MATERIALS AND METHODS

Sample preparation

EX00708 CHEAR Urine (n=390)- Ferguson
Add CHEER Urine controls.

Table I: IS stock solution - RP-LC and HILIC Recovery Standards

Internal Standards	Source	Cat#	IS stock solution concentration	Rt
L-^{15}N-Anthranalic acid			**200 uM**	
L-$^{15}N_2$- Tryptophan			**200 uM**	
L-D_4-Thymine			**200 uM**	
Gibberelic acid			**200 uM**	
L-Epibrassinolide			**200 uM**	

Table II: IS stock solution – RP-LC Injection Standards

Internal Standards	Source	Cat#	IS stock solution concentration	Rt
Zeatine			**200 uM**	

Sample Crash/Preparation

1. To precipitate proteins **400µL** MAA containing 2.5 µM Recovery standards is added to **100µL** of sample.
2. **Vortex** them at rate of 2000 RPM for 5 min in the shaker.
3. **Incubate** the samples at 4°C for 30 min.
4. **Vortex** and leave them at -20°C for 1 hr.
5. **Centrifuge** samples vials at 14,000RPM at 4°C for 10 min.
6. Transfer **200µL** precipitated supernatant of each sample to a clean vial and dry.
7. Repeat in new vial for 2nd phase.
8. Store in -20°C capped until ready for analysis.
9. Add **50 µL MeOH: Water (50:50) containing RP Injection standard**.
10. **Vortex** to mix for 2 min and centrifuge them for 5 min at 14,000RPM.
11. Transfer sample to an autovial containing glass insert.
12. Centrifuge autovial/insert containing sample volume at 4000RPM for 20 min in 4 °C to clarify sample and remove any bubbles.

LC-MS

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 13).

Table 1. 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.

Data analysis

Raw data processing was done using Agilent software (MassHunter Qual and ProFinder). Data analysis was performed with Agilent MassProfiler Pro package using recursive analysis workflow. Custom R scripts were used for data normalization.