ABSTRACT

Phytohormones play a key role in regulating development and growth, as well as acting on plant responses to biotic and abiotic stresses. The following protocol describes a target specific methodology to obtaining quantitative profiles of phytohormones from plant tissues by ultra-high-performance liquid chromatography (UHPLC) coupled to mass spectrometry.
**MANUSCRIPT CITATION:**
Coutinho, FS; Santos, DS; Lima, LL; Vital, CE; Santos, LA; Pimenta, MR; Silva, JC; Soares-Ramos, JRL; Mehta, A; Fontes, EPB; Ramos, HJO. Mechanism of the Drought Tolerance of a Transgenic Soybean Overexpressing the Molecular Chaperone BiP. Physiology and Molecular Biology of Plants 2019. DOI: 10.1007/s12298-019-00643-x

**License:** This is an open access protocol distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Protocol status:** Working
We use this protocol in our group and it is working. Developed in Center of Analysis of Biomolecules (NUBIOMOL), Universidade Federal de Viçosa-UFV, Viçosa-MG. Brazil.

**Created:** Jan 09, 2019
**Last Modified:** Feb 08, 2019

**PROTOCOL integer ID:** 19142

**Keywords:** Phytohormones, liquid chromatograph, mass, spectrometry, (cytokinin), abscisic acid, indoleacetic acid, salicylic acid, gibberellins, jasmonic acid

## MATERIALS

### REAGENTS

- Methanol LC-MS grade.
- Acetic Acid LC/MS grade.
- Isopropanol LC/MS grade.
- Acetonitrile LC/MS grade.
- Crystalline reference substances of the phytohormones -zeatin(cytokinin), 1-aminocyclopropane-1-carboxylic acid (ACC) (ethylene precursor), abscisic acid (ABA), 3-indoleacetic acid (IAA), salicylic acid (SA), gibberellins (GA<sub>3</sub> and GA<sub>4</sub>), jasmonic acid (JA), methyl jasmonate (MeJA)
- High pure water (18.2M Ωcm-1) provided by a Milli-Q system (Burlington, Massachusetts, USA).
- Liquid Nitrogen.

### EQUIPMENTS AND SUPPLIES

- Ultra-High-Performance Liquid Chromatography (UHPLC) coupled online to a mass spectrometer QQQ (triple quadrupole). Agilent 1200 Infinity LC System coupled to Agilent 6430 Triple Quadrupole LC/MS System (Agilent Technologies, Santa Clara, California, USA)
- Column Zorbax Eclipse Plus C18 (1.8 μm, 2.1 x 50mm) and a guard column Zorbax SB-C18, 1.8 μm (Agilent Technologies, Santa Clara, California, USA).
- Ultrasonic cleaners.
- Benchtop centrifuge.
- Ultra-freezer.
- Benchtop balance
- Mortar and pestle.
- Vials, caps and septa.
- Polyvinyl Difluoride (PVDF) Syringe Filters 13mm and 0.2 μm.
- Softwares: Skyline Targeted Mass Spec Environment version 4.1 (MacCoss Lab Software) and Microsoft Excel.

## PHYTOHORMONES EXTRACTION

1) Collect samples of plant tissues, immediately freeze in liquid nitrogen and store them in freezer -80°C until use.

2) Macerate the samples in liquid nitrogen using mortar and pestle. Do not allow to thaw. Weigh approximately 110mg of each sample into microtubes (2ml) and annotate the weight (used for absolute quantifications). Alternatively, the samples may be weighed into 1.5 ml tubes (eppendorff - round bottom) and macerate using glass or tungsten beads at 25 Hz / s for 3
minutes.

3) Add 400μl of a solution containing methanol:isopropanol:acetic acid (20:79:1).

4) Vortex samples 4 times for 20 seconds (keep on ice) and then sonicate for 10 minutes. Let the samples stand on ice for 30 minutes and again sonicate for 10 minutes.

5) Centrifuge for 13000g for 10 minutes at 4ºC and collect the supernatants in new tubes.

6) To the remaining pellet, repeat the procedures 3, 4 and 5 (supernatant 2) and then pool the supernatants.

7) Centrifuge the samples a 20000g for 5 minutes at 4ºC to remove debris and collect about 600μl for new tubes.

8) Filter the supernatant using disposable 0.2ml PVDF membrane.

9) Store the samples in freezer -80ºC until analyze them using LC-MS.

---

**LC/MS CONDITIONS**

2 1) UHPLC system containing vials for 1mL and loop for injection of 5μl.

2) Use a mass spectrometer triple quadrupole which enables product and MRM (multiple reaction monitoring) scans. The methods were optimized for an Ultra-High-Performance Liquid Chromatography (UHPLC) coupled online to a mass spectrometer QQQ (triple quadrupole).

3) Chromatographic separation is performed by reverse phase columns, such as an analytical Zorbax Eclipse Plus C18 (1.8 μm, 2.1 x 50mm) and a guard column Zorbax SB-C18, 1.8 μm.

4) The mobile phase consists of buffers A (water acetic acid 0.02%) and B (acetonitrile acetic acid 0.02%) and a gradient of %B: 5% x 0 min⁻¹; 60% x 11 min⁻¹; 95% x 13 min⁻¹; 95% x 17 min⁻¹; 5% x 19 min⁻¹; and 5% x 20 min⁻¹. The solvent flow rate is 0.3ml min⁻¹ in a column at 30ºC.

5) The ionization method used in the mass spectrometry was an ESI (Electrospray Ionization) under the conditions: gas temperature of 300ºC, nitrogen flow rate of 10L min⁻¹, nebulizer pressure of 35psi and capillary voltage of 4000V. The mass spectrometer is operated by positive or negative mode according to method for phytohormone detection.

6) INSERIR UMA TABELA OU COMO FOTO DO SETUP DO QQQ OU IDICANTO OS SEGMENTOS E O USO DO MRM DYNAMIC!!!

---

**STANDARD CURVES**

3 Note: For absolute quantification of phytohormones, a standard curve with known concentrations should be prepared.

1) Prepare a standard solution containing 1.0 μg/mL of each compound in methanol:isopropanol:acetic acid (20:79:1) and transfer it to vials.

2) Setup a product ion scan method for each compound and optimize the transmission and fragmentation parameters. This procedure may be executed manually or automatically. A second transition can be used to confirm the first transition used for the quantification, especially for low intensity chromatogram signals.

3) Select the higher intensity fragment ions to compose the transition list used in the scan mode
by MRM (Multiple Reaction Monitoring) as illustrated in the Table 1.

<table>
<thead>
<tr>
<th>Molecule List Name</th>
<th>Precursor Name</th>
<th>Precursor Charge</th>
<th>Precursor RT</th>
<th>Precursor CE</th>
<th>Precursor m/z</th>
<th>Polarinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>JA</td>
<td>Jasmonic Acid</td>
<td>-1</td>
<td>9,2</td>
<td>7</td>
<td>209</td>
<td>negative</td>
</tr>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
<td>-1</td>
<td>8,2</td>
<td>7</td>
<td>263</td>
<td>negative</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylic acid</td>
<td>-1</td>
<td>6</td>
<td>7</td>
<td>136,8</td>
<td>negative</td>
</tr>
<tr>
<td>IAA</td>
<td>Indoleacetic Acid</td>
<td>1</td>
<td>7,5</td>
<td>15</td>
<td>176</td>
<td>positive</td>
</tr>
<tr>
<td>ACC</td>
<td>ACC</td>
<td>1</td>
<td>0,6</td>
<td>7</td>
<td>102,1</td>
<td>positive</td>
</tr>
<tr>
<td>Zeatin</td>
<td>Zeatin</td>
<td>1</td>
<td>0,8</td>
<td>7</td>
<td>220</td>
<td>positive</td>
</tr>
<tr>
<td>MeJA</td>
<td>Methyl jasmonate</td>
<td>1</td>
<td>11,6</td>
<td>15</td>
<td>225,2</td>
<td>positive</td>
</tr>
<tr>
<td>GA4</td>
<td>Gibrell in 4</td>
<td>-1</td>
<td>10,4</td>
<td>15</td>
<td>331</td>
<td>negative</td>
</tr>
<tr>
<td>GA3</td>
<td>Gibrell in 3</td>
<td>-1</td>
<td>5,9</td>
<td>34</td>
<td>345,1</td>
<td>negative</td>
</tr>
</tbody>
</table>

Table 1. Mass spectrometric parameters used for analysis of nine target phytohormones and transition list used as input in the Skyline analysis.

4) Prepare serial dilutions of 0.1 ng/mL up to 400 ng/mL in according with the mass spectrometer sensitivity and linearity. Two replicate is enough to prepare the standard curves.
5) Inject 5.0 ul and perform the MRM method as setup in step 3.
6) Generate the area from XICs (extracted ion chromatograms) for each dilution using Skyline software.
Note: A complete tutorial for processing of the mass spectra data using Skyline software is described in the Supplementary Material 1.
7) Export the XICs to Microsoft Excel following the instructions of the Skyline tutorial (Supplementary Material 1).
8) Prepare the standard curves for each compound in ng/mL of fresh tissue. Use the XIC area versus phytohormones concentration (ng/mL) to generated linear curves (Figure 1).
Figure 1. Schematic diagram showing the steps to prepare the standard curves and to final quantification of phytohormones

SAMPLE ANALYSIS

4 1) Inject all sample randomly using the MRM method, which was used to generate the standard curves. Use the raw data to analyze mass spectra using Skyline software (section 3.3; step 6).
2) For each compound, use the XICs area exported to Microsoft Excel and convert to ng/mL using the standard linear curves (section 3.3; step 7).

Quantification of ABA for a Sample 1 with a XIC area of 6350:

ABA ; transition 263 >153; retention time 8.2 (Table 1).

Conversion to ng/mL:

Standard curve for ABA:
y=137.64x + 993.19; where x is the sample concentration (ng/mL) and y is the XIC area (arbitrary units)
ABA concentration in the sample 1: 38.91ng/mL

Conversion of ng/mL to ng/g of fresh tissue:
38.91ng ---- 1000ul
x------ 800ul
Then: 31.13 ng of ABA from 110mg of fresh tissue, 282.98 ng/g fresh tissue.
ACKNOWLEDGMENTS

The authors would like to thank the Núcleo de Análises de Biomoléculas (NuBioMol) of the Universidade Federal de Viçosa for providing the facilities for the data analysis. The authors also acknowledge the financial support provided by the following Brazilian agencies: Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Finance code 001), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (Finep), and Sistema Nacional de Laboratórios em Nanotecnologias (SisNANO)/Ministério da Ciência, Tecnologia e Informação (MCTI).

REFERENCES

Coutinho, FS; Santos, DS; Lima, LL; Vital, CE; Santos, LA; Pimenta, MR; Silva, JC; Soares-Ramos, JRL; Mehta, A; Fontes, EPB; Ramos, HJO. Mechanism of the Drought Tolerance of a Transgenic Soybean Overexpressing the Molecular Chaperone BiP. Physiology and Molecular Biology of Plants 2019. DOI : 10.1007/s12298-019-00643-x


SUPPLEMENTARY MATERIAL: SKYLINE TUTORIAL

Note: Tutorial for analysis of mass spectra from small molecules by skyline software. Adapted from tutorial “Skyline Small Molecule Targets”
https://skyline.ms/_webdav/home/software/Skyline/@files/tutorials/SmallMolecule-3_6.pdf

Install the Skyline Package (32 or 64 bits):
https://skyline.ms/wiki/home/software/Skyline/page.view?name=SkylineInstall_64_4-1&submit=false
https://skyline.ms/project/home/software/Skyline/begin.view

Generate a transition list table in accordance with the MRM parameters such as in the Table 1.

Open the transition list file in the OpenOffice software (Avoid language incompatibly). Select and Copy all lines, except the column heading.
Open Skyline package and click in *blank document*

Proceed *edit*>>>*insert*>>>*transition list*

Click in "columns" to edit. Select in accordance with the transition list create before. Use the mouse to dragging and to change the column order.

Paste the transition list information. Click in *check for errors*
Click in “insert”.

Open Setting >>> transitions setting and configure to processing the raw data from the QqQ mass spectrometry.
Open Setting >> Save Current.

Open File >> import >> Results

Click in Ok to import and save the Project as myprojectname. Sky

In the window import Results >> click in Ok!! Select and import all data from files .d, generated by LC/MS QqQ for all the samples.

After the data uploading, open View >>> retention times >>> and select Replicate Comparison

Open View >>> Peak Areas >>> and select replicatecomparison

Proceed a double-click in the tabs “Peak Areas” and “Retention Times”.

Click over the retention time (RT) bar to edit and correct the selected chromatogram area that was generated automatically. The dashed line could be moved!! Repeat for all compound and
samples!!!
Save the project.
Open *File* >>> *Export* >>> *report* >>> select *transition result* >>> OK, to export the peak areas of the all XICs as spreadsheet file. Open this file in the OpenOffice and use the XIC area for each compounds to obtain the quantitative information.