Liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis of Auxin Metabolites in Arabidopsis thaliana shoot tissues

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Method Article

Abstract

Auxin metabolome affects almost every facet of plant development from hormone transport and biosynthesis to degradation. It also triggers several environmental responses. Measuring auxin biosynthesis can enable a better understanding of metabolism and hormonal activities during plant growth and developmental phases. Here we optimised a solid phase extraction (SPE) sample preparation and liquid chromatography-high resolution mass spectrometry \(LC-HRMS) method for profiling auxin metabolites in the tissues \(shoot and leaves) of the model plant system (Arabidopsis thaliana). We combined a sensitivity SPE sample preparation protocol with accurate LC-HRMS analysis to profile auxin metabolites in plant tissues.

Introduction

Measurements of auxin metabolites at steady state as well as their metabolic flux can be of great importance to understand hormonal regulation of plant metabolism. The increasing use of LC-HRMS analysis in plant metabolomic analysis made specific and sensitive measurements of several metabolites possible. The HRMS analysis unlike triple quadruple methods required less method development and is more accurate and robust. So far there are several reports on analysis of indole acetate pathway using LC-triple quadruple MS or GC-MS instruments but very few reports on use of LC-HRMS.

Also, sensitivity of such analysis is hampered by methanol extraction protocols due to high matrix effect from plant tissues. Here, we optimised SPE sample extraction protocol for plant tissues to improve sensitivity and combined with accurate LC-HRMS analysis. The reported method is simple, robust, sensitive and accurate for the determination of auxin metabolites in Arabidopsis thaliana shoot tissues.

Reagents

MS grade Methanol (Biosolve BV, The Netherlands),

MilliQ Water

13C internal standards were purchased from OlChemim s.r.o. (https://www.olchemim.cz/Products.aspx?idc=2&idp=9)

Extraction buffer: (50 mM sodium phosphate buffer, pH 7.0, containing 0.1% diethyldithiocarbamate)

Loading buffer: Use 1 ml of the extraction buffer to test the amount of 1M HCl needed for its acidification to pH 2.7. Prepare the loading buffer by adding twice the amount of 1 M HCl per milliliter of extraction buffer. (Addition of 0.5 ml sample extract to 0.5 ml loading buffer must give a pH 2.7.)

Note: Keep diluents in chilled condition (0-4ºC)

Equipment

MM301 bead mill (Retsch GmbH (http://www.retsch.com))

Oasis HLB 1 cc Vac Cartridge, 30 mg Cartridge Sorbent (Waters; Catalogue number: 186003908)

Visiprep™ 12- and 24- Port Vacuum Manifold (Sigma Aldrich)

High resolution mass spectrometry (Q Exactive Plus MS, Thermo Scientific)

Agilent 1260 liquid chromatography (LC) system

LC column Kinetex C18 (100X 2.1; 2.6uM)

Eppendrof Concentrator plus

MICROVAP Microplate Evaporator (Catalog # 11801)

Procedure

Sample preparation and metabolite extraction:

Sample Extraction (For Indole acetate metabolites; IAA): 20 to 50mg of tissues was mixed with 1 ml cold extraction buffer and homogenised using a Mixer Mill MM301 bead mill at a frequency of 25 Hz for 5 min after adding 2 mm ceria-stabilised zirconium oxide beads. Add 1 µg of ^{13}C indole acetate (or other suitable internal standards) and vortex well. Incubated this plant extracts at 4°C with continuous shaking (20 min) and centrifuge (15 min, 23 000 g at 4°C). Transfer the supernatant to a new vial and adjust pH to 2.7 with 1 M HCl

SPE procedure:

a. Prepare SPE vacuum assembly with HLB (30mg) column cartridges.

b. Condition SPE column with 1 mL of methanol and 1mL of water

c. Equilibrate the column with 250 µl of 5 mM HCl. (Do not let column dry)

d. Load Equilibrate column with 0.5 ml loading buffer

e. Load 0.5 ml Sample onto the SPE column, and mix intensely with the loading buffer. Pass the mixture slowly through the HLB sorbent immediately.

f.   Wash the column with 2 ml of 5% methanol.

g. Put clean glass tubes into the manifold rack, and then elute the sample from the column with 2 ml of 80% methanol.

h. Collect sample eluent and evaporate the samples to dryness in a SpeedVac concentrator or under nitrogen stream at room temperature.

6.   Store samples at -80° C till further analysis or ship in dry ice.

7.   Sample are reconstituted in mobile phase (or 50% methanol) before analysis

8. Processed Blank sample was prepared in the same manner as above where sample tissue is replaced with 50uL of buffer

9. Pooled QC sample: equal mix of all samples prepared after processing

LC-HRMS analysis

<u>LC conditions:</u>

Column: Kinetex C18 (100X 2.1; 2.6uM) at temperature 40^0C

Mobile phase A: 0.1 % acetic acid in Water

Mobile phase B: 0.1 % acetic acid in Methanol

Flow rate: 0.26 ml/min

Mobile phase Gradient:

Time/ %B: 0/5, 3/ 5, 10/75, 12/98, 16/98, 16.1/5, 19/5

Injection volume: 10 uL

<u>MS parameters:</u>

ESI mode: Positive

Spray voltage 4.5 eV

Capillary Temp 300^{0}C

Probe Heater Temp 300^{0}C

Sheath Gas: 30 and Auxiliary Gas: 5 units

S-Lens RF Level 65v

m/z range: 60 to 900

Analysis sequence:

1. Mobile phase (A:B; 90:10) blank: to check the background from LC column and LC-MS system

2. Processed Blank: to check the contamination and background from sample processing

3. Auxin standard mix: for the system suitability of the method

3. Pooled QC: 5 injections or till the LC-MS system gets stabilised

4. Plant tissue samples

5. Inject Pooled QC after each 6 tissue samples

6. Blank

7. Auxin standard mix: for the system suitability of the method

Data analysis:

Thermo Xcalibur Quan software used for extracting peaks (as extracted ion chromatograms; EIC) for the metabolites of interest based on HRMS data with 5 ppm window for M+H adducts. The peaks were identified based on exact m/z and retention times in comparison with authentic standards. The peaks areas were used for relative quantification in the samples.

Targeted metabolites; chemical formulas:

Tryptophan; C11H12N2O2

Indole-3-acetaldoxime; C10H10N2O

Indole-3-acetonitrile; C10H8N2

Indole-3-acetamide; C10H10N2O

Indole-3-acetic acid; C10H9NO2

Indole glucosinolate; C16H19N2O9S2

Indole-3-pyruvic acid; C11H9NO3

The MS/MS data for above metabolites can be downloaded from EMBL-MCF spectral library
http://curatr.mcf.embl.de/MS2/

Troubleshooting

SPE Sample prep:

1. Early elution of metabolites during loading or washing step: make sure to have sample in 100 aqueous conditions with pH adjusted to acidic side as mentioned in protocol.

2. Verify SPE loading, wash and elution conditions using auxin metabolite standard mixtures.

Time Taken

For 10 samples:

Mobile phase Reagent preparations: 1hr

Sample preparation: 30 min

LC-MS set-up and stabilisation: 1hr

LC-MS run time: 20 min

Data analysis: 1-2 hrs

Anticipated Results

The representative chromatograms from standards is shown in Figure 1 .

The %CV for QC and ^{13}C internal standard should be more than 10%.

References

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2. Palmer A1, Phapale P1, Fay D1, Alexandrov T (2018) Curatr: a web application for creating, curating and sharing a mass spectral library. Bioinformatics. 15;34(8):1436-1438. doi: 10.1093/bioinformatics/btx786
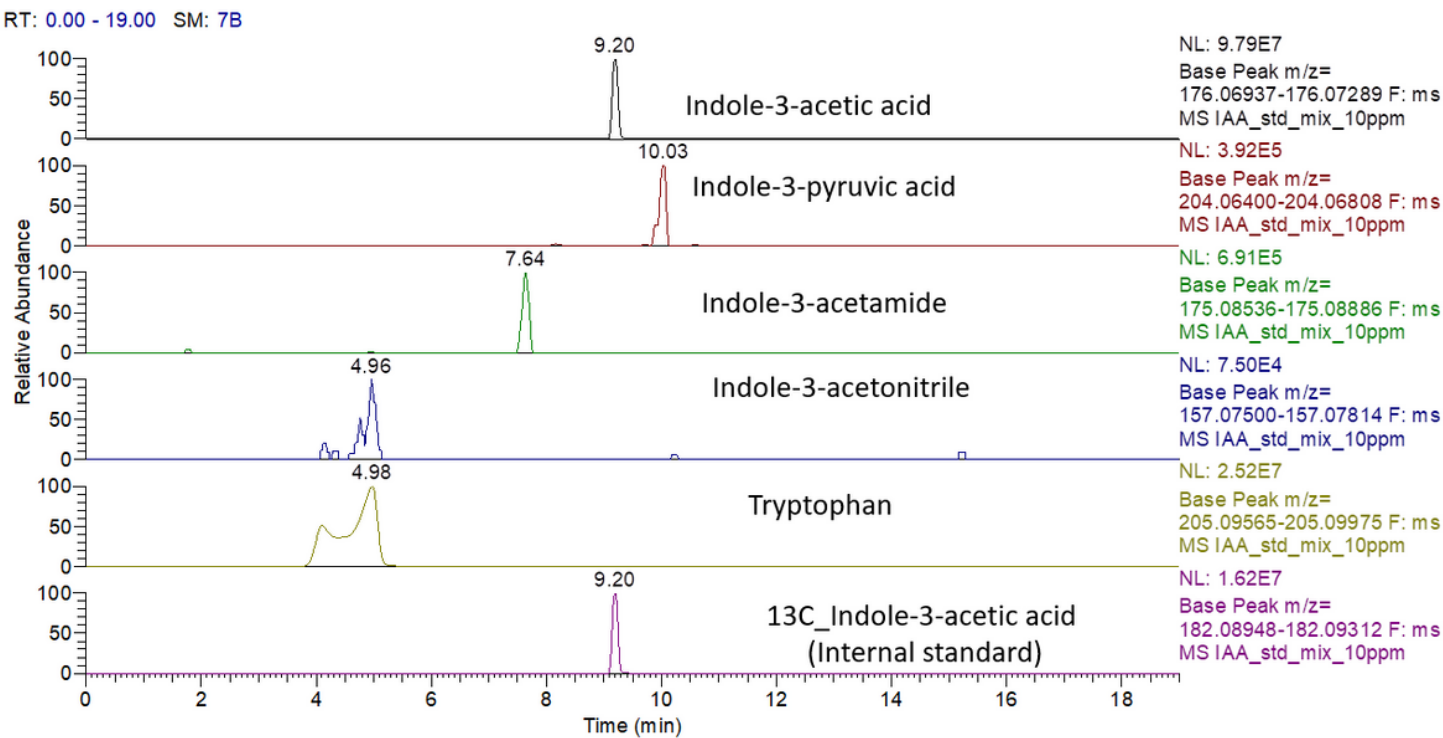
Acknowledgements

Figures

Figure 1

The representative extracted ion chromatograms (10 ppm window) from the analysis of standards.