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Optimisation of quantification of phytohormones in tree crops by UHPLC-MS/MS

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We use this protocol and it's working

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Abstract

Phytohormones such as auxin (IAA), cytokinins (CKs) abscisic acid (ABA), and gibberellins (GAs) play critical roles in regulating fruit development and stress responses in plants. Although phytohormone quantification in herbaceous model species such as *Arabidopsis thaliana* is well established, in tree crops such as mango, avocado and macadamia, accurate quantification is hindered by low endogenous concentrations and complex tissue matrices rich in lipids, phenolics and carbohydrates. In this study, we developed and optimised a robust method for extracting and quantifying seven key phytohormones (IAA, ABA, GA1, GA3, GA4, GA20 and GA29) from these tree species. Improvements to existing methods included using a combination of solid-phase extraction modified from the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method and chemical derivatisation with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC). The derivatisation strategy enabled detection in positive ion mode via UHPLC-MS/MS, significantly improving sensitivity and reducing matrix interference. The method achieved limits of detection ranging from 0.11 to 0.24 ng g⁻¹ and demonstrated high recovery (> 80%) and repeatability (RSD < 7.50%) across mango leaf, fruit and pedicel tissue. Validation in avocado and macadamia confirmed cross-species applicability. QuEChERS extraction alone enabled detection of multiple cytokinin classes, including trans zeatin (tZ), isopentenyladenine (iP), dihydrozeatin (DZ) and their corresponding riboside and nucleotide forms. This workflow provides a sensitive, reproducible, and scalable platform for hormone profiling in complex plant systems, supporting future research into developmental regulation and stress physiology in tree crops.

Materials

Chemicals and Reagents (all LC-MS grade):

- Methanol
- Ethanol
- Acetic Acid
- Acetonitrile
- QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) (Sourced from PM Separations, Australia)
- EDC (*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride) (Merck, Australia).
- Deuterated phytohormone standards (OlChemim, Czech Republic) for gibberellins (GAs A1, A3, A4, A20 and A29), indole-3-acetic acid (IAA) and abscisic acid (ABA).
- Non-deuterated phytohormone standards for gibberellins (A1, A3, A4, A20 and A29) (BioSynth), IAA and ABA (Merck, Australia).

Equipment and consumables:

- Pipette tips (Vertex branded, SSIbio)
Homogeniser (2010 GenoGrinder, SPEX SamplePrep, USA)
Weigh balance
- Benchtop centrifuge (temperature controlled)
- Rotational vacuum concentrator (MiVac Quattro Concentrator, GeneVac Ltd., United Kingdom)
- Sep-Pak tC18 cartridge (Waters)
- Vacuum SPE manifold (Phenomenex Inc., USA)
- Autosample vials (Agilent Technologies, USA)
- Autosample vial inserts (100 uL; Agilent Technologies, USA)
- Ultra-High-Performance Liquid Chromatography (UHPLC) coupled to a mass spectrometer QQQ (triple quadrupole). Nevera X2 UHPLC system (Shimadzu Corporation, Japan) coupled to a 5500 triple quadrupole linear ion trap (QTRAP) MS system equipped with an ESI (AB SCIEX, USA).
- Kinetex C18 reversed phase UPLC column (2.1 mm X 100 mm, 1.7 µm) and a guard column SecurityGuard ULTRA UHPLC C18, 2.1mm (Agilent Technologies, USA).

Softwares:

- SciexOS software (SCIEX)
- Analyst software (SCIEX)

Phytohormone extraction part 1

- 1 Plant tissue of interest (freeze-dried or frozen under liquid nitrogen) should be finely ground using a homogeniser (e.g. TissueLyzer, GenoGrinder).
- 2 Collect 20-50 mg of homogenised tissue and extract with 900 μ L of extraction solvent containing 1% acetic acid, 100% acetonitrile and 5 μ L of each internal standard (200 ng/mL). Vortex well.
- 2.1 Ahead of time, prepare internal standard mix by pooling individual working solutions (1 μ g/mL) of each phytohormone of interest to ensure 1 ng of each standard per sample.
- 3 For each sample, measure out 22.75 mg of QuEChERS mix and dissolve in 100 μ L of 1% acetic acid and acetonitrile. Add 100 μ L of dissolved mixture to each sample and vortex well. 
- 4 Centrifuge at 4,500g for 5 minutes at room temperature. 
- 5 Evaporate to dryness in rotational vacuum concentrator (e.g. MiVac Quattro, GeneVac) for 3 hours or overnight. 

Phytohormone extraction part 2

- 6 Dissolve dried pellets in 1 mL 1% acetic acid.
- 7 Using vacuum SPE manifold (e.g. VM24, Phenomenex): Wash C18 sep-pak cartridge with 1 mL of 100% methanol and then activate it with 1 mL of 1% acetic acid dissolved in MilliQ water. Ensure that cartridge is not drying out during all steps. Collect all flow products in 15 mL falcon tube for discarding.
- 8 Load the re-eluted samples from step 6 onto the cartridge, then wash the column with 1 mL of 1% acetic acid dissolved in MilliQ water.
- 9 Change flow-through tube rack to 2 mL tubes. Elute the cartridge with 1 mL of 80% acetonitrile and 1% acetic acid. Allow the cartridge to dry out for approximately 20-30 seconds after volume has drained.
- 9.1 If proceeding with hormones that DO NOT contain carboxyl groups (e.g. cytokinins), you can split the eluted sample here (500 μ L for cytokinins and 500 μ L for derivatisation) 



- 10 Evaporate to dryness in rotational vacuum concentrator (e.g. MiVac Quattro, GeneVac).
- 11 Dissolve dried pellet with 30 μ L 1% acetic acid dissolved in MilliQ water.
- 11.1 For non-derivatised samples, vortex re-suspended samples and centrifuge for 10 minutes at 4°C at top speed. Transfer to autosample vial with 100 μ L insert. 
- 12 For derivatising samples, transfer dissolved samples into 200 μ L microcentrifuge tube, and evaporate to dryness rotational vacuum concentrator (e.g. MiVac Quattro, GeneVac) for 1-2 hour.
- 13 To dried sample, add 50 μ L 200 mM EDC solution in 100% ethanol. 
- 14 Sonicate the samples for 1 minute at 40°C using an ultrasonic bath (e.g. BRAND DETAILS), and then incubate in PCR machine at 40°C for 5 hours or overnight.  
- 15 Evaporate to dryness in rotational vacuum concentrator (e.g. MiVac Quattro, GeneVac).
- 16 Re-dissolve with 15 μ L 1% acetic acid dissolved in MilliQ water, centrifuge and transfer to autosample vial with 100 μ L insert.
- 16.1 If non-derivatised samples were also extracted and transferred to an autosample vial, pool derivatised sample with non-derivatised sample. 
- 17 Wash out with another 15 μ L 1% acetic acid dissolved in MilliQ water, centrifuge and pool in autosample vial.
- 18 Store at -80°C until LC-MS run.

LC-MS run

- 19 Stock solutions of all deuterated and non-deuterated hormone internal standards should be prepared in 100% LC-MS grade methanol at a concentration of 100 μ g/mL and stored in autosample vials at -80°C. Working solutions should be prepared by mixing 10 μ L of 100 μ g/mL stock with 990 μ L 100% methanol to obtain 1 mL of 1 μ g/mL solution.

20 Prepare internal standard mix at differing concentrations for standard curve (e.g. 0, 0.1, 0.5, 1, 5, 10 and 20 ng/mL). Analyse 1 µL of each internal standard concentration. If peaks are acceptable, proceed to analyse 10 µL of sample.

20.1 Note that matrix calibration curves may be required for each individual tissue.

21 In the present protocol, Nevera X2 UHPLC system (Shimadzu Corporation, Japan) coupled with a 5500 triple quadrupole linear ion trap (QTRAP) MS system equipped with an ESI (AB Sciex, USA).

The mobile phases were (A) 0.5% formic acid in MilliQ water (v/v) and (B) 0.5% formic acid in acetonitrile (v/v).

Separate samples on a 1.7 µm Kinetex C18 reversed phase UPLC column (Phenomenex), with an initial flush of 95% B phase over 0.1 min, before using a gradient of 95% to 4% B over 0.1 min, 4% B over 0.8 min, 4% to 15% B over 5 min, 15% to 95% over 3 min, sustained 95% for 2 min, then returned to 4% over 0.1 min, followed by a 1 min column re-equilibration, at a flow rate of 0.4 mL/min.

An autosampler and a column compartment are set at 10°C and 40°C, respectively.

Mass spectrometry was performed in positive ion mode with the following ESI parameters: column temperature, 45°C; curtain gas, 20 psi; collision gas, medium; ion source temperature, 500°C; ion source gas, 80 psi; spray voltage, 4,500 V.

Acquire MS data in targeted positive ion monitoring mode using an electrospray ionisation mode.

22 Analyse raw data files using Analyst (SCIEX OS) software. Check that target ions are detected, and that the peaks are extracted correctly.

Phytohormone levels in tested samples were determined based on the peak area ratios of endogenous to corresponding ISTDs compounds calculated with the calibration curves of each standard.