Quantification of plant hormones by standard addition method

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Takuyu Hashiguchi[1], Masatsugu Hashiguchi[1], Hidenori Tanaka[1], Koki Fukushima[1], Takahiro Gondo[1], Ryo Akashi[1]

[1]Department of Animal and Grassland Sciences, University of Miyazaki

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Takuyu Hashiguchi

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ABSTRACT

Phytohormones are essential signaling molecules in multiple plant processes, including growth, development, and stress response. Simultaneous quantification of multiple plant hormones is required for understanding plant physiology because hormones typically change in tandem in response to external stimuli. Sensitive and quantitative analysis using liquid chromatography-linked mass spectrometry (LC-MS/MS) adopts stable isotope-labeled compounds in previously published works. However, a method for quantifying phytohormones when isotopically labeled chemicals are not available, remains to be established.

This protocol can be used for measuring plant hormone content by standard addition method. Standard addition method has been used for quantification of various compounds such as drugs and pesticides especially when it is difficult to obtain stable isotope-labeled compounds. Plant hormones are extracted from a plant material such as *Lotus japonicus* with 50% acetonitrile and partially purified with reversed-phase solid-phase extraction (SPE) and ready for LC-MS/MS analysis. Calibration curves are constructed by spiking pure standards to the matrix. The method allows us to quantify plant hormones in plant tissues of interest by correcting matrix effects.

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MATERIALS TEXT

- TissueLyzer II (QIAGEN)
- Plant hormone (gibberellin A_4, abscisic acid, salicylic acid, jasmonic acid, indole-3-acetic acid, *trans*-zeatin, *cis*-Zeatin, brassinolide)
- 2.0 ml polypropylene tube
- 15 ml polypropylene tube
- 0.2 ml Vial (1030-51028, GL Sciences)
- Screw cap for vial (1030-51228, GL Sciences)
- Methanol (LC-MS grade)
- Acetonitrile (LC-MS grade)
- Ultrapure water (LC-MS grade)
- Oasis HLB cartridge (WAT094225, Waters)
- Centrifuge (TOMY MX-301, TOMY SEIKO)
- Sonicator (1510J-MT, BRANSON ULTRASONICS)
- Tube rotator (SLRM-2M, SeouLin Bioscience)
- Vacuum concentrator (SPD140DDA, Thermo Fisher Scientific)
- Orbitrap Q-Exactive (Thermo Fisher Scientific)
- Xcalibur version 4.2.47 (Thermo Fisher Scientific)
- Accucore C18 LC Column (17126-152130, Thermo Fisher Scientific)
- Universal Directed-Connection and Stand Alone Holder (852-00, Thermo Fisher Scientific)
- Guard Cartridge (17126-012105, Thermo Fisher Scientific)

BEFORE STARTING

Extraction for plant hormones has been conducted utilizing a previously established protocol with minor modifications (Simura, J., et al. Plant Physiol, 177, 476-489, 2018).
In standard addition method, some experimental conditions such as the amount of starting material and dilution ratio have to be optimized when other tissues or plant species are used.

1 Plant tissues of interest such as leaves, roots, and stems are disrupted in liquid nitrogen using a homogenizer (e.g. TissueLyzer II, QIAGEN).

2 Collect 50 mg of the tissue and extract with 1 ml of cold 50 % acetonitrile. Prepare the tissues for both a sample and standards. For example, when you make a calibration curve with 5 different spiking solutions, prepare six homogenized-tissues in total (one for a sample and five for standards).

3 When you adopt a pre-spike method, add pure standards to the tissue with 1ml cold 50% acetonitrile and then move to the next step.

4 Sonicate the solutions for 5 min at 4°C using a ultrasonicator (e.g. 1510J-MT, BRANSON) and then incubate on a tube rotator for 60 min at 4°C.

5 After centrifuge at 15,000 × g for 15 min, collect the supernatant to a new 2 ml tube.

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6 Activate Oasis HLB cartridge (Waters) with 1 ml of 100 % methanol and then wash 1 ml of ultrapure water.

7 Equilibrate the cartridge with 1 ml of 50 % acetonitrile. Load the sample collected at step 5 onto the cartridge. Collect the flow-through into a 15 ml tube .

8 Elute with 1 ml of 30 % acetonitrile into the tube and divide into two 2 ml tubes. Evaporate to dryness in vacuum concentrator (e.g. SPD140DDA, Thermo Fisher Scientific) for 3 hours or overnight.

9 Dissolve dried pellets in 100 µl of 30 % acetonitrile. Adjust the volume when you need to dilute or concentrate it.

10 Prepare stock solutions by adding each plant hormone into one tube. When you make five working solutions, prepare five stock solutions (e.g. 0, 20, 200, 1000, 2000 ng/ml). In the case of a pre-spike method, you need to make those before adding to the matrix.

11 Make matrix calibration curves by diluting the stock solutions with the matrix. For example, add 2.5 µl of the stock solutions to 47.5 µl of the matrix obtained at step 9.
Note that a dilution factor depends on a plant material. If a sample contains high concentration of plant hormones, the sample can be diluted more.

12 Analyze 10 µl of the sample by LC-MS/MS . In the present protocol, orbitrap Q-Exactive-linked to an UltiMate 3000RSLC (Thermo Fisher Scientific) is used.

13 Separate samples on a 2.6 µm Accucore C18 LC column (Thermo Fisher Scientific) using a linear methanol gradient of 1-100 % for 10 min at a flow rate of 0.5 ml/min. An autosampler and a column compartment are set at 10°C and 40°C, respectively.

14 Acquire MS data in targeted selected ion monitoring (t-SIM) mode using an electrospray ionization mode.

15 Set mass spectrometric conditions as follows: polarity, positive and negative ionization modes; spray voltage for positive, 3.5 kV; spray voltage for negative, 2.0 kV; sheath gas flow rate, 50; auxiliary gas, 10; sweep gas, 0; heated capillary temperature, 380 °C; S-lens RF level, 50; and auxiliary gas heater temperature, 350 °C; resolution, 70,000; AGC target, 5E4; maximum injection time, 200ms.

16 When a stable isotope is also monitored, set isolation window at 10.0 m/z and the offset at 2.0 m/z.

17 Analyze raw data files using Qual Browser in Xcalibur software. Check that target ions are detected.

18 Analyze a sequence file using Quan Browser in Xcalibur software. Check that the peaks for target ions are extracted correctly.

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19 Subtract the peak areas of the standards by the peak areas of the matrix and obtain the equation including the y-intercept and the slope of the calibration curve. Calculate the concentration of plant hormones by substituting the peak area of the sample for the equation.

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