Chapter 2

Analytical Determination of Auxins and Cytokinins

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Abstract

Parallel determination of auxin and cytokinin levels within plant organs and tissues represents an invaluable tool for studies of their physiological effects and mutual interactions. Thanks to their different chemical structures, auxins, cytokinins and their metabolites are often determined separately, using specialized procedures of sample purification, extraction, and quantification. However, recent progress in the sensitivity of analytical methods of liquid chromatography coupled to mass spectrometry (LC-MS) allows parallel analysis of multiple compounds. Here we describe a method that is based on single step purification protocol followed by LC-MS separation and detection for parallel analysis of auxins, cytokinins and their metabolites in various plant tissues and cell cultures.

Key words Auxin, Cytokinins, Phytohormones, Liquid chromatography, Mass spectrometry

1 Introduction

Plant hormones as small molecules occurring in minute concentrations are of decisive importance for major physiological processes in plants. Of those, auxin and cytokinins through their specific cross talk are important cell fate modulators [1]. Indole-3-acetic acid (IAA), the major endogenous auxin, is an indole derivative with weak acidity due to the exocyclic carboxylic acid group (Fig. 1a). IAA-associated metabolites include its precursors, i.e., tryptophan, indole pyruvic acid, indole acetonitrile, indole acetamide, conjugates with amino acids (mostly with aspartate and glutamate) and carbohydrates (IAA-glucosyl ester), and oxidative catabolites represented by oxindole-3-acetic acid [2]. Cytokinins, with their common natural representative *trans*-zeatin (*t*Z, Fig. 1a) are adenine derivatives with five carbon side chain connected to the exocyclic amino group. The exocyclic amino group determines the alkaline character of cytokinins. Common cytokinin metabolites include *N*9-ribosides, *N*9-ribotides, *N*7-glucosides, and *O*-glucosides [3].

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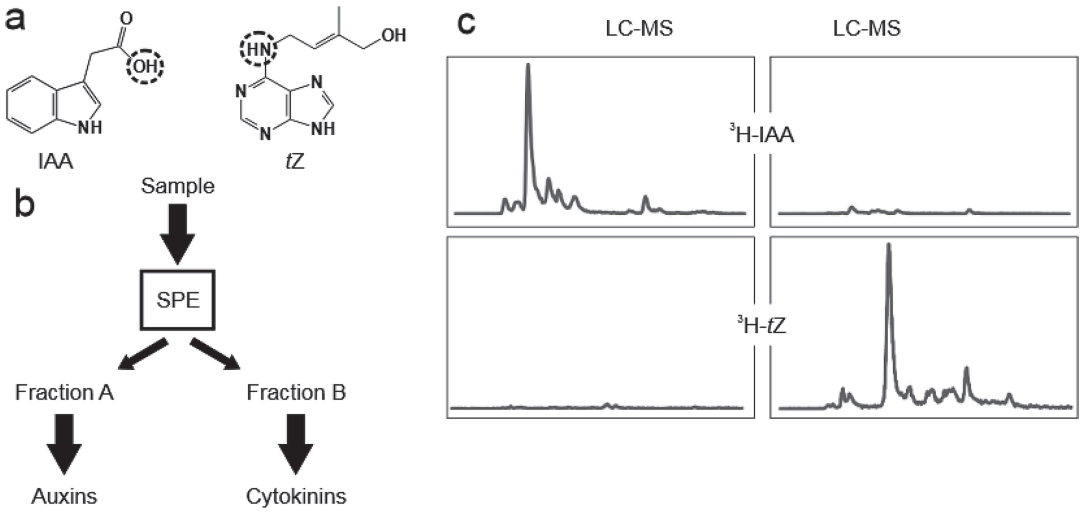

Fig. 1 Structures of IAA and *tZ*. (**a**) Structures of IAA and *tZ* with depicted ionisable parts that affect their isolation. (**b**) Simplified purification scheme (SPE, solid phase extraction) with splitting of plant extract into two fractions, fraction A containing auxins, and fraction B containing cytokinins. (**c**) HPLC separation of metabolites of ^{3}H-IAA and ^{3}H-*tZ* applied to 14-day-old *Arabidopsis thaliana* seedlings for 4 h from fractions A and B according to the scheme above. Each fraction was run on HPLC coupled with online radiodetector

The determination of endogenous levels of auxin and cytokinins underwent significant progress in recent years. The trend of plant hormone analysis is to switch from tedious multistep purification, derivatization, and immunodetection or nonselective instrumental detection of limited number of compounds, to minimalistic purification, consisting of few or even one step, followed by sensitive and selective detection of many compounds [4–9]. This "metabolite profiling" relies on the LC-MS and allows tracking the whole metabolic paths and networks of phytohormones.

Here we describe the procedure that is applied in our laboratory for determination of auxin, cytokinins and their metabolites in plants and cell cultures. It is based on single step purification, followed by LC-MS set at multiple reaction monitoring (MRM) mode for simultaneous measurement of hormonal precursors, active forms, and metabolites in single sample. Simplified scheme of the procedure is shown in Fig. 1b. The purification step splits the extract into two fractions, fraction A containing compounds of neutral and acidic character, and fraction B containing basic compounds. Since auxin and its major metabolites are acidic or neutral, they remain in fraction A. On the other hand, cytokinins as weak bases elute in fraction B. As shown here for samples purified from *Arabidopsis thaliana* seedlings incubated with either ^{3}H-*tZ* or ^{3}H-IAA for 4 h, the majority of IAA metabolites are found in fraction A, without any appreciable radioactivity in fraction B, and vice versa for the *tZ* (Fig. 1c). This separation of auxin and cytokinins and their metabolites is crucial for their metabolic profiling by LC-MS, as documented in our studies [7, 10].

2 Materials

Prepare all water solutions using MilliQ deionized water. For in vitro prepared plant material, all culture media should be sterilized as well as equipment for handling cells, seeds, or seedlings (*see* **Note 1**).

2.1 Plant Material

The methods of sampling, extraction or purification may differ with respect to particular material. Leaf tissue of approximately 100 mg FW is most common representative of processed samples thus the method of its extraction and purification is described in detail in Subheadings 3.2 and 3.3. For procedures analyzing plant hormones in liquid media or tiny materials of low weights, e.g., root tips, parts of very young seedlings or etiolated ones (*see* **Note 6**).

2.2 Sampling, Extraction, and Purification

1. Liquid nitrogen.
2. Methanol, deionized water, ammonium hydroxide 25%, formic acid, all p.a. grade.
3. Mixer mill MM301 Retsch, Teflon adapter racks for 2 mL vials, 5 mm zirconium oxide grinding balls.
4. Pipettes, 50 µL, 1 mL.
5. Analytical balances.
6. Stable isotope labeled internal standards, 0.2 µM dissolved into 50% methanol in water (*see* **Note 2**).
7. 2 mL microcentifuge tubes.
8. Benchtop cooled centrifuge.
9. Freezers (−20 °C, −80 °C).
10. Solid phase extraction columns (SPE Oasis MCX), 1 mL/30 mg, Waters.
11. SPE vacuum manifold, 12 or 24 port.
12. Rotary vacuum evaporator, SpeedVac.

2.3 LC-MS

1. Acetic acid and acetonitrile, both LC-MS grade.
2. Deionized water, MilliQ.
3. Autosampler vials, 0.5 mL.
4. HPLC column, e.g., Luna C18, 3 µm, 100 × 2 mm, Phenomenex.
5. HPLC system, e.g., Ultimate 3000, Dionex.
6. MS detector, e.g., 3200 QTRAP LC/MS/MS, AB Sciex.

2.4 Solvent Preparation

1. Extraction solvent: mix the following p.a. grade solvents: methanol–water–formic acid = 15/4/1, v/v/v, store at −20 °C.
2. SPE load solvent: 1 M formic acid, dilute 3.8 mL of 99% formic acid with water to 100 mL.

3. SPE elute 1 solvent: methanol, 100%, p.a. grade.
4. SPE elute 2 solvent: 0.35 M ammonium hydroxide in 70% methanol: to 70 mL methanol add 2.5 mL 25% ammonium hydroxide and fill to 100 mL with water; prepare fresh.
5. LC-MS solvent A: 5 mM acetic acid in 5% acetonitrile, to 50 mL acetonitrile for LC-MS add 286 µL acetic acid (99%, for LC-MS) and fill to 1000 mL with MilliQ water.
6. LC-MS solvent B: 5 mM acetic acid in 95% acetonitrile, to 50 mL MilliQ water add 286 µL acetic acid (99%, for LC-MS) and fill to 1000 mL with acetonitrile for LC-MS.

3 Methods

3.1 Plant Material Sampling

1. Collect about 100 mg FW plant material and put it into labeled and tared 2 mL microcentrifuge tube. Weigh and record the precise weight of sample (*see* **Note 3**).
2. Add one 5 mm zirconium oxide grinding ball (*see* **Note 4**) and close the tube.
3. Quickly freeze the sample into liquid nitrogen bath (*see* **Note 5**). Store samples at −80 °C.
4. Extremely small and tiny samples is worth to collect directly into microcentrifuge tube with methanol (about 300 µL) precisely weighted before sample addition. In certain cases the collecting of the samples may cause a loss of methanol due to the quick evaporation then the counting must be related to the number of collected parts instead of FW.

3.2 Homogenization and Extraction

Homogenization and extraction can be skipped in certain special cases (*see* **Note 6**).

1. Place samples into Teflon adapter and leave at −80 °C to cool down, at least for 15 min.
2. Mount the cold adapter with tubes into the arms of mixer mill MM 301. Apply frequency 25 Hz for 2 min. Repeat if tissue is not fully homogenized. Add 0.5 mL cold (−20 °C) extraction solvent (*see* **Note 7**).
3. Add 50 µL internal standards (*see* **Note 8**), mix and leave at −20 °C for 1 h. Centrifuge at 20,000 × *g* at 4 °C for 20 min. Transfer the supernatant into new 2 mL tube.
4. Re-extract the pellet with additional 0.5 mL extraction solvent for 30 min (*see* **Note 9**) and centrifuge as above.
5. Evaporate pooled supernatants in SpeedVac at 10 mBar and 40 °C to ¼ of initial volume (less than 0.25 mL, *see* **Note 10**).

3.3 Purification (See Note 11)

Purification can be skipped in certain special cases (*see* **Note 6**).

1. Mount Oasis MCX column on SPE vacuum manifold. Equilibrate the column by washing it with 1 mL methanol, followed by 1 mL SPE load solvent.
2. Dilute partially evaporated sample extract in 0.5 mL of SPE load solvent and apply to column. Discard flow-through.
3. Wash the column with 0.5 mL SPE load solvent, followed by 1 mL water. Discard flow-through.
4. Apply 0.5 mL SPE elute 1 solvent, collect the flow-through into new 2 mL microcentrifuge tube, which represents fraction A.
5. Apply 0.5 mL SPE elute 2 solvent, collect the flow-through into new 2 mL microcentrifuge tube, which represents fraction B.
6. Evaporate the collected fractions in SpeedVac at 10 mBar and 40 °C to dryness. Store dried fractions at −20 °C till LC-MS analysis.

3.4 Quantification by LC-MS (See Note 12)

3.4.1 Fraction A

1. Dissolve dried sample into 30 µL 15% acetonitrile in water. Centrifuge at 20,000 × *g*, 4 °C for 20 min. Transfer supernatant into autosampler vial.
2. Inject an aliquot, usually 1/10, of sample into LC-MS (*see* **Note 13**).
3. Run linear gradient of LC-MS solvents A and B; 10–50% LC-MS solvent B in 15 min at flow rate 0.3 mL/min. Flush column with 100% solvent B for 5 min and equilibrate with 10% solvent B for 10 min.
4. Set MS in negative electrospray mode. Ion source parameters: ion spray voltage −4000 V, nebulizer gas 50 psi, heater gas 50 psi, curtain gas 20 psi, gas heater 500 °C.
5. Set MS analyzer in MRM mode with the optimal compound specific parameters like declustering potential, precursor ion *m/z*, product ion *m/z*, collision energy, adjusted for each compound and internal standard.

3.4.2 Fraction B

1. Dissolve dried sample into 30 µL 5% methanol in water. Centrifuge at 20,000 × *g*, 4 °C for 20 min. Transfer supernatant into autosampler vial.
2. Inject an aliquot, usually 1/10, of sample into LC-MS.
3. Run linear gradient of LC-MS solvents A and B; 5–40% LC-MS solvent B in 15 min at flow rate 0.3 mL/min. Flush column with 100% solvent B for 5 min and equilibrate at 10% solvent B for 10 min (*see* **Note 14**).
4. Set MS in positive electrospray mode. Ion source parameters: ion spray voltage +4000 V, nebulizer gas 50 psi, heater gas 50 psi, curtain gas 20 psi, gas heater 500 °C.

5. Set MS analyzer in MRM mode with the optimal compound specific parameters like declustering potential, precursor ion *m/z*, product ion *m/z*, collision energy; adjusted for each compound and internal standard.
6. Inject an appropriate number of calibration standards for determination of the parameters of calibration curve. Quantify the samples based on isotope dilution with calibration curve (*see* **Note 15**).

4 Notes

1. For the reliable analyses of auxins and cytokinins in in vitro cultured plants or cell cultures it is absolutely essential to keep this material in strictly aseptic conditions, i.e., all media and materials should be sterilized before use. Since bacteria and fungi can themselves be a source of auxins and cytokinins, they could greatly influence their levels in analyzed plant samples.
2. For list of internal standards, *see* ref. 7. It should be stressed that chromatographic and MS optimalization of standards is instrument-dependent and should be carefully optimized.
3. The amount collected depends on the sensitivity of quantitation method and on the losses during purification. For our procedure an optimal amount is about 100 mg of fresh plant material. Although most commonly the results are presented in units of hormone amount per gram fresh weight (i.e., pmol/g FW) there are some cases where one has to consider different units. Other alternatives can be hormone amount per gram dry weight, per organ, per cell, per organ distance, etc. Choosing different units of data presentation or even better, having more alternatives, could improve for example the data variability, or could give more biological sense of results. The collected material should be representative of the experiment, and as homogenous as possible. If the amount of plant material is not limited, it is desirable to collect large representative batch from which an aliquot is taken. Extremely small and tiny samples are worth to collect directly into microcentrifuge tube with methanol (about 300 μL) precisely weighted before sample addition. In certain cases the collecting of the samples may cause a loss of methanol due to the quick evaporation then the counting must be related to the number of collected parts instead of FW.
4. We found that zirconium oxide balls are most appropriate for homogenization. The use of wolfram or stainless steel balls leads to significant loses of indole compounds, i.e., auxin and its metabolites.

5. The sample is frozen in liquid nitrogen to stop any metabolic processes.
6. In case of determination of phytohormones secreted into liquid growth media, extraction step is omitted and the medium (up to 1 mL) is acidified below pH 3 with formic acid. Purification starts after addition of internal standards to each sample with application on the SPE column (Subheading 3.3, **step 2**).

   Similarly, in case of tiny plant material (root tips, etiolated parts of seedlings, etc.) and amounts below 50 mg FW per sample the whole purification procedure might be skipped. Internal standards are added to samples collected in methanol and evaporated to dryness as described in Subheading 3.3, **step 6**.
7. The extraction is a process of quantitative release of phytohormones from plant tissue into extraction solvent. Concurrently, the extraction solvent should ideally fix the tissue, i.e., stop any metabolic conversions during extraction. In our protocol the extraction solvent contains organic solvent (methanol, 75% by volume) and formic acid. High organic and low pH deactivate and precipitate enzymes in the tissue. Low temperature of extraction also diminishes post-extraction reactions.
8. Very important step is the addition of internal standards in the beginning of extraction. Internal standards are compounds with known purity and concentration, which ideally are chemically closest to the compounds of interest thus having same behavior during extraction and purification. The quantitation step should be able to discriminate between the target compound and the internal standard and measure them separately. The best internal standard is the stable isotope labeled equivalent of the target compound that differs only by mass, which can be distinguished by mass spectrometer. Since the concentration of internal standard is used for quantitation calculations, it is important to add precisely known and equal amounts.
9. The primary extraction recovers about 90% of hormones with re-extraction adding another 8–9%.
10. It is important to remove the organic solvent from the extraction mixture, because it could interfere with the following purification. Cool down samples at −80 °C before placing them into SpeedVac to avoid sample losses due to splash.
11. Ideally, the purification should remove all unwanted compounds that have been extracted and leave a solution where target compounds dominate. To achieve this, several purification steps can be applied, such as reversed phase, cation exchange, and anion exchange, preferably with orthogonal modes of action. Purification columns used here contain the so-called dual-mode sorbent, having two functionalities: reversed phase and cation exchange. This permits to use single purification column, and by stepwise elution with appropriate

solvents, to separate different groups of hormones into individual fractions. As opposed to the silica based sorbents, the sorbent we use can be air flushed without losing its activity and capacity. We apply air flush between individual elutions to increase the sample recovery and to better split them. The sample is applied to column in aqueous acidic solvent, allowing the hormones to bind to the sorbent by means of hydrophobic (auxins, CKs) and ion exchange (CKs) interactions. Following is the first elution with methanol, which releases from sorbent hydrophobically bound hormones, i.e., auxins. The second elution with ammonia and methanol increases abruptly pH, thus weakening the electrostatic binding of CKs to sorbent and releasing them in solution. As shown in Fig. 1c the metabolites of auxin and cytokinin at least in *Arabidopsis* have the same affinities towards the sorbent, thus elute in the same fraction with their precursor, i.e., the auxin metabolites in first elution (A-fraction) and CK metabolites in second elution (B-fraction). It should be noted that the order of elution solvents is important and should be observed. To avoid significant sample loss due to column overload, the maximal extracted plant material should be below 0.5 g FW.

12. The resulting two fractions of purification step are each analyzed individually on LC-MS. Fraction A containing the auxin and its metabolites are separated on reversed phase HPLC column using gradient of acidified aqueous mobile phase and acetonitrile. The mass spectrometer utilizes electrospray (ESI) interface set at negative mode, the most appropriate for the auxins. The fraction B, containing cytokinins, utilizes essentially same chromatographic conditions, with exception of slightly different gradient and the ESI in positive mode.
13. An aliquot of 1/10 corresponds to an extract from 100 mg FW (typically *Arabidopsis* seedlings or tobacco cell cultures). This aliquot can be adjusted up or down depending on the plant sample amount or the expected hormonal content.
14. Cytokinin metabolites are more demanding chromatographically, because there are several positional isomers like *cis*-Zeatin vs. *trans*-Zeatin, *N7*- vs. *N9*- vs. *O*-glucosides that must be separated.
15. In order to have statistically robust results, it is recommended to have at least three biological repetitions, with optimal five and more repetitions.

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References

1. Chandler JW, Werr W (2015) Cytokinin–auxin crosstalk in cell type specification. Trends Plant Sci 20:291–300
2. Tivendale ND, Ross JJ, Cohen JD (2014) The shifting paradigms of auxin biosynthesis. Trends Plant Sci 19:44–51
3. Kaminek M (2015) Tracking the story of cytokinin research. J Plant Growth Regul 34: 723–739
4. Novák O, Hényková E, Sairanen I et al (2012) Tissue-specific profiling of the Arabidopsis thaliana auxin metabolome. Plant J 72: 523–536
5. Kojima M, Sakakibara H (2012) Highly sensitive high-throughput profiling of six phytohormones using MS-probe modification and liquid chromatography-tandem mass spectrometry. In: Normanly J (ed) High-throughput phenotyping in plants: methods and protocols, vol 918, Methods in molecular biology. Springer Science + Business Media, Dordrecht
6. Antoniadi I, Plackova L, Simonovik B, Dolezal K, Turnbull C, Ljung K, Novak O (2015) Cell-type-specific cytokinin distribution within the Arabidopsis primary root apex. Plant Cell 27: 1955–1967
7. Dobrev PI, Vankova R (2012) Quantification of abscisic acid, cytokinin, and auxin content in salt-stressed plant tissues. In: Shabala S, Cuin TA (eds) Plant salt tolerance: methods and protocols, vol 913, Methods in molecular biology. Springer Science + Business Media, Dordrecht
8. Kojima M, Kamada-Nobusada T, Kamatsu H, Takei K, Kuroha T, Mizutani M, Ashikari M, Ueguchi-Tanaka M, Matsuoka M, Suzuki K, Sakakibara H (2009) Highly sensitive and high-throughput analysis of plant hormones using MS-probe modification and liquid chromatogramy-tandem mass spectrometry: an application for hormone profiling in Oryza sativa. Plant Cell Physiol 50:1201–1214
9. Pan X, Welti R, Wang X (2008) Simultaneous quantification of major phytohormones and related compounds in crude plant extracts by liquid chromatography-electrospray tandem mass spectrometry. Phytochemistry 69:1773–1781
10. Djilianov DL, Dobrev PI, Moyankova DP, Vankova R, Georgieva DT, Gajdosova S, Motyka V (2013) Dynamics of endogenous phytohormones during desiccation and recovery of the resurrection plant species Haberlea rhodopensis. J Plant Growth Regul 32:564–574

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