Chemicals needed:

- Precipitate solution, 80% Methanol
- Reconstitution Solution, H_20 with 0.1% Formic Acid LC/MS grade
- Mobile phases, H_20 with 0.1% Formic Acid LC/MS grade, and Acetonitrile LC/MS grade
- Internal Standard

Materials needed:

- Labeled 2 mL Eppendorf tubes
- Labeled 15 mL conical tubes
- Repeater Pipette
- Calibrated Micropipettes in various volumes* (see table below)
- Appropriate Micropipette tips* (see table below)
- Refrigerated Centrifuge
- N_2 Dryer
- Labeled LC vials with appropriate caps
- LC-MS
- ACE Excel 2 C18-PFP Column (100 x 2.1mm, 2 µm particle size)
- Positive Calibration Solution
- Negative Calibration Solution
- Personal Protective Equipment

Type	Volumes (µL)	Tip color
P10	0.5 – 10	white
P20	2 – 20	yellow
P200	20 – 200	yellow
P1000	200 – 1000	blue

Precise Micropipette Volume and Transfer capabilities

Instrumentation:

Centrifugation, Set centrifuge to 5°C. Load samples making sure samples and/or weights are evenly distributed among the wheel.

N_2 Dryer, Organomation Asscociates, Inc- MultiVap 118: Flip green power switch to “on” (located on bottom left of display). Of the three black switches, set the start/reset switch to neutral. Set the heat switch to neutral. Set the gas switch to Manual. To obtain gas flow, turn the gas nozzle on right side of hood. Turn the Harris valve in hood to open position. Adjust LPM air to no more than 15.

Place samples in drying tray. Open/close N_2 flow lines depending on where samples are placed. Lower N_2 lines to enable drying.

UHPLC, Thermo Scientific-Dionex Ultimate 3000: While setting up sequence, ensure that these initial conditions for analysis are as follows: 5 uL injection, 0.350mL/min flow rate, gradient of 100% pump A . Check the lines for air bubbles and purge line if present.

Mass Spectrometer, Thermo Scientific- Q Exactive: Divert valve set to position 2

To calibrate in positive ion mode: Correctly set up positive ion syringe filled with positive ion calibration solution and attach to MS using positive ion calibration tubing. Open Tuner→ File→ Load Tune File→ Click on StabilityTestMStune. Go to Instrument control tab and ensure conditions are as follows:

For Scan Parameter

Scan Type	Full MS
Scan Range	70-1,000
Fragmentation	In-Source CID 2 eV
Resolution	70,000
Polarity	Positive
Microscans	1
Lock Masses	Best
AGC target	3e6
Max Inject time	200ms

Go to calibrate tab → source auto → default settings. Ensure settings are as follow

Set syringe to dispense positive ion calibration solution of a 500uL volume at 3µL /min. Begin dispensing. Turn MS on. Begin calibration. Negative calibration completed in similar fashion using negative ion calibration solution. Calibration should only be performed by trained staff.

Protein Precipitation Procedure:

1- To cell pellet in 15 mL concial tube, add 2µL Isotopically-labeled Daily Working Standard Solution as an Internal Standard.
2- Add 1 mL precipitate solution (ice cold 80% methanol) with repeater pipette.
3- Gently pipet up and down to disrupt cellular membrane.
4- Vortex sample to ensure mixing.
5- Incubate on ice for 10 minutes to further precipitate proteins.
6- Centrifuge at 2000 rpm for 5 min. to separate proteins.

7- Transfer 500 µL of supernatant to new, labeled 2 mL Eppendorf tube making sure to leave behind protein pellet.
8- Dry liquid sample using Nitrogen gas in Organomation Associates MultiVap.
9- Reconstitute sample by adding 30 µL H_20 with 0.1% Formic Acid. Vortex.
10- Place on an ice bath for 10-15 minutes. Centrifuge again.
11- Transfer supernatant to labeled, glass LC vial with insert cap.
12- Load samples into auto sampler.

Data Collection:

1- Turn on UHPLC and MS and set to starting conditions by loading method. Calibrate MS if not yet performed. Ensure that Column is an ACE Excel 2 C18-PFP with dimensions of 100 x 2.1mm.
2- Create file and name it following protocol (QE1_ThreeInitials_Run#).
3- Set up sequence starting with 2 blanks, 1 neat QC and 1 pooled cell pellet QC. Enter samples with one blank and one QC following every 8-10 samples.
4- Name samples following protocol, verify location of samples, ensure method is correct and injection volume is correct.
5- Check MS settings to make sure they are correct.

For Scan Parameter

Scan Type	Full MS
Scan Range	70-1,000
Fragmentation	In-Source CID 2 eV
Resolution	70,000
Polarity	Positive
Microscans	1
Lock Masses	Off
AGC target	3e6
Max Inject time	100

For HESI Source in positive (negative)

Sheath Gas Flow Rate	40
Aux Gas Flow Rate	5
Sweep Gas Flow Rate	1
Spray Voltage (kV)	3.3 (3.5)
Spray Current (uA)	-
Capillary Temp (°C)	300 (350)

S-Lens RF Level (%)	35.0
Heater Temp (°C)	350

Gradient Information

- Duration of run is 22 minutes
- Initial conditions are 100% Pump A (0.1% FA in Water)
- Beginning at Run Time 1 minute and ending at Run Time 18 minutes, begin a ramp gradient up to 95% pump B (Acetonitrile)
- Hold conditions at 95% pump B from Run Time 18 minutes to Run Time 20 minutes
- Beginning at Run Time 20 minutes, return to initial conditions at ending at Run Time 22 minutes

Time (min)	%A	%B
0	100	0
1	100	0
11	35	65
13	35	65
18	5	95
20	5	95
21	100	0
22	100	0

6- Begin Sequence

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