PROTOCOL MSU_MSMC_003 version 1.1

PROTOCOL: Extraction of phytohormones for subsequent LC/MS/MS analysis

Contributed by staff of MSU Mass Spectrometry and Metabolomics Core

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Method based on Zeng *et al* (2011) PLoS Pathogens, written by Bethany Huot on 4/29/14, modified by MSMC

Standard Preparation

NOTE: May be prepared prior to use.

Materials needed:

Methanol (MeOH, HPLC grade)
Salicylic Acid (Sigma-Aldrich, Cat#247588; MW = 138.12 g/mol)
Indole-3-Acetic Acid (Sigma, Cat#I2886; MW = 175.2 g/mol)
Abscisic Acid (Sigma-Aldrich, Cat#A1049; MW = 264.32 g/mol)
Jasmonic Acid (JA; Sigma-Aldrich, Cat#J2500, Cayman Chemical, 88300); MW = 210.3 g/mol)
JA-Isoleucine (JA-Ile; Toronto Research Chemicals, J210550; Cayman Chemical, 10740) MW = 323.4 g/mol)
Methyl JA (MeJA; Aldrich, Cat# 392707; MW = 224.3; 4.24 M)
N-(3-Indolylacetyl)-L-alanine (Sigma, 345911-250MG)
Salicylic acid glycoside (Santa Cruz Biotechnology, sc-473013)
Coronatine (Sigma, C8115-1MG)
12-OPDA (Cayman Chemical, 88520)

Labeled internal standards that are available:

Salicylic Acid-13C6 (Santa Cruz Biotechnology, sc-220088)
Abscisic acid (ABA)-d6 (Toronto Research Chemicals, A110002)

MSMC has stocks of these labeled internal standards that can be purchased by individual labs to reduce the cost.

Labeled internal standards that TRC lists on backorder (not currently available):

Jasmonic Acid-d3 (Toronto Research Chemical, J210522)
Indole acetic acid-13C6 (Toronto Research Chemical, I577342)
Jasmonyl-L-isoleucine-d3 (Toronto Research Chemicals, J210552)

Preparation of concentrated Stocks:

- **Only need to prepare stocks for compounds that you are interested in quantifying**

1. Weigh out compound by adding to polypropylene microcentrifuge tube (1.5 ml) tared on a balance. Depends on accuracy of balance, but generally can measure out 10-50 mg accurately. For chemicals purchased in very small quantities (1 mg or less), may need to just dissolve total amount purchased in original vial and calculate concentration based on purchased weight and amount of solvent used.
2. Add HPLC grade methanol (1 ml). 10 mg/ml will be in the range of 30-70 mM for most of the phytohormones.
3. Cap and vortex to dissolve.
4. Calculate final concentration for each.
5. Make appropriate dilution in methanol to give 1 mM final concentration to use for preparing standard curve.
6. Make 1/10 dilution of 1mM in methanol to give 100 μM stock
7. Store at -20°C.

Sample Collection

Materials needed:

Liquid nitrogen
Sharp scissors
Small forceps (not sharp)
Labeled 2.0 mL cryo tubes (USA Scientific, Cat# 1420-9600)
3 mm Zirconium beads (Glen Mills, Cat# VHD ZrO) or stainless steel ball bearings (VXB.com, Cat #Kit12064)
analytical balance
sample sheet for recording mass
Labeled freezer sample box

Before Harvest: Label tubes and put 3 beads in each tube.

Harvesting:

1. Tare balance with pre-labeled tube containing beads.
2. Use sharp scissors to remove 2-4 fully expanded Arabidopsis leaves avoiding the petiole, carefully place into tared tube using forceps and place on balance. **NOTE:** Two fully expanded Arabidopsis leaves will weigh ~0.05 g.
3. Record mass, cap and flash freeze in liquid nitrogen as quickly as possible.
4. Store frozen plant materials in -80 ºC or process them as described below.

Solution Preparation

Cold Extraction Buffer: 80:20 v/v methanol: water, 0.1% formic acid, 0.1 g/L butylated hydroxytoluene (BHT)

NOTE: Add internal standards to the EB just prior to use.

Materials needed:

MeOH (HPLC grade)
water
formic acid
butylated hydroxytoluene (BHT; Aldrich, Cat#W218405)
100 µM Abscisic acid (ABA)-d6 (Toronto Research Chemicals, A110002) and 100 µM of any other internal standards
250 ml bottle
15, 50 ml polypropylene Falcon tubes
10, 25 ml stripettes and pipet aid
p100 pipet and tip
weigh paper and spatula
Parafilm

Preparation of EB*:

1. Add 0.005 g of BHT to 10 ml of MeOH in a 15 ml polypropylene Falcon tube and vortex to mix.
2. In fume hood, add 30 ml MeOH to 250 ml clean glass bottle.
3. Pour MeOH with BHT into bottle with MeOH.
4. Rinse Falcon tube with 10 ml water and add to bottle.
5. Add 50 µl of formic acid (or 56.8 µl of 88% formic acid). This is the Extraction Solution (ES).
6. Prepare ES*:
   a. Multiply the number of samples by volume ES* to be added to each.
      Ex. 25 samples * 0.5 ml/sample = 12.5 ml ES*.
   b. Round up to next full ml + 2 ml. Ex. 12.5 ml –> 13 ml + 2 ml = 15 ml
   c. Transfer appropriate volume of ES to a 50 ml Falcon tube (or bottle if larger volume is needed).
   d. Add internal standards to ES to a final concentration of 100 nM (**<u>should be the same concentration as used in the standard curve samples</u>). From 100 µM stocks, make 1/1,000 dilution to give 100 nM final concentration. For example, add 15 µL of 100 µM labeled standards to 15 mL of ES.

Clean glass bottles used for EB appropriately or use new bottles that have not been used for preparing other media. Avoid rubber lined caps on bottles, especially if using organic solvents.

Phytohormone Extraction

Day 1:

Materials needed:

Frozen tissue in cryo tubes with beads
Adapters for bead beater, pre-frozen
Tissue Lyser II
liquid nitrogen
cold ES*
ice
p100 and 1000 pipets and tips
rotator at 4°C

NOTE: Keep ES* on ice if processing a lot of samples.

Steps:

1. Place samples in pre-frozen bead beater adaptors (be sure they are balanced) and beat at 30/s for 30 sec. Rotate top and bottom lids on adaptors 180°, replace in Tissue Lyser and beat for an additional 30 sec at 30/s. Confirm all tissues are completely ground.
2. Briefly (20 s) centrifuge at 4°C to pull ground tissue to bottom of tubes. Transport samples in liquid nitrogen to avoid thawing.
3. Add an equal volume of cold ES* to each sample. Around 1 ml for each 100 mg tissue works well. **NOTE:** The 2 ml tubes comfortably hold ~1.7 ml.
4. Vortex samples to suspend tissue and place in box.
5. Incubate on rocking platform at 5,000 rpm at 4°C for 16-24 h.
6. Label centrifugal filter units and HPLC vials for next day.

If no bead beater (Tissue Lyser) is available to grind the frozen tissue, can use other methods for grinding frozen tissue (polypropylene pestle that fits extraction tube for example).

Phytohormone Extraction

Day 2:

Materials needed:

Vortexer
chilled microcentrifuge
0.2 µM PTFE centrifugal filter units (Millipore, Cat#UFC30LG25)
p1000 pipet and tips
autosampler HPLC vials (BMB stores)
250 µl inserts (BMB stores, Agilent, Cat#5183-2085)
vial caps (BMB stores)
HPLC vial rack

Steps:

NOTE: chill the centrifuge to 4°C at the beginning of Day 2.

1. Vortex samples to mix and then centrifuge at 12,000 *xg* for 10 min at 4°C.
2. Transfer 0.4 ml of each sample to centrifugal filter units and centrifuge at 5,000 *xg* for 1 min at room temp. **NOTE:** the maximum volume for these filter units is 0.5 ml.
3. Transfer flow through to labeled autosampler HPLC vials with inserts. **NOTE:** For guaranteed sampling, need a minimum volume of 500 µl if using vials without inserts or 50 µl if using the vials with 250 µl inserts.

Phytohormone standard curve

From concentrated stocks of unlabeled standards, prepare a mixture of each standard in 80% MeOH, 0.1% formic acid at 2 µM final concentration.

Make serial 1:4 dilutions of 2 µM unlabeled standard solution down to ~ 1 nM. (5 dilutions to give 6 points on std curve) (for example: 200 µL of 2 uM solution + 600 µL of 80% MeOH, 0.1% FA).

Prepare a sample of 80% MeOH, 0.1% FA containing 200 nM concentration of labeled internal standards.

Dilute each of the serial dilutions of unlabeled standards 1:2 with the 200 nM solution of labeled internal standards. (for example: add 100 µL of unlabeled standard solution + 100 µL of 200 nM internal standard solution)

- This will give a set of standard curve solutions starting at 1 µM, with a fixed concentration (100 nM) of internal standards.
- Make aliquots of the standard curve in LC autosampler vials and store at -20 or -80 until needed.