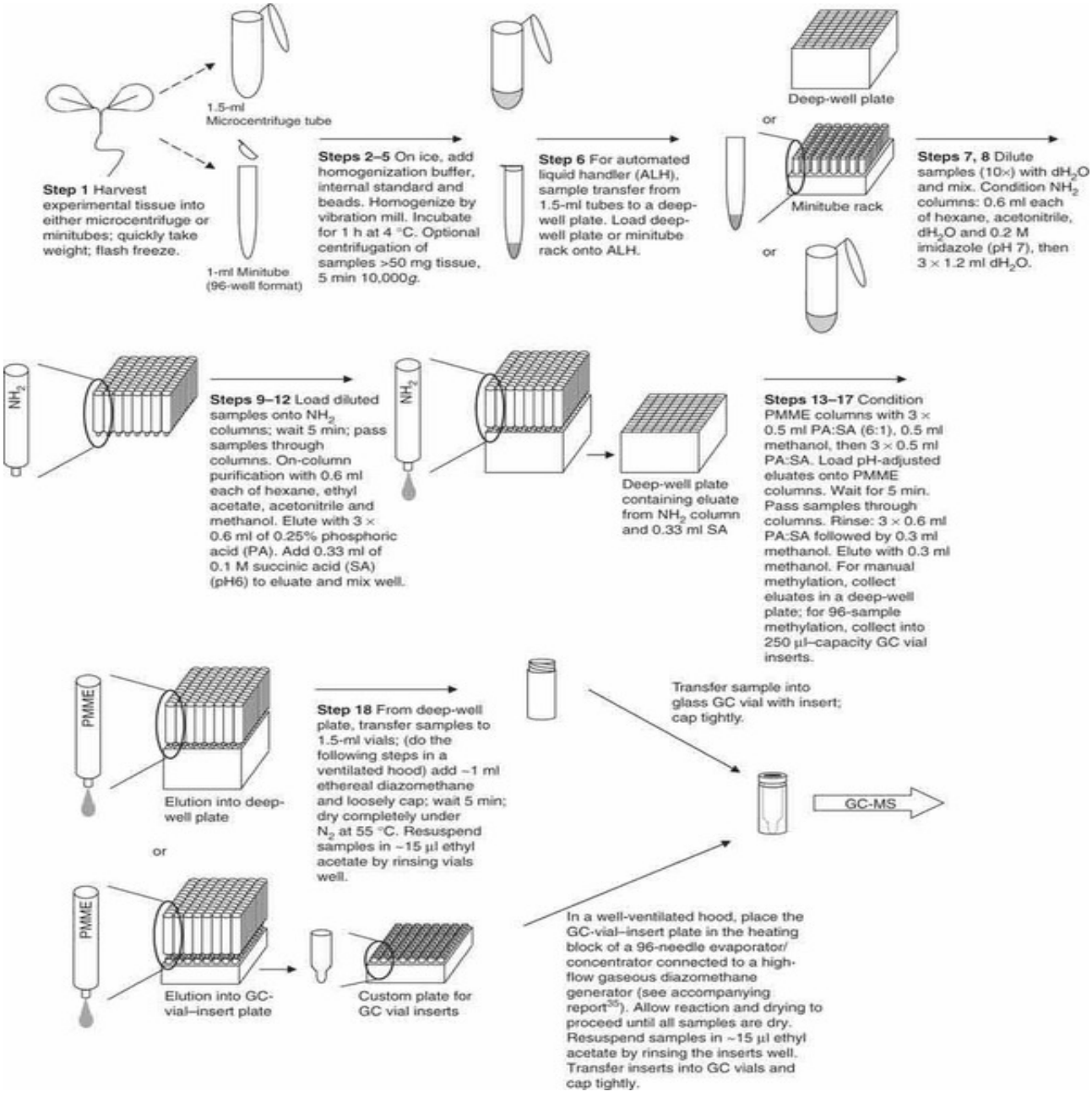
1.5-ml
Microcentrifuge tube
1-ml Minitube
(96-well format)
Step 1 Harvest experimental tissue into either microcentrifuge or minitubes; quickly take weight; flash freeze.
Steps 2–5 On ice, add homogenization buffer, internal standard and beads. Homogenize by vibration mill. Incubate for 1 h at 4 °C. 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.
Deep-well plate
or
Minitube rack
or
Steps 7, 8 Dilute samples (10×) with dH2O and mix. Condition NH2 columns: 0.6 ml each of hexane, acetonitrile, dH2O and 0.2 M imidazole (pH 7), then 3 × 1.2 ml dH2O.
NH2
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 × 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.
NH2
Deep-well plate containing eluate from NH2 column and 0.33 ml SA
Steps 13–17 Condition PMME columns with 3 × 0.5 ml PA:SA (6:1), 0.5 ml methanol, then 3 × 0.5 ml PA:SA. Load pH-adjusted eluates onto PMME columns. Wait for 5 min. Pass samples through columns. Rinse: 3 × 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.
PMME
Elution into deep-well plate
or
PMME
Elution into GC-vial–insert plate
Custom plate for 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.
Transfer sample into glass GC vial with insert; cap tightly.
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 report[35]). 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.
GC-MS