**GC-MS Protocols for plant samples (6 mg dry)**

**Metabolite extraction (6.0 mg dry tissue)**

1. Freeze tissue in liquid nitrogen.
2. Lyophilize frozen tissue about 2-3 days (or longer if larger quantities are being prepared). The dried tissues should be stored at -80 ºC.
3. Homogenize tissue (with glass rod or with mortar and pestle)
4. Weigh dried and ground samples accurately to 6.00-6.05 mg, and transfer to 1-dram (~4ml) glass vial.
5. Add 1.5mL of chloroform containing 10.0 μg/mL docosanol (non-polar internal standard) using a glass syringe. DO NOT TRANSFER USING PLASTIC PIPPETTE TIPS as this can result in serve sample contamination! Cap the vials tightly, vortex for 1 minute and incubate at 50°C for 45 minutes in an oven with periodical shaking (shake every 10-15 minutes).
6. Remove samples from the oven, allow samples to equilibrate to room temperature.
7. Add 1.5ml of water containing 25 μg/mL ribitol (polar internal standard). Cap the vials tightly, vortex for 1 minute and incubate at 50°C for 45 minutes.
8. Remove samples from the oven, allow samples to equilibrate to room temperature.
9. Centrifuge at 3000x*g* for 30 minutes at 4°C to separate the solution into two layers.
10. Use a glass and stainless syringe to transfer 1ml of each layer into 2.0mL auto-sampler vials. Wash syringe 3x in between samples using chloroform (for organic layer) and methanol (for aqueous layer)
11. Dry the aqueous polar layer (upper layer) in a rotary evaporator and the organic (chloroform) layer under nitrogen. Samples are stored at -80°C until further processing.

**Derivatization and analysis polar metabolites (6 mg dry tissue)**

1. Prepare methoxyoxamine reagent (i.e. methoxyamine HCl in pyridine, 15mg/mL). The reagent needs to be prepared fresh each day. It may require some shaking to dissolve methoxyamine in pyridine. Return methoxyamine HCl bottle to the desiccator after use. 🕱Note, the reagent is EXTREMELY TOXIC and should be handled in the fume hood or under a snorkel. 🕱
2. Use a glass and stainless syringe to add 50μL of freshly made methoxyamine reagent into the samples from step 11, cap tightly, briefly sonicate until crystallized metabolites are suspended in solution and incubate at 50°C for 1h (shake briefly every 10-15 minutes).
3. Remove the sample solutions from the oven and allow them to equilibrate to room temperature.
4. Score glass ampoule and break open ampoule of MSTFA+1%TMCS. Use a dry glass and stainless syringe to add 50μL MSTFA + 1%TMCS to the sample solutions and incubate for 1h at 50°C (shake briefly every 10-15 minutes). 🕱Note, the reagent is EXTREMELY TOXIC and should be handled in the fume hood or under a snorkel. 🕱
5. Remove the sample solutions from the oven and allow them to cool down to room temperature.

1.0 μL of the solution is injected at 15:1 split ratio onto a HP 6890N GC equipped with a 60M DB-5-MS column coupled to a HP 5973N MS. The injection port and transfer arm is held at 280°C, Separation is achieved with a temperature program of 80ºC for 2 min, then ramped at 5ºC min-1 to 315ºC and held for 12 min, a 60 m DB-5MS column (J&W Scientific, 0.25 mm ID, 0.25 mm film thickness) and a constant flow of 1.0 mL/min. The MS source is held at 250ºC and the quadrupole at 150 ºC and scanned from m/z 50-650.

**Derivatization and analysis of non-polar metabolites (6 mg of tissue)**

1. Re-suspend the non-polar layer samples (from step 11) in 0.8 ml of chloroform and hydrolyze by adding 0.5 mL 1.25 M HCl in MeOH. Cap tightly and incubate at 50°C for 4 hours. Shake occasionally.
2. Evaporate the solvents and HCl under nitrogen.
3. Re-suspend dried samples in 70 μL pyridine, briefly sonicate until crystallized metabolites are re-suspended in pyridine, and incubate at 50°C until residue is dissolved.
4. Add 30 μL of MSTFA+ 1% TMCS and incubate 1hr at 50°C.
5. Equilibrate samples to room temperature, transferred to a 200 μL glass insert using glass pipette or syringe and analyze using an Agilent 6890 GC coupled to 5973 MSD scanning from *m/z* 50-650. 1.0 μL of the solution is injected at 1:1 split ratio. The injection port and transfer arm is held at 280°C, separation was achieved with a temperature program of 80°C, for 2 min, then ramped at 5°C/min to 315°C and held for 12 min, and a constant flow of 1.0 mL/min.

Note.

1. Samples should be analyzed within 24 hours of derivatization as moisture/humidity can hydrolyze derivatized samples. So plan your sample preparation accordingly. Avoid preparing more than 24 samples at one time.
2. Avoid exposing samples and reagents to air as moisture/humidity can hydrolyze derivatized samples. Work fast when adding reagents. Cap tightly after adding reagents. Warm or cool samples to room temperature after removing them from freezer or oven.