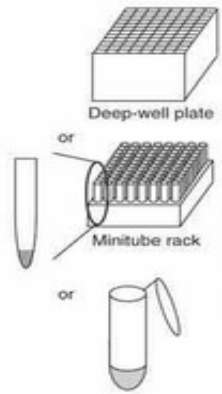


Step 1 Harvest experimental tissue into either microcentrifuge or minitubes; quickly take weight; flash freeze.

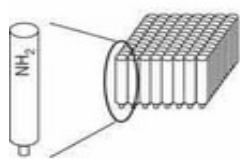
Steps 2-5 On ice, add homogenization buffer, internal standard and beads. Homogenize by vibration mill. Incubate for 1 h at 4 °C. Optional centrifugation of samples >50 mg tissue, 5 min 10,000g.



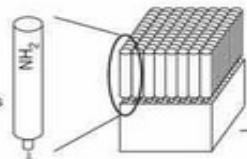
Step 6 For automated liquid handler (ALH), sample transfer from 1.5-ml tubes to a deep-well plate. Load deep-well plate or minitube rack onto ALH.



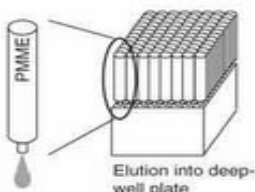
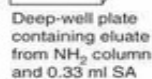
Steps 7, 8 Dilute samples (10x) with dH₂O and mix. Condition NH₂ columns: 0.6 ml each of hexane, acetonitrile, dH₂O and 0.2 M imidazole (pH 7), then 3 x 1.2 ml dH₂O.



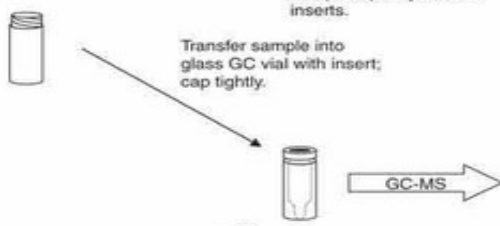
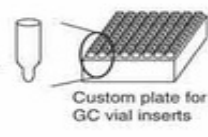
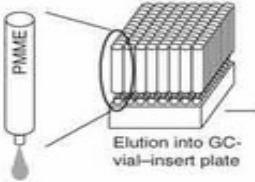
Steps 9-12 Load diluted samples onto NH₂ columns; wait 5 min; pass samples through columns. On-column purification with 0.6 ml each of hexane, ethyl acetate, acetonitrile and methanol. Elute with 3 x 0.6 ml of 0.25% phosphoric acid (PA). Add 0.33 ml of 0.1 M succinic acid (SA) (pH6) to eluate and mix well.



Steps 13-17 Condition PMME columns with 3 x 0.5 ml PA:SA (6:1), 0.5 ml methanol, then 3 x 0.5 ml PA:SA. Load pH-adjusted eluates onto PMME columns. Wait for 5 min. Pass samples through columns. Rinse: 3 x 0.6 ml PA:SA followed by 0.3 ml methanol. Elute with 0.3 ml methanol. For manual methylation, collect eluates in a deep-well plate; for 96-sample methylation, collect into 250 µl-capacity GC vial inserts.



Step 18 From deep-well plate, transfer samples to 1.5-ml vials; (do the following steps in a ventilated hood) add ~1 ml ethereal diazomethane and loosely cap; wait 5 min; dry completely under N₂ at 55 °C. Resuspend samples in ~15 µl ethyl acetate by rinsing vials well.



In a well-ventilated hood, place the GC-vial-insert plate in the heating block of a 96-needle evaporator/concentrator connected to a high-flow gaseous diazomethane generator (see accompanying report³⁵). Allow reaction and drying to proceed until all samples are dry. Resuspend samples in ~15 µl ethyl acetate by rinsing the inserts well. Transfer inserts into GC vials and cap tightly.