Chromatography protocol

The samples were analysed using high resolution mass spectrometer in Orbitrap Q Exactive Plus (Thermo) equipped with a SeQuant ZIC-pHILIC column (150 mm x 2.1 mm x 5 micron packing, Merck) and a ZIC-pHILIC guard column (20 mm x 2.1 mm x 5 micron packing, Merck) under alkaline mobile phase conditions with ESI ion source. The ESI was operated in positive (M+H) and negative (M-H) modes separately. MS1 parent ion were used for quantification purpose and MS2 was used for secondary validation of metabolites. The spray voltage was set at 4.2 kV and 3.5 kV for the positive and negative mode respectively. The temperature was maintained at 300°C and 320°C for the ion transfer capillary and probe heater respectively. A heated electrospray ionization probe II (H-ESI-II) probe was used with the following tune parameters: sheath gas: 29; auxiliary gas: 7; sweep gas: 0; S-lens at 45 arbitrary units. A full scan range of 66.7 to 1000 m/z was applied for positive as well as negative modes and the spectrum data type was set to profile mode. The automatic gain control (AGC) target was set at 1e6 with resolution of 70,000 at 200 m/z. Prior to analysis, cleaning of LC-MS system and ion transfer capillary (ITC) was performed. Mass calibration was done for both positive and negative ESI polarities by using Thermo Calmix solution along with MS contaminants to take into account lower mass ranges. The signals of compounds 83.06037 m/z (2xACN+H) and 119.03498 m/z (2 x Acetate-H) were selected as lock masses for positive and negative mode respectively with lock mass tolerance of 5 ppm (Zhang et al. 2014).

The mobile phase for chromatographic separation comprised of non-polar phase A (acetonitrile: water mixed in the ratio 9:1, 10 mM ammonium acetate, pH 9.23 using ammonium hydroxide) and polar phase B (acetonitrile: water mixed in the ratio 1:9, 10 mM ammonium acetate, pH 9.23 ammonium hydroxide). A linear gradient with flow rate of 200 ul/min was set as follows: 0-1 min: 0% B, 1- 32 min: 77.5% B, 32- 36 min: 77.5% B to 100%

B, 36-40 min: hold at 100% B, 40-50 min: 100% B to 0% B, 50-65 min: re-equilibration with 0% B (Teleki et al. 2015). Injection volume of 5 μL was used for all the samples and standards. Sample blanks composed of acetonitrile: buffered water (60:40) was run in triplicates before injection of standard or quality control (QC) and in between samples to monitor carry-over effects. Accurate determination of metabolites with their retention time was validated by running QC samples under the same mobile phase conditions. QC samples consisted of either a mix of fixed amount of ^{13}C internal standard and external standards (25 μM concentration) or solely ^{13}C internal standards, reconstituted in acetonitrile: buffered water (60:40). The volume of the pooled internal standard was accepted only if the external standard and internal standard peak height differed less than 5-fold (Bennett et al. 2009). This fixed volume of ^{13}C internal standard was used as spike-in at the earliest stage of metabolite extraction. To determine absolute intracellular metabolite concentrations, ^{12}C standard mixes were used for external calibration along with the same fixed amount of ^{13}C internal standards.