCl. Then add the acidified sample and pull it through the PPL cartridge. Target flow rate should be less than 40 ml min⁻¹.
- 15 Rinse the PPL cartridge with one cartridge volume of 0.01 M hydrochloric acid.
- 16 Dry for about 5 minutes.
- 17 Put a fresh glass test tube under the outlet of each sample
- 18 Add one cartridge volume of 100% methanol and elute the sample off the PPL. The flow rate should be less than 2 ml min⁻¹, and gravity is usually sufficient.
- 19 Transfer to an 8 ml amber vial with a Pasteur pipette.



- 20 Vacufuge (almost to dryness) and bring up to 250 μ L in a new vial with 95:5 water:acetonitrile (or you can add the acetonitrile first and then water if it doesn't go into solution well).
- 21 Add 200 μ L to a glass insert & LC vial for the FT.

Set up pooled sample for conditioning column and QC

- 22 To create a pooled sample: add 45 μ L of each sample in to an LC vial (without an insert) and vortex it. This should be run as a partial loop between blanks and samples and as a full loop approximately every 6 samples throughout the run. If the volume you have is adequate for that many replicates you can leave it in the 2 ml vial, otherwise you can put 200 μ L of the pooled sample in to multiple vials with inserts to make the volume go further.