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TITLE:

Plant Sample Preparation for Nucleoside/Nucleotide Content Measurement with An HPLC-MS/MS

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KEYWORDS:

nucleoside, nucleotide, plant, HPLC-MS/MS, quantitative analyses

SUMMARY:

A precise and reproducible method for in vivo nucleosides/nucleotides quantification in plants is described here. This method employs an HPLC-MS/MS.

ABSTRACT:

Nucleosides/nucleotides are building blocks of nucleic acids, parts of cosubstrates and coenzymes, cell signaling molecules, and energy carriers, which are involved in many cell activities. Here, we describe a rapid and reliable method for the absolute qualification of nucleoside/nucleotide contents in plants. Briefly, 100 mg of homogenized plant material was extracted with 1 mL of extraction buffer (methanol, acetonitrile, and water at a ratio of 2:2:1). Later, the sample was concentrated five times in a freeze dryer and then injected into an HPLC-

MS/MS. Nucleotides were separated on a porous graphitic carbon (PGC) column and nucleosides were separated on a C18 column. The mass transitions of each nucleoside and nucleotide were monitored by mass spectrometry. The contents of the nucleosides and nucleotides were quantified against their external standards (ESTDs). Using this method, therefore, researchers can easily quantify nucleosides/nucleotides in different plants.

INTRODUCTION:

Nucleosides/Nucleotides are central metabolic components in all living organisms, which are the precursors for nucleic acids and many coenzymes, such as nicotinamide adenine dinucleotide (NAD), and important in the synthesis of macromolecules such as phospholipids, glycolipids, and polysaccharides. Structurally, nucleoside contains a nucleobase, which can be an adenine, guanine, uracil, cytosine, or thymine, and a sugar moiety, which can be a ribose or a deoxyribose[1,2]. Nucleotides have up to three phosphate groups binding to the 5-carbon position of the sugar moiety of the nucleosides[3]. The metabolism of nucleotides in plants is essential for seed germination and leaf growth[4–6]. To better understand their physiological roles in plant development, the methods for the absolute quantification of different nucleosides/nucleotides in vivo should be established.

One of the most commonly used approaches to measure nucleosides/nucleotides employs a high-performance liquid chromatography (HPLC) coupled with an ultraviolet-visible (UV-VIS) detector[4,7–11]. In 2013, using HPLC, Dahncke and Witte quantified several types of the nucleosides in *Arabidopsis thaliana*[7]. They identified an enhanced guanosine content in a T-DNA insertion mutant targeting in the guanosine deaminase gene compared to the wild-type plant. Another pyrimidine nucleoside, cytidine, was also quantitatively detected in plants employing this method, which resulted in the identification of a *bona fide* cytidine deaminase gene[4]. Based on the UV detector, this method, however, cannot easily distinguish the nucleosides which have similar spectrums and retention times, e.g., guanosine or xanthosine. The detection limit of HPLC method is relatively high, therefore, it is frequently used for the measurement of high content of nucleosides in vivo, such as cytidine, uridine, and guanosine.

In addition, gas chromatography coupled to mass spectrometry (GC-MS) can also be used in nucleoside measurement. Benefiting from it, Hauck et. al. successfully detected uridine and uric acid, which is a downstream metabolite of nucleoside catabolic pathway, in the seeds of *A. thaliana*[12]. However, GC is normally used to separate volatile compounds but not suitable for the thermally labile substances. Therefore, a liquid chromatography coupled to mass spectrometry (LC-MS/MS) is probably a more suitable and accurate analytical technique for the in vivo identification, separation, and quantification of the nucleosides/nucleotides[13,14]. Several previous studies reported that a HILIC column can be used for nucleosides and nucleotides separation[15,16] and isotopically labeled internal standards were employed for the compound quantification[17]. However, both components are relatively expensive, especially the commercial isotope-labeled standards. Here, we report an economically applicable LC-MS/MS approach for nucleosides/nucleotides measurement. This method has been already successfully used for the quantitation of diverse nucleosides/nucleotides, including ATP, N^6-methyl-AMP, AMP, GMP, uridine, cytidine, and pseudouridine[1,5–6,18], in plants and *Drosophila*. Moreover, the method we

report here can be used in other organisms as well.

PROTOCOL:

1 Plant growth and materials collection

1.1 Ensure that *Arabidopsis* seeds are sterilized in 70% ethanol for 10 min and sowed on the agar plates, which were prepared with one-half-strength Murashige and Skoog nutrients.

1.2 Incubate the plates containing *Arabidopsis* seeds under dark at 4 °C for 48 h, and then transfer them into a controlled growth chamber under 16 h light of 55 µmol m^{-2} s^{-1} at 22 °C and 8 h dark at 20 °C.

1.3 Harvest 100 mg of 2-week seedlings (fresh weight) and freeze in liquid nitrogen for metabolites extraction.

CAUTION: Researchers should appropriately wear gloves, protective glasses, and a lab coat to avoid the human-tissue contamination during the materials collection.

2 Nucleosides/Nucleotides extraction

2.1 Ground 100 mg of frozen plant tissues with 7–8 steel beads in a pre-cold mixer mill for 5 min at a frequency of 60 Hz.

2.2 Prepare the extraction solution, which contains methanol, acetonitrile, and water in a ratio of 2:2:1.

2.3 Resuspend the homogenized materials (including most metabolites but not proteins) with 1 mL of extraction solution.

2.4 Centrifuge the resulting solution at 12,000 x *g* for 15 min at 4 °C.

2.5 Transfer 0.5 mL of the suspension to a new 1.5 mL tube and freeze in the liquid nitrogen.

2.6 Evaporate the frozen sample in a freeze dyer and resuspend in 0.1 mL of 5% acetonitrile and 95% water.

2.7 Centrifuge the resulting solution (0.1 mL) at 40,000 x *g* for 10 min at 4 °C. Load the supernatant in a vial for LC-MS/MS measurement.

3 LC-MS/MS measurement

3.1 Prepare a 10 mM ammonium acetate buffer by dissolving 1.1 g of ammonium acetate in 2 L of double deionized water (Mobile phase A). Adjust the pH to 9.5 by 10% ammonium and

acetate acid.

3.2 Prepare 2 L of ultrapure 100% methanol (Mobile phase B1) for nucleosides measurement. Also, prepare 2 L of ultrapure 100% acetonitrile (Mobile phase B2) for nucleotides measurement.

3.3 Inject 0.02 mL of pre-treated metabolites extraction of each sample from step 2.7 into a HPLC system with binary pumps (LC) coupled with a triple quadrupole mass spectrometer (MS).

CAUTION: HPLC system employs a C18 column (50 x 4.6 mm, particle size 5 µm; working at 25 °C) buffering with mobile phase A and B1 (**Figure 1A**) for the nucleosides separation and use a porous graphitic carbon (PGC) column (50 x 4.6 mm, particle size 5 µm; working at 25 °C) with mobile phase A and B2 (**Figure 1B**) for the nucleotides separation. Each sample was injected three times for the technical replication.

3.4 Program the method as shown in **Table 1** for the C18 column, and the method as shown in **Table 2** for the PGC column. Set a flow rate of 0.65 mL min^{-1}.

NOTE: The mass transitions (**Table 3**) were monitored by mass spectrometer. The mass spectrum analysis conditions of eight nucleosides and five nucleotides containing canonical ones and modified ones are listed in **Table 3**.

3.5 Record the peak areas of every target compound (**Figure 1**).

4 Generation of the standard calibration curves

4.1 Pool six sample extractions together, which were produced following the description in section 2, and vortex it. Then, aliquot it to six extractions (same volume) again to get each background.

4.2 Add six different concentrations of each standard to these six extractions, respectively, and inject them one by one following step 3.2.

4.3 Record the peak areas of each standard at different concentrations via the mass transitions as described in steps 3.5 and 3.6.

4.4 Plot the peak area against the nominal concentration of each standard to generate a six-point curve.

NOTE: The peak areas of nucleosides/nucleotides recorded in the step 3.6 should fall in the range of standard calibration curves.

4.5 Calculate the equation of a straight line for each standard compound: $Y = aX + b$

5 Metabolites' quantification

5.1. Calculate the metabolites' contents using the peak area recorded in step 3.6 and the equation from step 4.5.

REPRESENTATIVE RESULTS:

Here, we show the identification and quantification of N^1-methyladenosine, a known modified nucleoside, in 2-week-old *Arabidopsis* wild type (Col-0) seedlings as an example. Mass spectrometry profile indicates that the product ions generated from the N^1-methyladenosine standard are 150 m/z and 133 m/z (**Figure 2A**), and the same profile is also observed in Col-0 extraction (**Figure 2B**). Due to high abundance of the product ion of 150 m/z, the mass transition of 282.1 to 150 (m/z) is selected for the N^1-methyladenosine identification. In addition, the retention time (RT) of target peak (**Figure 3B**) is 7.05 min, which is same as the RT of N^1-methyladenosine standard (**Figure 3A**). Considering the data mentioned above, we demonstrate that wild type seedlings contain in vivo N^1-methyladenosine pool.

A concentration series of N^1-methyladenosine standards (0, 1, 2.5, 5, 10, and 50 ng / mL) was added into six sample extractions produced following steps 4.1 and 4.2, respectively (**Figure 4A**). 0.02 mL of each standard samples was injected into the LC-MS/MS, and the increased peak areas of N^1-methyladenosine were plotted against the nominal concentrations of N^1-methyadenosine standards. The equation of the straight line is Y = 0.0004X - 0.163 (**Figure 4B**).

Three replicates of Col-0 seedlings were extracted and pre-treated as described above. The peak area of N^1-methyladenosine in these three samples were recorded as 8,659, 12,147, and 12,711. Considering the five times enrichment during the extraction (see steps 2.5 and 2.6) and using the equation Y = 0.0004X - 0.163, N^1-methyladenosine concentration were calculated in three wild type lines to be 0.66, 0.94, and 0.98 ng / mL, respectively. Hence, 100 mg of each wild type seedlings were used for extraction and resuspended in 1 mL extraction buffer. Therefore, 8.6 ± 1.7 ng of N^1-methyladenosine was quantified in 1 g of 2-week-old *Arabidopsis* wild type seedlings.

FIGURE AND TABLE LEGENDS:

Table 1: The method for the C18 column. Schematic representation of solvent changes for the equilibration of C18 column. Mobile phase A = 10 mM ammonium acetate, pH 9.5. Mobile phase B = 100% methanol.

Table 2: The method for the PGC column. Schematic representation of solvent changes for the equilibration of PGC column. Mobile phase A = 10mM ammonium acetate, pH 9.5. Mobile phase B = 100% acetonitrile.

Table 3: MS analysis conditions of nucleosides and nucleotides detected by mass spectrometer. The precursor ion and product ion of eight nucleosides and six nucleotides are listed here and can be monitored by MS for compound identification and quantification.

Figure 1: The chromatographic peaks of eight nucleosides and five nucleotides. The separation

profiles of eight nucleosides by the C18 column (**A**), and five nucleotides by the PGC column (**B**).

Figure 2: Identification of N^1-methyladenosine by mass transition. MS/MS spectra of precursor ion m/z 282.1 and product ions m/z 150 and m/z 133 detected from N^1-methyladenosine standard (**A**) and Col-0 samples (**B**).

Figure 3: The chromatographic peak of N^1-methyladenosine. Mass transition of 282.1 to 150 was monitored for N^1-methyladenosine quantification. The retention times of N^1-methyladenosine peaks in the standard and sample measurement were similar.

Figure 4: Generation of the N^1-methyladenosine standard curve. (**A**) Six different concentrations of N^1-methyladenosine were added into six sample extraction matrixes, respectively. And the resulted increase peak areas were recorded. (**B**) The calibration curve of N^1-methyladenosine.

DISCUSSION:

Organisms contain various nucleosides/nucleotides, including canonical and aberrant ones. However, the origin and metabolic endpoints of them, especially modified nucleosides, are still obscure. Furthermore, the current understanding of the function and homeostasis of nucleosides/nucleotides metabolism remain to be explored and expanded. To investigate them, a precise and gold-standard method for these metabolites identification and quantification needs to be employed. Here, we described a protocol using the mass spectrum for nucleosides/nucleotides detection. Taking N^1-methyladenosine as an example, this method could detect as low as 0.02 ng standard, and the accuracy of the calibration curve is quite high (R^2 = 0.999; **Figure 4B**). Compared with the HPLC method, an MS-based protocol provides much better detection limit and accuracy. More importantly, this method can be easily performed by researchers in a biological laboratory that has a LC-MS/MS. Moreover, it can also be used for the identification of other structures known metabolites in plants.

For the in vivo absolute quantification of nucleosides/nucleotides content, commercial standard chemicals are required. They produce the straight standard curves, which allow to calculate the target metabolites in samples through peak areas recorded by mass spectrometry. It is important that the range of peak areas in standard calibration curves should cover the peak area of target metabolite read in MS. Moreover, a concentration series of standards should be added into the sample extractions but not dissolved in water for calibration curve generation. This is because it will avoid the matrix effect, which is tremendously significant for quantification accuracy.

The method described here provides a powerful tool for nucleosides/nucleotides quantification. Its application can extend to all plants and even other organisms. The whole procedure of samples' pre-treatment needs to stay cold and fast to avoid metabolites degradation, although the extraction buffer contains 80% organic chemicals, which could precipitate most of the proteins (enzymes). However, this method is not suitable for unknown target identification. The identification and quantification of target chemical in this method largely depends on the commercial chemical standards. Another limitation of this method is that the measurement of nucleosides and nucleotides has to be done separately by employing a C18 column and a PGC

column, respectively. It is because that the performance of the C18 column, although, is more stable and reproducible than PGC column, the latter could especially distinguish nucleotides much better (**Figure 1B**).

In conclusion, the presented method allows in vivo quantification of nucleosides/nucleotides in plants. From seedlings growth to obtaining the final results, the experiments can be completed within 3 weeks. Complete samples pre-treatments and LC-MS/MS analyses take about 2 days for a set of 10 to 20 samples.

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DISCLOSURES:

The authors have no conflict of interest to disclose.

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Figure 1

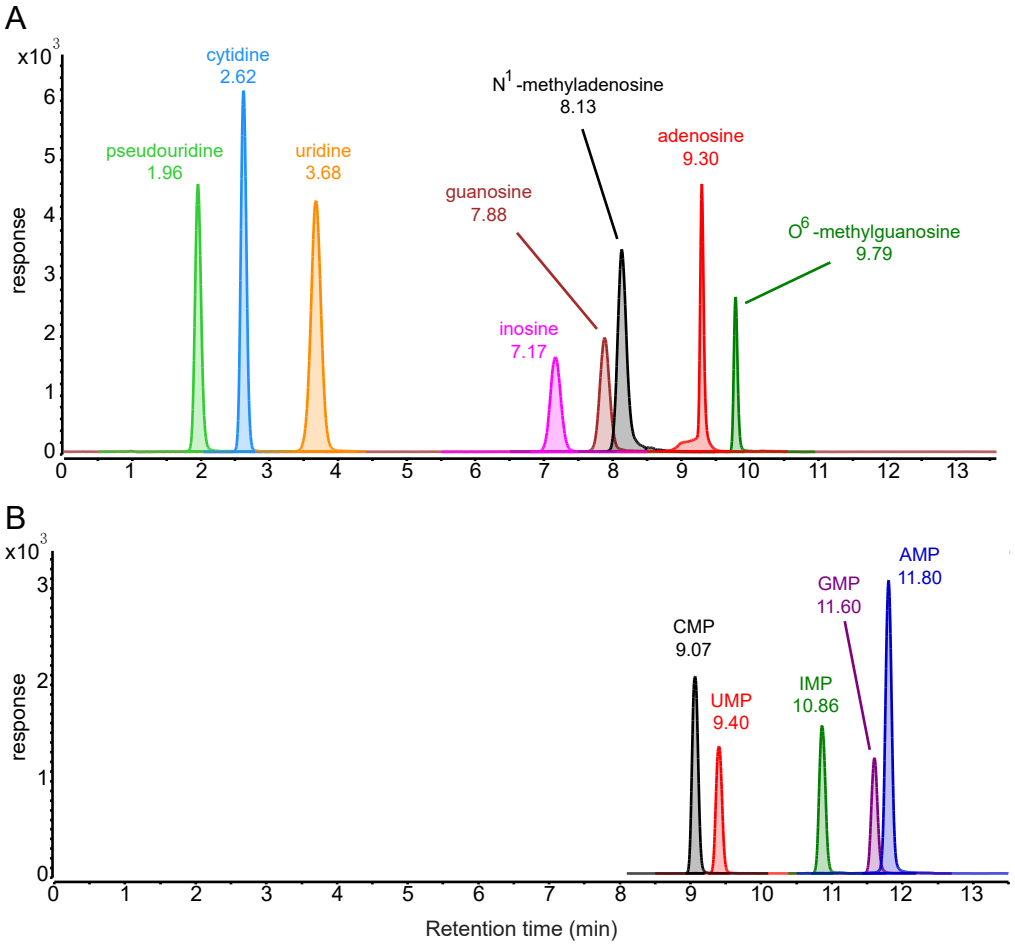

Figure 2

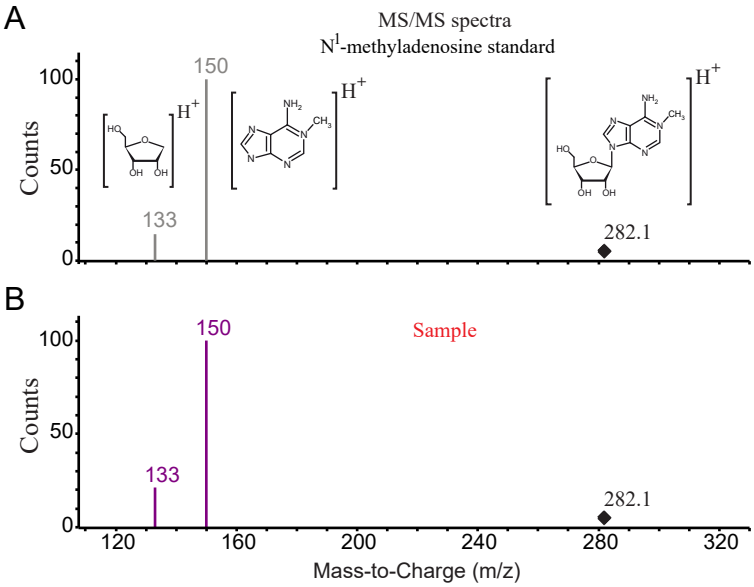

Figure 3

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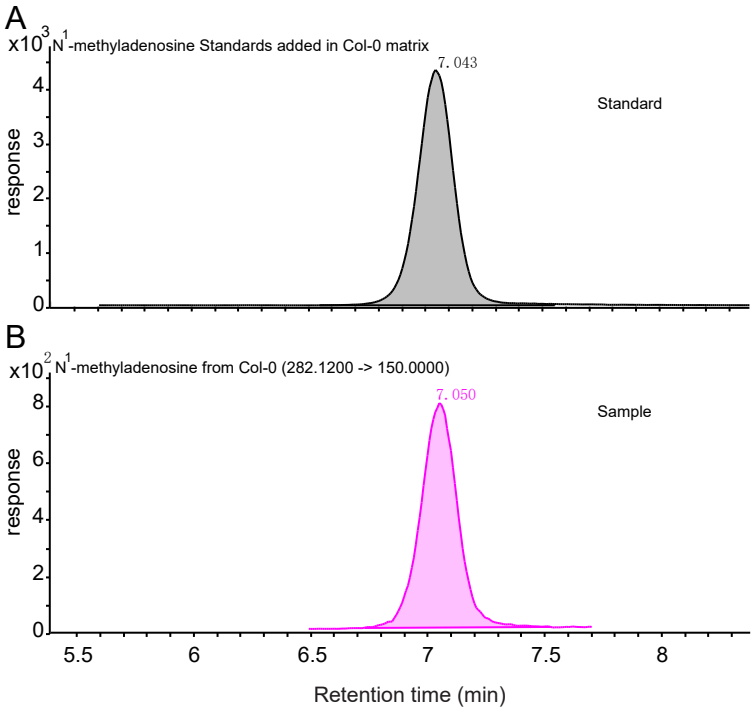
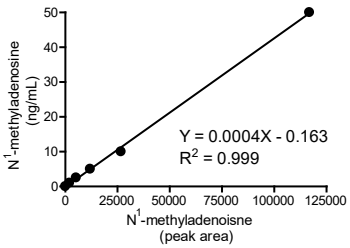


A

N^1-methyladenosine (peak area)	N^1-methyladenosine (ng / mL)
0	0
1998	1
5168	2.5
11938	5
26658	10
116738	50

B



Time	Flow rate (mL min^{-1})	Mobile phase A (%)	Mobile phase B (methanol %)
0	0.65	95	5
2	0.65	95	5
5.5	0.65	85	15
9.5	0.65	15	85
11	0.65	15	85
11.1	0.65	95	5
20	0.65	95	5

Table 1. The method for the C18 column. Schematic representation of solvent c

:hanges for the equilibration of Polaris 5 C18A column. Mobile phase A: 10 mM ammonium acet

:ate, pH 9.5. Mobile phase B1: 100 % methanol.

Time	Flow rate (mL min^{-1})	Mobile phase A (%)	Mobile phase B (acetonitrile %)
0	0.65	90	10
9	0.65	0	100
10.4	0.65	0	100
10.6	0.65	90	10
21	0.65	90	10

Table 2. The method for the PGC column. Schematic representation of solvent chan

ges for the equilibration of Hypercarb column. Mobile phase A: 10mM ammonium acetate, pH

9.5. Mobile phase B2: 100 % acetonitrile.

Nucleosides/nucleotides	Mass transition (m/z)		Polarity	Fragmentor
	precursor ion	Product ion		
adenosine	268.1	136	Positive	86
N^1-methyladenosine	282.12	150	Positive	88
guanosine	284.1	135	Positive	90
O^6-methylguanosine	298.12	166	Positive	68
inosine	269.1	136.9	Positive	55
uridine	245.21	133	Positive	85
pseudouridine	245.21	125	Positive	68
cytidine	244.2	112	Positive	150
AMP	348.07	136	Positive	111
GMP	364.07	152	Positive	80
IMP	348.9	137	Positive	79
UMP	325.01	212.9	Positive	98
CMP	324	112	Positive	90

Table 3. Mass transitions of nucleosides and nucleotides detected by mass spectrom

Collision energy (eV)	Cell Accelerator voltage	Retention time (min)	Retention Window (min)	Monitoring Mode
15	4	9.3	1.5	MRM
19	4	8.1	1.5	MRM
45	4	7.9	1.5	MRM
19	4	9.8	1.5	MRM
14	4	7.2	1.5	MRM
14	4	3.7	1.5	MRM
15	4	1.9	1.5	MRM
10	4	2.6	1.5	MRM
17	4	11.8	2	MRM
45	4	11.6	2	MRM
15	4	10.9	2	MRM
3	4	9.4	2	MRM
12	4	9.1	2	MRM

eter. The mother ion and product ion of eight nucleosides and five nucleotides are listed here and car

ɲ be monitored by MS for compound identification and quantification.

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
acetonitrile	Sigma-Aldrich	1000291000	
adenosine	Sigma-Aldrich	A9251-1G	
ammonium acetate	Sigma-Aldrich	73594-100G-F	
AMP	Sigma-Aldrich	01930-5G	
CMP	Sigma-Aldrich	C1006-500MG	
cytidine	Sigma-Aldrich	C122106-1G	
GMP	Sigma-Aldrich	G8377-500MG	
guanosine	Sigma-Aldrich	G6752-1G	
Hypercarb column	Thermo Fisher Scientific GmbH	35005-054630	
IMP	Sigma-Aldrich	57510-5G	
inosine	Sigma-Aldrich	I4125-1G	
methanol	Sigma-Aldrich	34860-1L-R	
N^1-methyladenosine	Carbosynth	NM03697	
O^6-methylguanosine	Carbosynth	NM02922	
Murashige and Skoog Medium	Duchefa Biochemie	M0255.005	
Polaris 5 C18A column	Agilent Technologies	A2000050X046	
pseudouridine	Carbosynth	NP11297	
UMP	Sigma-Aldrich	U6375-1G	
uridine	Sigma-Aldrich	U3750-1G	

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9. 4.1: What were the concentrations? Please add the information in line 175 to this step.
REPLY: Done.

10. 4.2: Please specify that this is done via the HPLC component of your LC-MS setup.
REPLY: No, it is done by Mass spectrum, and we add this note in Step 4.2 now.

11. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.
REPLY: Done.

12. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account as vector image files to ensure high resolution throughout production: (.psd, ai, .eps.). Only the title and a description should appear after the Representative Results of the manuscript text.
REPLY: We removed the embedded Figures.

13. As we are a methods journal, please add to the Discussion the following with citations:
a) Any modifications and troubleshooting of the technique
b) The significance with respect to existing methods
REPLY: We added.

14. Please sort the Materials Table alphabetically by the name of the material.
REPLY: Done

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This paper deals with an analytical method of 13 nucleosides and nucleotides in plant materials. It describes all the steps : from plant collection to metabolites extraction, lyophilisation, resuspension and LC-MS/MS analysis MRM mode. The samples are analyzed using two LC-MS/MS methods, nucleosides are separated on a C18 column, whereas nucleotides are separated on a Hypercarb column. MRM areas are measured for each peak, calibration is performed by matrix-matched calibration to avoid matrix effect biases.

Warnings were displayed for the main steps and risks. Concerns about the accuracy of quantification by using standards, matrix-matched calibration curves and their ranges were appropriately raised. The method seems to be efficient, and has already been used in several papers by the authors. But it is not clear if it is the most efficient method, when comparing it to literature.

Major Concerns:

The method is not clearly presented in the context of the existing literature : plenty of papers have been published specifically about the separation and quantification of nucleosides/nucleotides by LC-MS/MS in MRM mode (Seifar et al, Anal Biochem, 2018 ; Guo et al, J Chromatogr A, 2013 ; Moravcova et al, J Chromatogr, 2014 ; Zong et al, Molecules, 2015 ; Zhou et al, J Sep Sci, 2019 ; Qu et al, J Sep Sci, 2019). The authors should have cited some of these technical papers; a presentation of the various approaches that were used and their pros and cons was expected: isotopically-labelled internal standards or not, chromatography types (HILIC, Hypercarb, C18, ion pair chromatography) and retention, MS analyzers (triple quadrupole or triple quadrupole linear ion trap).

REPLY: Thanks for your suggestion. We cited and briefly presented several new technical papers in “INTRODUCTION” section.

More specifically, it is not clear why the authors use two different chromatography analyses, one for nucleotides, the other for nucleosides, whereas several papers propose a one-shot approach. Please justify, as it doubles the time of use of an expensive LC-MS/MS.

REPLY: Because C18 column is relatively cheap and the performance of it is quite stable, it is, in our hand, used for nucleosides separation. However, it is not good at nucleotides separation. For example, AMP and IMP can not be separated in C18 but can be distinguished in Hypercarb column. More importantly, these two compounds have similar precursor ions, so it is also difficult to distinguish by the mass spectrum. Similar case is UMP and CMP. So, we normally employ an hypercarb column to quantify nucleotides in our samples.

Minor Concerns:

Fig 1: please replace « profiles » with « spectra » and « mother ions » with « precursor ion »

REPLY: Done.

Please replace the sentence « MS/MS profiles of mother ions and product ions detected… » with « MS/MS spectra of precursor ion m/z 282, detected … »
REPLY: Done

Table 3: One decimal digit should be enough for mother ion mass
REPLY: Done

Reviewer #2:
Manuscript Summary:
In the summary, the author used statistical terminology "precise" and "reproducible", however the methods and results presented were not clearly demonstrating it.

Major Concerns:
1) Do you used ambient temperature centrifuge or refrigerated one?

REPLY: The metabolites were extracted at 4℃. We add this information in the protocol.

Thanks for reminding.

2) Why a reverse phase column C18 for nucleosides was chosen and a Hypercarb for nucleotides?
REPLY: Because C18 column is relatively cheap and the performance of it is quite stable, it is, in our hand, used for nucleosides separation. However, it is not good at nucleotides separation. For example, AMP and IMP can not be separated in C18 but can be distinguished in Hypercarb column. More importantly, these two compounds have similar precursor ions, so it is also difficult to distinguish by the mass spectrum. Similar case is UMP and CMP. So, we normally employ an hypercarb column to quantify nucleotides in our samples.

3) It is not clear whether the two column used separately or coupled. Please show the separation profiles of these two columns.
REPLY: Two column are used separately. Now, we add the separation profiles in Fig.1.

4) Please detail the condition for HPLC, e.g. column temperature, type of solvent (ultrapure grade or MS/MS grade? MS/MS grade is usually hypergrade for LC-MS, while ultrapure is for normal HPLC).
REPLY: The information was added in the protocol.

5) Please detail the MS interface condition and MS analysis condition that produced such results, e.g. ionization method, range of detection, type of scan (Q1/Q3, or SIM), positive or negative modes, collision energy, event time, scan speed, etc., since this is a method journal.
REPLY: We add the more details in Table 3.

6) How do you get the Figure 2? Which chromatography condition do you used?
REPLY: We used C18 column for separation. The chromatography condition was shown in Table 1.

7) Calibration curve is for showing the instrument response. Please clearly discuss sensitivity, accuracy (%), precision (% CV), stability (temperature wise), and % recovery, in order to be consistent with manuscript summary
REPLY: Done.

Minor Concerns:
1) Please clearly define between buffer (pH is sustained) and solvent mixtures (no additional compound that sustains pH).
REPLY: Done, we revised.

2) To ensure wide-range readers understand what is going on, please briefly clarify what do we expect from initial separation step in sample preparation? What is in the suspension?
REPLY: We add information in step 2.2.

3) line 85, please specify "metabolites", since only nucleoside/nucleotide is discussed
REPLY: We changed.

4) Give technical detail, e.g. ratio v/v/v
REPLY: Done.

5) line 117/118/124/125... "Mobile phase A: 10 mM..." please use "=" in order to differentiate the use punctuation for addressing ratio
REPLY: We changed.