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Liquid Chromatography/Mass Spectrometry (LC/MS) based Untargeted Metabolite Analysis of Hydrolysate

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We use this protocol and it's working

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Abstract

This protocol describes a standardized workflow for untargeted metabolomics profiling of hydrolysate samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Hydrolysate powders were reconstituted in water, followed by metabolite extraction using a methanol-acetonitrile-formic acid solution with isotope-labeled internal standards. Extracted metabolites were separated using a Zorbax SB-CN analytical column under a defined chromatographic gradient and analyzed on a Q Exactive Orbitrap mass spectrometer operating in data-dependent acquisition (DDA) mode. MS1 scans were acquired at a resolution of 35,000, and MS2 scans were triggered for the top precursor ions. This method enables the comprehensive detection of polar and semi-polar small molecules in complex biological matrices and supports high-throughput, reproducible metabolomic profiling for bioprocess and biotechnology research applications.

Materials

General Materials

- Deionized water
- Microcentrifuge tubes (1.5 mL or 2.0 mL)
- Vortex mixer
- Pipettes (10 µL, 100 µL, 1000 µL) and pipette tips
- Centrifuge capable of reaching 12,000 × g
- SpeedVac centrifuge or vacuum concentrator

Reagents

Extraction solution:

- Methanol
- Acetonitrile
- 1% formic acid in water (40:40:20 ratio)
- Isotope-labeled amino acid standard (e.g., Cambridge Isotopes, CAT#MSK-A2)

Equipment for Mass Spectrometry

- QExactive mass spectrometer
- Vanquish liquid chromatography (LC) system
- Analytical column: Zorbax SB-CN Rapid Resolution HD column (1.8 µm, 2.1 × 150 mm, Agilent)

Solvents for LC-MS

- Mobile phase A: 0.1% formic acid in water
- Mobile phase B: 0.1% formic acid in methanol

Consumables

- LC-MS grade solvents (methanol, acetonitrile, formic acid, and water)
- LC-MS vials with inserts

Miscellaneous

- Freezer (-20°C) for sample incubation
- Lab timer for incubation and centrifugation steps

Preparation of Powdered Hydrolysate

1 1. Weigh the powdered hydrolysate sample.
2. Reconstitute the powdered hydrolysate in deionized water to achieve a final concentration of **1 µg/µL**.
3. Vortex to ensure complete dissolution.

Metabolite Extraction

2 **Resuspension Sampling:**
- Transfer **60 µL** of the reconstituted hydrolysate solution into a microcentrifuge tube.

3 **Add Extraction Solution:**
- Add **240 µL** of the extraction solution (40:40:20 methanol/acetonitrile/1% formic acid) to the tube.
- Include **3 µL** of a 2X diluted isotope-labeled amino acid standard (e.g., Cambridge Isotopes, CAT#MSK-A2).

4
- **Drying**:
- Dry the collected supernatant in a SpeedVac centrifuge until completely desiccated.

5 **Reconstitution:**
- **Re**constitute the dried extract in **100 µL of deionized water**.

Vortex **to mix.**

Sample Preparation for MS Analysis

6 **Dilution:**
- Transfer **10 µL** of the reconstituted sample to a vial suitable for mass spectrometry.
- Dilute with **40 µL of deionized water**.

7 **Pooling Verification:**
- Ensure the dilution level aligns with prior analysis of the pooled sample to maintain consistency.

Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis

8 **Instrument Setup:**
- Use a **QExactive mass spectrometer** interfaced with a **Vanquish liquid chromatography system**.

Chromatographic Separation:

- Employ a **Zorbax SB-CN Rapid Resolution HD column** (1.8 µm, 2.1 × 150 mm, Agilent).
- Set the flow rate to **0.2 mL/min**.
- Apply the following gradient elution program:
- Hold at **1% mobile phase B** (0.1% formic acid in methanol) for **2 minutes**.
- Ramp to **15% B** over **10 minutes**.
- Increase to **60% B** in **3 minutes**.
- Ramp to **100% B** in **4.9 minutes** and hold for **2 minutes**.
- Return to **1% B** in **0.1 minutes** and hold for **5 minutes**.
- Mobile phase A: **0.1% formic acid in water**.

Mass Spectrometry Settings:

MS1 Scans:
- Resolution: **35,000**
- Automatic gain control (AGC)target: **3e6**
- Max injection time: **100 ms**
- Scan range: **75–900 m/z**

MS2 Scans:
- Resolution: **17,500**
- Automatic gain control (AGC) target: **1e5**
- Max injection time: **50 ms**
- Isolation window: **2 m/z**
- Loop count: **10**
- Normalized collision energy: **20 and 35**
- Scan range: **200–2000 m/z**

Ion Source Parameters:
- Positive ion spray voltage: **3,700 V**
- Negative ion spray voltage: **-3,500 V**
- Capillary temperature: **300°C**
- Sheath gas: **45**
- Auxiliary gas: **15**
- Spare gas: **2**
- S-Lens RF level: **45**
- Probe heater temperature: **250°C**

Acquisition Modes:
- MS1: Profile mode.
- MS2: Centroid mode.

Data Analysis

9 1. Utilize a data-dependent acquisition scheme.
2. Apply dynamic exclusion settings of **10 seconds** to improve spectral quality.
3. Export data for downstream processing and untargeted metabolomics analysis.

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