

Phytohormones - LC/MS Protocol (ABA, JA, SA, JA-Ile)

Ethylacetate spiked with internal standards

You will need 1ml ethylacetate spiked with labeled phytohormones per sample. The final concentration should be adapted to the samples. For a meaningful quantification the peak area of the phytohormone must be in the same range to the labeled standard. For *Nicotina attenuata* leaf tissue and the described protocol use D₆-ABA, D₄-SA and JA-¹³C₆-Ile 40 ng/ml and D₂-JA 200 ng/ml (4µl PH-ISD per ml ethylacetate).

Harvest:

1. weight approximately 200 mg frozen grinded plant tissue in Eppendorf tubes and store it at - 70°C until use.

Extraction FastPrep[®]:

2. cool down centrifuge to 4°C
3. transfer frozen tissue to FastPrep[®] tubes containing 900 mg FastPrep[®] matrix and 1 ml extraction solvent (ethylacetate spiked with internal standards),
4. homogenize by reciprocal shaking (FastPrep[®] speed 6.5) twice for 45 sec
5. centrifuge at 13,000 rpm (16,100g) for 20 min at 4°C
6. transfer supernatant to 2ml Eppendorf tubes
7. repeat the extraction by adding 0.5 ml ethylacetate without internal standards to the tissue and vortex for 5 min
8. centrifuge at 13,000 rpm (16,100g) for 20 min at 4°C
9. combine supernatants
10. evaporate using a vacuum concentrator until dryness at 30°C
11. dissolve the dried samples in 500µl 70% MeOH, vortex for at least 5 min and centrifuge at 13,000 rpm for 10 min
12. transfer 400µl of the supernatant into a HPLC vials.
13. clean the FastPrep[®] matrix and tubes with acetone and water!!!

Alternative Extraction GenoGrinder[®]:

1. Use 2 ml Eppendorf tubes for step 1
2. add two steel balls and put them in a N₂ cooled rack
3. grind the tissue with 250 stokes/per minute for 30 sec. Make sure that the two positions for genogrinder racks are balanced
4. add 1 ml spiked ethylacetate and vortex for 10 min
5. centrifuge at 13,000 rpm (16,100g) for 20 min at 4°C
6. transfer supernatant to 2ml Eppendorf tubes
7. repeat the extraction by adding 0.5 ml ethylacetate without internal standards and vortex for 5 min
8. centrifuge at 13,000 rpm (16,100g) for 20 min at 4°C
9. combine supernatants

10. evaporate using a vacuum concentrator until dryness at 30°C
11. dissolve the dried samples in 500µl 70% MeOH, vortex for at least 5 min and centrifuge at 13,000 rpm for 10 min
12. transfer 400µl of the supernatant into a HPLC vial.
13. Collect the metal balls in the hood lab 007!!!

Instrument parameters

Instrument: Varian 1200L Triple-Quadrupol-MS
 Injection volume: 10 µl
 Column: ProntoSIL C18-ace-EPS, 50x2mm, 5µm, 120 Å
 Solvent A: 0.05% formic acid in water
 Solvent B: MeOH
 Gradient:

Time	%A	%B	Flow (ml/min)
0	85	15	0.4
1			0.4
1:30			0.2
2:30			0.2
4:30	2	98	0.2
10			0.2
10:30			0.4
11			0.4
12	85	15	0.4
15			0.4

MS: ESI, negative
 Collision Gas : 2.1mTorr
 API Drying Gas : 19 psi, 300°C
 API Nebulizing Gas : 60 psi
 Needle : 4500 V
 Shield : 600V
 Detektor : 1800V

Method:	Comp.	Capillary	Q1FM	Q3FM	Collision Energy
	SA	35.0V	137.0	93.0	15.0V
	D4-SA	35.0V	141.0	97.0	15.0V
	JA	35.0V	209.0	59.0	12.0V
	D2-JA	35.0V	213.0	59.0	12.0V
	ABA	35.0V	263.0	153.0	9.0V
	D6-ABA	35.0V	269.0	159.0	9.0V
	JA-Ile	45.0V	322.0	130.0	19.0V
	JA- ¹³ C ₆ -Ile	45.0V	328.0	136.0	19.0V

Method for data acquisition and data Analysis: Phytohormones_MeOH.

Approximate retention times

SA	6.0 min
D4-SA	6.0 min
JA	5.9 min
D2-JA	6.6 min
ABA	5.6 min
D6-ABA	5.6 min
JA-Ile	7.3 min
¹³ C ₆ -JA-Ile	6.8 min and 7.3 min (area of both synthetic diastereoisomers must be added)

Analysis

The automatic integration requires still a careful look through the chromatograms. Sometimes a manual integration or correction of the integration parameters is necessary!!

$$\text{Amount [ng/g tissue]} = \text{area/area ISD} * \text{amount ISD [ng]} / \text{sample weight [g]}$$