Untargeted metabolomics sample preparation and data acquisition protocol

REVERSE PHASE LC-MS

Sample preparation

Extraction solvent: MAA (Methanol : Acetonitrile : Acetone 1:1:1) with internal standards; for 200 samples 100 ml extraction solvent and 4 ml internal standard mixture (Table 1).

Reconstitution solvent: Methanol : H2O 1 : 1

Table 1Reverse phase LCMS internal standards

Internal standard	Stock concentration
Epibrassinolide	200 µM
L-[15N] Anthranilic acid	200 µM
L-Arginine-(guanidineimino-15N2)	200 µM
Thymine-d4 (methyl-d3,6-d1)	200 µM
Zeatin	200 µM

10 µL of reverse phase IS stock is added per 400 µL of the extraction buffer.

Procedure:

- Pipette 100µL of sample from the vials into a labeled 1.5 ml micro-centrifuge tube
- Add 400µL of extraction solvent to all tubes.
- Vortex for 5 min, then let sit 30 min at 4°C. Vortex again and let sit at -20°C for 1 h, then centrifuge 10 min at 15,000 rpm.
- Transfer 250 µL of supernatant to labeled labelled 1.5 ml micro-centrifuge tube.
- Dry all samples under nitrogen stream at room temperature.
- Reconstitute in 100 µL of reconstitution solvent.
- Vortex for 5 min, centrifuge 5 min at 15,000 rpm.
- Transfer supernatant to auto-sampler vials with inserts, discard pellets.

CHROMATOGRAPHY

1290 Infinity Binary LC System from Agilent is used for chromatographic separation together with Waters Acquity UPLC HSS T3 1.8 µm 2.1 x 100 mm column in connection with a Water Acquity UPLC HSS T3 1.8 µm VanGuard pre-column.

- Data acquisition: time 27 min
- System equilibration time: 7 min
- Total run length: 34 min
- Flow rate: 0.45 ml/min
- Solvent A: 0.1% formic acid in water
- Solvent B: 0.1% formic acid in methanol
- Column temperature: 55°C
- Flow rate 0.45 ml/min

Same chromatography is used for both positive and negative mode (Table 2).

Table 2. LC gradient timetable

Time	Solvent composition
0 min	98%A : 2%B
20 min	25%A : 75%B
22 min	2%A : 98%B
30 min	2%A : 98%B
30.1 min	98%A : 2%B
37 min	98%A : 2%B

MASS SPECTROSCOPY

Agilent Technologies 6530 Accurate-Mass Q-TOF with a dual ASJ ESI ion source was used as the mass detector. Mass spectrometer settings were as follows: Ion source: gas temperature - 325℃, drying gas flow - 10 l/min, nebulizer pressure - 45 psig, sheath gas temperature - 400℃, sheath gas flow - 12 l/ml, capillary voltage - 4000 V. fragmentor voltage - 140 V, skimmer voltage - 65 V, mass range 50-1000 m/z, acquisition rate 2 spectra/s. Inline mass calibration was performed using debrisoquine sulfate (m/z 176.1182) and HP-0921 from Agilent (m/z 922.0098) in positive mode and 4-NBA (m/z 166.0146) and HP-0921 from Agilent (m/z 966.0007, formate adduct) in negative mode.

Lipidomics sample preparation and data acquisition protocol

Sample preparation

Samples are stored at -80^{o}C until analyzed. The lipids are extracted using a modified Bligh-Dyer method. The extraction is carried out using 2:2:2 (v/v/v) water/methanol/dichloromethane at room temperature after spiking internal standards (Table 1). The organic layer is collected and dried completely under the stream of nitrogen. Dried extract is re-suspended in 100 µL of solvent B (see Table 1 below).

Table 1. Internal standards for lipidomics analysis

Name	Abbreviation	CAS	FORMULA	Exact mass
1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine	lysoPC 17:0	50930-23-9	C25H52NO7P	509.34814
1,2-diheptadecanoyl-sn-glycero-3-phosphocholine	PC 34:0	70897-27-7	C42H84NO8P	761.59345
1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine	PE 34:0	140219-78-9	C39H78NO8P	719.54651
1,2-diheptadecanoyl-sn-glycero-3-phospho-L-serine	PS 34:0	799268-51-2	C40H78NO10P	763.53633
N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine	SM 35:1	121999-64-2	C40H81N2O6P	716.58322
cholest-5-en-3ß-yl heptadecanoate	CE 17:0	24365-37-5	C44H78O2	638.60018
1-palmitoyl-2-oleoyl-sn-glycerol	DG 34:1	29541-66-0	C37H70O5	594.52232
1-heptadecanoyl-rac-glycerol	MG 17:0	5638-14-2	C20H40O4	344.29266
N-heptadecanoyl-D-erythro-sphingosine	Ceramide	67492-16-4	C35H69NO3	551.52774
1,2-diheptadecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol)	PG 34:0	799268-52-3	C40H79O10P	750.541085
1,3(d5)-dinonadecanoyl-glycerol	1,3-19:0 D5 DG	1246523-66-9	C41H75D5O5	657.632
Glyceryl tri(hexadecanoate-d31)	D-31 TAG	241157-04-0	C51D93H5O6	900.89

LC-MS

Chromatographic separation was performed on a Shimadzu CTO-20A Nexera X2 UHPLC systems equipped with a degasser, binary pump, thermostated autosampler, and column oven (all from Shimadzu). The column heater temperature was maintained at 55^{o}C. The injection volume was

5µL for all analyses. For lipid separation, the lipid extract is injected onto a 1.8µm particle 50 × 2.1 mm id Waters Acquity HSS T3 column (Waters, Milford, MA) which is heated to 55°C. Elution is performed using acetonitrile / water (40:60, v/v) with 10mM ammonium acetate as solvent A and acetonitrile / water / isopropanol (10 : 5 : 85 v/v) with 10mM ammonium acetate as solvent B. Column is equilibrated before the next injection making total run time 20 min. The flow rate is 0.400µL/min.

Data are acquired in positive and negative mode using data-dependent MSMS with dynamic mass exclusion. Pooled human plasma sample and pooled experimental sample (prepared by combining small aliquots of all client's samples) are used to control the quality of sample preparation and analysis. Randomization scheme is used to distribute pooled samples within the set.