# Cytokinin Analysis: Sample Preparation and Quantification

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**[Abstract]** Cytokinins are a group of phytohormones discovered about half a decade ago by Miller *et al.* (1955) and Skoog *et al.* (1965). Since then they were found to participate in many plant physiological processes, including the regulation of the source/sink transitions, plant growth and organ development, responses to environmental conditions such as light, nutrient and water availability and biotic interactions with mutualists, pathogens and herbivores (Werner and Schmülling, 2009; Giron *et al.*, 2013). To aid the quantification of cytokinins for analyzing their changes after environmental stress conditions, we developed this cytokinin extraction and analysis method. This protocol is based on the cytokinin extraction with an acidic methanol-water solution and purification with a mixed-mode solid phase extraction procedure described by Dobrev and Kamínek (2002) and the modifications of Kojima *et al.* (2009). The protocol was successfully used to verify cytokinin overproduction in transgenic *Nicotiana attenuata* plants expressing the cytokinin biosynthesis gene *Tumor morphology root* (*Tmr*) from *Agrobacterium tumefaciens* under the control of the chemical inducible expression system pOp6/LhGR in the glasshouse and under field conditions (Schäfer *et al.*, 2013) to study the role of cytokinins in plant-herbivore interactions.

## Materials and Reagents

1. Plant tissue
2. MeOH
3. 1 N HCOOH
4. 0.35 N $NH_4OH$
5. 0.35 N $NH_4OH$ in 60% MeOH
6. 0.1% (v/v) acetic acid
7. 0.05% (v/v) HCOOH (for mass spectrometry) in Milli-Q $H_2O$
8. Acetonitrile (gradient grade)
9. [$^2H_5$] *t*Z (Olchemim, catalog number: 030 0301)
10. [$^2H_5$] *t*ZR (Olchemim, catalog number: 030 0311)
11. [$^2H_5$] *t*ZROG (Olchemim, catalog number: 030 5131)
12. [$^2H_5$] *t*Z7G (Olchemim, catalog number: 030 5111)

13. Extraction buffer (see Recipes)
14. Extraction buffer + deuterated standards (see Recipes)

## Equipment

1. 96-well BioTubes (1.1 ml individual tubes) (Arctic White LLC, catalog number: AWTS-X22100)
2. Steel balls (ASKUBAL, catalog number: 3 MM-G100-1.4034)
3. Caps for 96-well BioTubes (strips of 8 plug caps) (Arctic White LLC, catalog number: AWSM-T100-30)
4. Pipet
5. ArctiSeal 96 Round Well Sealing Mats for 96-well BioTubes (Arctic White LLC, catalog number: AWSM-2002RB)
6. Nunc 96-well Deep Well Plates (Thermo Fisher Scientific, catalog number: 278752)
7. Nunc 96-Well Cap Mats (Thermo Fisher Scientific, catalog number: 276002)
8. 96-well PCR plates (Kaneka Corporation, Eurogentec, catalog number: RT-PL96-MQ)
9. Sealing film (OMNILAB-LABORZENTRUM, Schubert & Weiss, catalog number: 5420203)
10. Machery Nagel Multi 96 HR-X (96 x 25 mg) (MACHEREY-NAGEL, catalog number: 738530.025M)
11. Machery Nagel Multi 96 HR-XC (96 x 25 mg) (MACHEREY-NAGEL, catalog number: 738540.025M)
12. Chromabond Multi 96 vacuum manifold (MACHEREY-NAGEL, catalog number: 738630.M)
13. Evaporator system (Glas-Col, catalog number: 099A EV9624S)
14. Geno/Grinder 2000 (SPEX SamplePrep)
15. Eppendorf Centrifuge 5804 R equipped with a Swing-bucket rotor A-2-DWP (Eppendorf)
16. Ultrasonic bath Bransonic Models 1200 (BRANDSON™))
17. Agilent 1200 HPLC system (Agilent))
18. Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm) (Agilent)
19. API 5000 tandem mass spectrometer (Applied Biosystems®) equipped with a Turbospray ion source

## Procedure

A. Preparation of plant material
   1. Collect plant tissue and immediately freeze it in liquid nitrogen. Stored at -80 °C.
   2. Homogenize plant tissue under liquid nitrogen.

 

3. Aliquot 100 mg plant tissue per sample in liquid nitrogen-precooled 96-well BioTubes (with two steel balls per tube, closed with caps).
   *Note: Make small holes in the caps to prevent tubes from exploding.*
4. Keep at least for 30 min at -20 °C (for longer storage at -80 °C) before starting with the extraction to evaporate liquefied gases from the tubes.

B. Extraction

*Note: In-between the steps, while buffer addition and sample collection samples are kept on ice.*

1. Add 800 µl precooled (-20 °C) extraction buffer + deuterated standards to each tube, and cover with a precooled sealing mat.
2. Shake in Geno/Grinder for 60 s (1,150 strokes *$min^{-1}$).
3. Incubate over night at -20 °C.
4. Shake in the Geno/Grinder for 60 s (1,150 strokes *$min^{-1}$).
5. Centrifuge (20 min, 1,913 *x g*, 4 °C).
6. Collect 600 µl supernatant in 96-well BioTubes and keep it at -20 °C.
7. Add 600 µl extraction buffer to the pellet.
8. Shake in the Geno/Grinder for 60 s (1,150 strokes *$min^{-1}$).
9. Incubate for 30 min at -20 °C.
10. Centrifuge (20 min, 1,913 *x g*, 4 °C).
11. Collect 600 µl supernatant and combine it with the supernatant from the first extraction steps. Cover samples with a sealing mat.
12. Centrifuge supernatants (20 min, 1,913 *x g*, 4 °C) and continue with the sample purification.

C. Purification

*Note: If not further mentioned the following steps are performed at room temperature.*

***HR-X***

1. Condition the HR-X column with 0.6 ml MeOH. Discard flow-through.
   *Note: Use the Vacuum manifold to suck the samples and buffers through the column. Same accounts for the following steps.*
2. Condition the column with 0.6 ml extraction buffer. Discard flow-through.
3. Load the samples to the column (collect flow-through) and subsequent wash with 0.2 ml extraction buffer (collect flow-through). Collection of both flow-through can be done in a Nunc 96-well Deep Well Plate.

4. Evaporate the samples at 45 °C under a constant nitrogen stream to remove MeOH from the samples, utilizing the evaporator system (see Equipment).
   *Note: MeOH evaporates fastest and after evaporation of the MeOH, approximately 350 µl liquid should be left, which can be tested with a pipet. Samples only have to be evaporated until this point.*
5. Add 850 µl 1 N HCOOH per sample.
6. Cover the plate with a sealing mat and shake in the Geno/Grinder for 30 s (1,000 strokes */min).
7. Centrifuge (20 min, 1,913 *x g*, 4 °C).

***HR-XC***

8. Condition the HR-XC column with 0.6 ml MeOH. Discard flow-through.
9. Condition the column with 0.6 ml 1 M HCOOH. Discard flow-through.
10. Load the samples to the column. Discard flow-through.
11. Wash column with 1 ml 1 M HCOOH. Discard flow-through.
12. Wash column with 1 ml MeOH. Discard flow-through.
13. Wash column with 1 ml 0.35 N $NH_4OH$. Discard flow-through.
14. Elute cytokinin-nucleobases, -ribosides and -glucosides from the column with 0.35 N $NH_4OH$ in 60% MeOH. Collect flow-through in 96-well BioTubes.
15. Completely evaporate the samples at 45 °C under a constant nitrogen stream utilizing the evaporator system.
16. Reconstitute samples in 50 µl 0.1% (v/v) acetic acid.
17. Cover the plate with a sealing mat and shake in the Geno/Grinder for 60 s (1,000 strokes */min).
18. Sonicate the samples for 10 min in an Ultrasonic bath.
    *Note: Remove the bottom plate of the 96-well rack and place the whole rack in the Ultrasonic bath. Do not use 96-well racks without bottom plate for shaking and centrifugation!*
19. Centrifuge (20 min, 1,913 *x g*, 4 °C).
20. Fill samples in 96-well PCR plates and cover with the sealing film.
21. Centrifuge (20 min, 1,913 *x g*, 4 °C) and continue with Ultra-performance LC coupled MS/MS.

D. Ultra-performance LC coupled MS/MS

Inject 2 µl per sample in an Agilent 1200 HPLC system equipped with a Zorbax Eclipse XDB-C18 column. The mobile phase comprises of 0.05% (v/v) HCOOH in Milli-Q $H_2O$ as solvent A and acetonitrile as solvent B with following settings:

 

Flow rate: 1.1 ml/min

Column temperature: 25 °C

Solvent gradient for chromatographic separation:

| Time [min] | solvent A | solvent B |
|---|---|---|
| 0 | 95% | 5% |
| 0.5 | 95% | 5% |
| 5 | 68.5% | 31.5% |
| 5.01 | 0% | 100% |
| 6.5 | 0% | 100% |
| 6.51 | 95% | 5% |
| 9 | 95% | 5% |

Analysis is done with an API 5000 tandem mass spectrometer equipped with a Turbospray ion source. Multiple-reaction-monitoring mode is used with following settings for quantification:

Ionization mode: positive

Ion spray voltage: 5,500 eV

Turbo gas temperature: 700 °C

Nebulizing gas: 70 psi

Curtain gas: 25 psi

Heating gas: 60 psi

Collision gas: 6 psi

Precursor-to-product ion transitions used for cytokinin analysis:

| Analyte | Q1 [m/z] | Q3 [m/z] | DP[1] | CE[2] | CXP[3] |
|---|---|---|---|---|---|
| *t*Z | 220.2 | 136.3 | 26 | 25 | 16 |
| *t*ZR | 352.2 | 220.3 | 76 | 25 | 30 |
| *t*ZROG | 514.1 | 382.1 | 96 | 25 | 16 |
| *t*Z7G | 382.1 | 220.0 | 71 | 31 | 16 |
| [$^2H_5$] *t*Z | 225.2 | 136.3 | 26 | 25 | 16 |
| [$^2H_5$] *t*ZR | 357.2 | 225.3 | 76 | 25 | 30 |
| [$^2H_5$] *t*ZROG | 519.1 | 387.1 | 96 | 25 | 16 |
| [$^2H_5$] *t*Z7G | 387.1 | 225.0 | 71 | 31 | 16 |

*t*Z, *trans*-zeatin, *t*ZR, *trans*-zeatin riboside, *t*ZROG, *trans*-zeatin riboside *O*-glucoside, *t*Z7G, *trans*-zeatin $N^7$-glucoside

[1]Declustering potential, [2]Collision energy, [3]Collision cell exit potential

## Representative data

1. Chromatograms

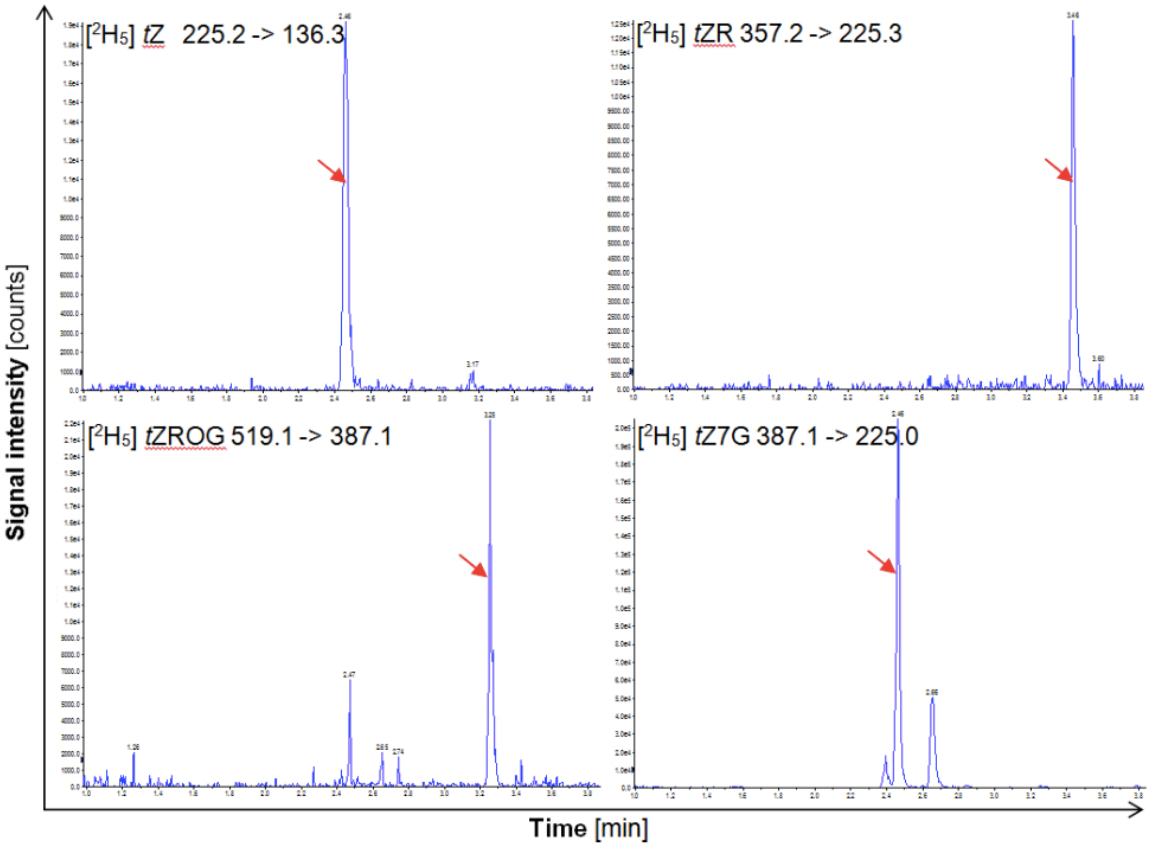


**Figure 1. Separation of deuterated internal standards in the ultra-performance LC coupled MS/MS analysis.** The red arrows indicate the respective peaks.

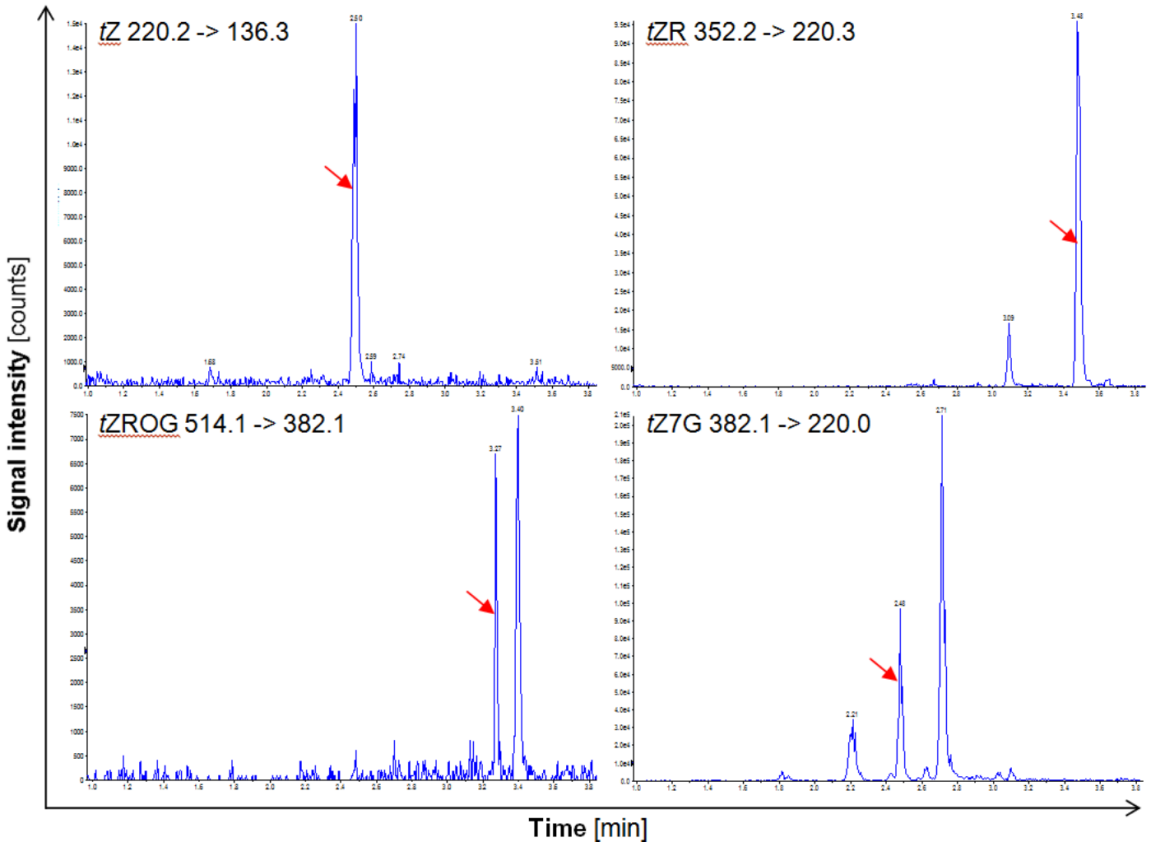


**Figure 2. Separation of cytokinins from the plant tissue in the ultra-performance LC coupled MS/MS analysis.** The red arrows indicate the respective peaks.

2. Calculation

   Exemplary *t*Z quantification in 0.1 g leaf tissue, supplemented with 1 ng deuterated *t*Z as internal standard. The obtained peak areas were 23,700 counts and 38,400 counts for *t*Z and [$^2H_5$] *t*Z, respectively.

   $Concentration_{Compound}$ = $Peak\ area_{Compound}/Peak\ area_{Internal\ Standard}/m_{Extracted\ tissue} * m_{Internal\ Standard}$

   $Concentration_{tZ}$ = $Peak\ area_{tZ}/Peak\ area_{[^2H5]\ tZ}/m_{Leaf\ tissue} * m_{[2H5]\ tZ}$

   $Concentration_{tZ}$ = 23,700 counts/38,400 counts/0.1 g * 1 ng = 6.2 ng *t*Z per g leaf tissue

## Recipes

1. Extraction buffer

   750 ml MeOH

   200 ml $ddH_2O$

   50 ml HCOOH

2. Extraction buffer + deuterated standards

Prepare Extraction buffer spiked with 1 ng [$^2H_5$] *t*Z, 0.1 ng [$^2H_5$] *t*ZR, 4 ng [$^2H_5$] *t*ZROG and 2 ng [$^2H_5$] *t*Z7G per 800 µl extraction buffer.

*Note: Deuterated cytokinins are used as internal standards for quantification.*

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