This is an Accepted Manuscript, which has been through the RSC Publishing peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, which is prior to technical editing, formatting and proof reading. This free service from RSC Publishing allows authors to make their results available to the community, in citable form, before publication of the edited article. This Accepted Manuscript will be replaced by the edited and formatted Advance Article as soon as this is available.

To cite this manuscript please use its permanent Digital Object Identifier (DOI®), which is identical for all formats of publication.

More information about Accepted Manuscripts can be found in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics contained in the manuscript submitted by the author(s) which may alter content, and that the standard Terms & Conditions and the ethical guidelines that apply to the journal are still applicable. In no event shall the RSC be held responsible for any errors or omissions in these Accepted Manuscript manuscripts or any consequences arising from the use of any information contained in them.
Simultaneous Quantitative Determination of Major Plant Hormones in Pear Flowers and Fruit by UPLC/ESI–MS/MS

Qingfeng Niu, Yu Zong, Minjie Qian, Fengxia Yang and Yuanwen Teng

Plant hormones play a significant role in regulating growth and development during the entire life of a plant, and in responses to biotic and abiotic stress. The determination of concentrations of hormones in flowers and fruit is essential to understand the role of hormones in the regulation of physiological and biochemical processes associated with flowering and fruit development. Based on high-performance liquid chromatography, coupled with tandem mass spectrometry, we developed and established a novel method to quantify four distinct endogenous hormones through ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/ESI–MS/MS), which achieves higher throughput screening and improved resolution than HPLC or HPLC/ESI–MS/MS. Crude plant extracts were prepared by extraction with extraction solvents I and II, then purified with a Sep-Pak™ C18 reverse-phase extraction cartridge, and subsequently the purified extracts were analyzed by UPLC/ESI–MS/MS. Plant hormones, comprising indole-3-acetic acid, abscisic acid, gibberellin A4, and trans-zeatin riboside, were separated and quantified in 6 min. The method was simple, rapid, and precise, and was applied for determination of plant hormones in pear tissue with recovery ranging from 70.11% to 89.84% and relative standard deviation ranging from 4.25% to 14.96%. In conclusion, sample preparation, extraction, purification, and UPLC/ESI–MS/MS conditions were optimized for quantitative analysis of four major plant hormones in pear tissues.
Introduction

Plant hormones are physiologically active substances that play pivotal roles in physiological processes during a plant’s life cycle. They influence a diversity of processes including plant growth, flowering, fruit set and fruit development, maturity, and senescence.1, 2 Recent studies have focused on elucidating how hormone actions create a signaling network and mutually regulate multiple metabolic and signaling systems. For example, gibberellin A₄ (GA₄) is the active gibberellin in the regulation of leaf transcription and Arabidopsis floral initiation. The levels of GA₄ and sucrose increase dramatically in the shoot apex shortly before floral initiation, and the regulation of genes involved in GA metabolism suggests that this increase is possibly due to transport of GAs and sucrose to the shoot apex. These findings demonstrate that GA₄ is the active GA in the regulation of both shoot elongation and flower initiation.3 During reproductive development, auxins specify the site of flower initiation and subsequently regulate organ growth and patterning as well as later events that determine reproductive success.4, 5 Cytokinins are plant hormones that might play essential roles in the regulation of both shoot elongation and flower initiation.6 Cytokinin deficiency results in diminished activity of the vegetative and floral shoot apical meristems and leaf primordia, indicating an absolute requirement for cytokinins.7 FCA, an RNA-binding protein involved in flowering, binds abscisic acid (ABA) with high affinity in an interaction that is stereospecific and conforms to receptor kinetics.8 The application of exogenous auxin to developing apple fruit increases cell expansion, suggesting that the endogenous auxin concentration is one of the limiting factors that control fruit size.9 Furthermore, different plant hormones regulate similar processes through largely non-overlapping transcriptional responses.1, 10-12 Therefore, focusing only on a single endogenous plant hormone is inadequate to evaluate hormone-regulated physiological or biological phenomena.

To better understand the hormone regulatory network, an ideal analytical method should be established that provides a measure of multiple hormone concentrations from one experimental sample. A variety of methods for simultaneous quantification of multiple plant hormones has been reported. For example, high-performance liquid chromatography (HPLC) with electrochemical detection13, 14 performs poorly for quantitative analysis of endogenous plant hormones at nanomolar levels in plant tissue samples because the purification process is complicated and the limit of quantitation (LOQ) that meets the determination requirements is high and difficult to achieve with small amounts of sample.15 Gas chromatography combined with tandem mass spectrometry (GC–MS/MS) is limited to analysis of volatile compounds, and purification and derivatization require high temperatures, which can degrade thermally labile compounds.16 Therefore, HPLC coupled with tandem mass spectrometry (HPLC–MS/MS), which shows higher sensitivity and selectivity of detection, is used for quantification of plant hormones. Several methods for simultaneous quantification of multiple plant hormones using HPLC–MS/MS have been reported recently.17-19 In addition, an HPLC–electrospray ionization tandem mass spectrometry (HPLC/ESI–MS/MS) method to analyze plant hormones in Arabidopsis thaliana (L.) Heynh.20 and an ultra-performance liquid chromatography (UPLC)/ESI–MS/MS technique to analyze endogenous hormones in Arabidopsis leaves have been described.21 However, these analytical methods were only applied to leaf tissue of Arabidopsis21, 21 and oilseed rape.22 Although such analytical techniques allow simultaneous analysis of multiple endogenous plant hormones, no previous report has simultaneously quantified all plant hormones in one sample precisely. A published protocol18 was only described as a process without method validation. The UPLC/ESI–MS/MS method21 is more complex than ours, which uses different extraction solvents to extract the different hormones and no recovery data are presented. Many approaches have been described for detection of plant hormones in leaf tissue, whereas methods suitable for flower or fruit tissue are less common. Furthermore, development of an extraction solution for improved efficiency, and optimization of the extraction process to ensure maximal resolution and signal with a minimal run time are required. In theory, compounds could be ionized in both negative and positive modes. However, different compounds have a preference for a certain ionization mode. In order to improve ionization efficiency, using a polarity-switching mode enables analysis of compounds with different preferred ionization modes.

In this study, we optimized and established an UPLC/ESI–MS/MS approach for analysis of hormones in ‘Cuiguan’ pear (Pyrus pyrifolia Nakai cv. Cuiguan) flower and fruit tissues. We performed UPLC to separate hormones under isocratic elution conditions on an Acquity® BEH C₁₈ column, followed by detection by tandem mass spectrometry with multiple reaction monitoring (MRM). We aimed to develop an optimal, sensitive analytical protocol for simple and rapid detection of endogenous hormones in pear flower and fruit tissues.

Materials and methods
Materials and reagents

Samples from the Chinese sand pear cultivar ‘Cuiguan’ were obtained from the orchard in ‘Guoqingtang’ (Wenling city, Zhejiang province, China). The pear trees were 12 years old and were considered to be in the generative phase. Trees used in the experiment were not chemically treated. The samples were collected from three trees at each developmental stage. Flowers on the current season’s growth were collected on 20 March, 2012 (1 day before anthesis), and fruit were collected on 29 March, 2012 (8 days after anthesis) and 6 April, 2012 (16 days after anthesis). The flowers were divided into three groups of 10 flowers each. The fruit at each stage were also divided into three groups of 10 fruit each. After collection, lateral flower and fruit samples were placed immediately in liquid nitrogen and stored at −80°C until extraction of plant hormones. Abscisic acid (≥98.5%), indole-3-acetic acid (IAA; 98%), GA₄ (≥90%), trans-zearin riboside (t-ZR; ~95%), and acetoniitrile (HPLC grade) were purchased from the Sigma Chemical Company (St Louis, MO, USA). Water (HPLC grade) was obtained from a Milli-Q Reagent Water System (Millipore, Bedford, MA, USA). Other reagents and chemicals used were of analytical grade.

Chromatographic and mass spectrometric conditions

The Acquity® UPLC system features a novel liquid chromatography technology that uses 1.7-µm stationary-phase pressure-tolerant particles. UPLC was performed under isocratic elution conditions on an Acquity® BEH C₁₈ column (100 mm × 2.1 mm, 1.7-µm particle size; Waters) with a VanGuard Pre-column (5 mm × 2.1 mm, 1.7-µm particle size; Waters), followed by detection with a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA). The mobile phase, consisting of acetoniitrile (solvent A) and 0.02% (v/v) glacial acetic acid in water (solvent B), was pumped at a flow rate of 0.15 mL min⁻¹ with a volume ratio (A:B) of 60:40. Five microliters was injected into the column at a column temperature of 40°C and separated for 6 min.

The mass spectrometer was operated in the ESI negative/positive mode and data were acquired in the MRM mode. The MS parameters were set as follows: capillary voltage (−) 2800 V/+(+) 3500 V, desolvation gas (nitrogen) flow rate 800 L h⁻¹, cone gas (nitrogen) flow rate 80 L h⁻¹, source temperature 120°C, and desolvation temperature 350°C. The specific transitions for the analytes were monitored using the MRM mode. The MS collision parameters for each compound were optimized.

Preparation of working solutions and calibration curves

Standard stock solutions were prepared by dissolving hormone standards in methanol (200 µg mL⁻¹). The hormone stock solutions were each diluted with methanol to obtain working solutions of six different concentrations (0, 1.6, 8, 40, 200, and 1000 ng mL⁻¹). All working solutions were stored at −40°C. Five microliters of each working solution was analyzed by UPLC/ESI–MS/MS to construct calibration curves.

Extraction solvent preparation

Concentrated hydrochloric acid (100 µL) was added to 100 mL 2-propanol and 50 mL distilled water to prepare the extraction solvent I, which consisted of 2-propanol:H₂O:concentrated HCl (2:1:0.002, v/v/v). The solution was stable for 2 months.¹⁸ The extraction solvent II was dichloromethane.

Method validation study

Frozen flower (4.0 g) and fruit (4.0 g) tissues were combined and ground to a powder in liquid nitrogen with a mortar and pestle. The sample was divided into four groups of each have five duplication randomly. Under the conditions of protection away from light, the sample powder (300 mg) was transferred to 10 mL screw-cap tubes, to which 3 mL extraction solvent I was added. The relative standard deviation (RSD) and percentage recovery were determined, and the matrix effect (ME) was calculated by dividing the peak area obtained from analysis of a blank matrix spiked with a hormone analyte, by the peak area obtained in the absence of a matrix. The analyses were performed using samples spiked with 0, 20, 40, or 80 ng kg⁻¹ of analyte. After ultrasonic extraction for 5 min, the tubes were placed on a shaker at a speed of 100 rpm for 30 min at 4°C. Extraction solvent II (5 mL) was added to the tubes and after further ultrasonic extraction for 5 min, the tubes were placed on a shaker at a speed of 100 rpm for 30 min at 4°C. After centrifugation of the resulting solution at 4°C at 10,000 ×g for 8 min, two liquid phases were formed with the plant solids between the phases. Five microliters of the solvent from the lower phase (solvent II) was transferred to a new tube. By aeration of the solvent mixture using a nitrogen evaporator with nitrogen gas, the sample was concentrated rapidly in the dark. Samples were redissolved in 1 mL methanol and purified with a Sep-Pak™ C₁₈ reverse-phase extraction cartridge. The samples were dried completely under a nitrogen stream and redissolved in 200 µL methanol, centrifuged at 10,000 ×g for 5 min at 4°C, then filtered through a 0.22 µm PTFE filter (Waters). Five microliters of each sample solution was analyzed by UPLC/ESI–MS/MS.
Sample preparation
Frozen pear flowers (ovary about 1.5 g) or fruit material (endocarp about 1.5 g) was ground into powder in liquid nitrogen with a mortar and pestle. Under the conditions of protection away from light, the sample powder (300 mg) was transferred to 10 mL screw-cap tubes, to which 3 mL extraction solvent I was added. After ultrasonic extraction for 5 min, the tubes were placed on a shaker at a speed of 100 rpm for 30 min at 4°C. Extraction solvent II (5 mL) was added to the tubes and after further ultrasonic extraction for 5 min, the tubes were placed on a shaker at a speed of 100 rpm for 30 min at 4°C. After centrifugation of the resulting solution at 4°C at 10,000 ×g for 8 min, two liquid phases were formed with the plant solids between the phases. Five milliliters of the solvent from the lower phase (solution II) were transferred to a new tube. By aeration of the solvent mixture using a nitrogen evaporator with nitrogen gas, the sample was concentrated rapidly in the dark. Samples were redissolved in 1.0 mL methanol and then purified with a Sep-Pak™ C18 reverse-phase extraction cartridge. The samples were dried completely under a nitrogen stream and redissolved in 200 μL methanol, centrifuged at 10,000 ×g for 5 min at 4°C, then filtered through a 0.22-μm PTFE filter (Waters). The samples solutions (5 μL) were injected and then analyzed by UPLC/ESI-MS/MS.

Results and discussion

Method development
In this study, ESI was chosen as the ionization source. To identify and quantify the analytes in plant tissue extracts at trace levels, the MRM-associated acquisition parameters were optimized for the maximum abundance of fragmented ions under ESI positive/negative mode conditions by confining standard solutions of the compounds into the MS. Identification of the parent ion as well as the choice of ionization mode for each analyte was performed in the full-scan mode by recording mass spectra from 50 to 400 m/z. For each analyte the deprotonated or protonated molecular ion ([M-H]⁻ or [M+H]⁺) and MS/MS fragment in the reference standards were determined and chosen as the precursor ion (Figure 1) and product ion, respectively. Different fragmentor voltages were tested for each precursor ion to find the most suitable settings. Then, dissociation was induced and different collision energies were tested to find the most abundant product ion. The most sensitive transition in MRM mode was selected for quantification in the screening method. The optimum values for each condition for each compound were summarized, and the most intense characteristic MRM transitions were chosen for each analyte (Table 1). The most important condition to be satisfied for identification of a target compound was that at least two ion transitions gave signals distinguishable from the background ion current when MS/MS detection was performed. Figure 2A shows the total ion chromatogram of the MRM mode for the four plant hormone standards at the concentration of 50 ng mL⁻¹. The total ion chromatogram of the MRM mode for plant hormones in pear flowers collected at 1 day before anthesis is shown in Figure 2B.

The UPLC conditions were optimized to ensure maximal resolution and signal with a minimal run time. To obtain the optimal mobile phase that is most compatible with ESI-MS/MS and to achieve good resolution and symmetric peak shapes for the analysis, different ratios of organic solvents and water were compared. Different combinations of methanol, acetonitrile, and water with different concentrations of glacial acetic acid were compared to identify the optimal mobile phase that produced the highest sensitivity, efficiency, and peak shape. Acetonitrile as an organic solvent resulted in superior sensitivity compared with methanol. A low water content resulted in a better peak shape, higher sensitivity, and shorter running time for the analytes. A mobile phase consisting of acetonitrile (A)–water (B) (containing either 0.02% glacial acetic acid (Figure 3A), 0.2% glacial acetic acid (Figure 3B), or 0.6% glacial acetic acid (Figure 3C)) was used and the A:B volume ratio of 60:40 was optimal. The analysis time and resolution between peaks were compared using the Acquity® BEH C18 column (100 mm × 2.1 mm, 1.7-µm particle size; Waters) at 40°C, and the mobile phase consisting of acetonitrile–water (containing 0.02% glacial acetic acid) was optimal for UPLC.

The quality of extraction and purification of plant hormones critically influenced the quality of the results. After optimizing the parameters for UPLC and MS/MS, we tested methanol:H₂O (85:15, v/v),22 methanol:isopropanol:glacial acetic acid (79:20:1, v/v/v),23 and 2-propanol:H₂O:concentrated HCl (2:1:0.002, v/v/v)24 mixtures with the aim of minimizing the number of extraction steps required for adequate recovery. In addition, we sought to achieve high reproducibility. Depending on the chemical properties of the plant hormones, the solvent 2-propanol:H₂O:concentrated HCl (2:1:0.002, v/v/v) is recommended as an extraction solvent. A summary of the extraction and analysis protocol is depicted in Figure 4. Purification with a Sep-Pak™ C18 reverse-phase column was an indispensable step in the procedure.

Method validation
Calibration curves for the four compounds were obtained with relatively wide concentration ranges. The correlation coefficients for all of the calibration curves were higher than 0.99. For the different components, the limit of detection (LOD) was in the range of 0.1–1.3 ng mL⁻¹ and the LOQ was in the range of 0.26–3 ng mL⁻¹. Sensitivity parameters are listed in Table 2. The LOD and
LOQ, based on a signal-to-noise ratio of 3:1 and 10:1, respectively, were calculated from the standard addition curves. The RSD ranged from 4.25% to 14.96% and percentage recovery was 70.11–89.84% (Table 3). Given that the potential ME is a concern with a rapid isocratic system, the co-elution effect and potential ion suppression or enhancement were evaluated. The ME was calculated with the formula \((B−C)/A \times 100\), where A is the peak area of the hormone standard dissolved in methanol, B is the corresponding peak area of the hormone in pear tissue spiked post-extraction, and C is the corresponding peak area of the hormone in pear tissue samples not spiked with hormone analyte. An ME value of 100% indicated that the response in the mobile phase and in the hormone extracts was identical and no absolute ME was observed (Table 4). The absolute ME values were 85.28–96.81%, which indicated that no significant ion suppression or enhancement effect was observed. The variability was acceptable with RSD values <15% at the different concentrations. These data confirm that the relative ME for plant hormones was not significant in pear samples comprising mixed flowers and fruit. Thus, no ion suppression or enhancement effect was observed and the present analytical method was considered to be reliable.

Sample analysis

All the samples were collected from three trees at each stage. Pear flowers were collected at 1 day before anthesis (Mar. 20, 2012; 1 DAA) and fruits were collected at 8 (Mar. 29, 2012; 8 DAA) and 16 days after anthesis (Apr. 6, 2012; 16 DAA). Samples were randomly selected for plant hormone concentration detection. Fruits of each stage were divided into three groups. Each group contained 10 fruits. Flowers were treated as above. After picking, lateral flower and fruit samples were stored immediately in liquid nitrogen and then at –80°C until plant hormones extraction after picking. Representative examples of the plant hormone concentrations measured in pear flowers and fruit are shown in Figure 5. The endogenous IAA concentration rose sharply in the ovary tissue at 8 days after pollination. A2 Auxins, primarily IAA, appear to participate in the coordination of processes within and between floral organs during flower and fruit development, such as anther dehiscence, pollen maturation, gynoecium development, fertilization, fruit initiation, seed development, and fruit ripening. The present results demonstrated that our method showed excellent performance for quantification of major plant hormones in pear flower and fruit tissues. The results of the methodology validation test encourage us to believe that the method is suitable for use in further hormone-related research.

Conclusions

In this study, sample preparation, extraction, purification, and UPLC-MS/MS conditions were optimized for quantitative analysis of plant hormones in flowers and fruit. Based on an HPLC-MS/MS method reported previously, we developed and established a method to analyze quantitatively the four major plant hormones by UPLC/ESI-MS/MS, which enables higher throughput and chromatographic resolution. Simultaneous quantitative determination of the plant hormones IAA, ABA, GAs, and t-ZR were achieved within 10 min using this method. The method is simple, rapid, and precise, and was applied for determination of plant hormone levels in pear flower and fruit tissues with average recovery of 80.55% and RSD of 9.38%.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. 31272141) and the Earmarked Fund for Modern Agro-industry Technology Research Systems (nyctx-29-14).

References

* Department of Horticulture, State Agricultural Ministry Key Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Zhejiang University, Hangzhou 310029, China. Fax: +86-571-88982803; Tel: +86-571-88982803;
* Author for correspondence: ywteng@zju.edu.cn

### Tables

#### Table 1 Mass spectrometric parameters and fragment spectra of the plant hormones analyzed

<table>
<thead>
<tr>
<th>Ionization mode</th>
<th>Endogenous hormone</th>
<th>Parent ion (m/z)</th>
<th>Quantitative ion (m/z)</th>
<th>Cone voltage (V)</th>
<th>Collision energy (eV)</th>
<th>Dwell time (s)</th>
<th>Time window (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESI(−)</td>
<td>GA₄</td>
<td>331.0</td>
<td>243.2/225.1</td>
<td>22</td>
<td>13</td>
<td>0.1</td>
<td>4.80–4.95</td>
</tr>
<tr>
<td>ESI(−)</td>
<td>ABA</td>
<td>263.0</td>
<td>152.9</td>
<td>25</td>
<td>12</td>
<td>0.1</td>
<td>2.60–2.70</td>
</tr>
<tr>
<td>ESI(−)</td>
<td>IAA</td>
<td>174.4</td>
<td>129.9</td>
<td>22</td>
<td>13</td>
<td>0.1</td>
<td>2.45–2.55</td>
</tr>
<tr>
<td>ESI(+)</td>
<td>t-ZR</td>
<td>352.0</td>
<td>219.7</td>
<td>40</td>
<td>18</td>
<td>0.1</td>
<td>2.35–2.50</td>
</tr>
</tbody>
</table>

ABA, abscisic acid; GA₄, gibberellin A₄; IAA, indole-3-acetic acid; t-ZR, trans-zeatin riboside

#### Table 2 UPLC/ESI-MS/MS data for the calibration curves, limit of detection (LOD), and limit of quantitation (LOQ)

<table>
<thead>
<tr>
<th>Endogenous hormone</th>
<th>Regression equation</th>
<th>Correlation coefficient ($R^2$)</th>
<th>Linear range (µg L⁻¹)</th>
<th>LOD (µg L⁻¹)</th>
<th>LOQ (µg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-ZR</td>
<td>$y = 631.75x + 246.32$</td>
<td>0.9942</td>
<td>8–1000</td>
<td>0.1</td>
<td>0.26</td>
</tr>
<tr>
<td>GA₄</td>
<td>$y = 31.814x - 77.885$</td>
<td>0.9906</td>
<td>1.6–1000</td>
<td>0.25</td>
<td>1.3</td>
</tr>
<tr>
<td>ABA</td>
<td>$y = 45.985x + 178.23$</td>
<td>0.9961</td>
<td>8–1000</td>
<td>0.1</td>
<td>0.26</td>
</tr>
<tr>
<td>IAA</td>
<td>$y = 5.1437x + 82.049$</td>
<td>0.9999</td>
<td>0.32–1000</td>
<td>1.3</td>
<td>3</td>
</tr>
</tbody>
</table>

ABA, abscisic acid; GA₄, gibberellin A₄; IAA, indole-3-acetic acid; t-ZR, trans-zeatin riboside

#### Table 3 Repeatability and recovery of four plant hormones from spiked extractions (n = 5)

<table>
<thead>
<tr>
<th>Endogenous hormone</th>
<th>Spiked concentration (µg kg⁻¹)</th>
<th>Repeatability (RSD; %)</th>
<th>Recovery mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-ZR</td>
<td>20</td>
<td>7.56</td>
<td>81.36</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>12.25</td>
<td>72.33</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>9.47</td>
<td>84.15</td>
</tr>
<tr>
<td>GA₄</td>
<td>20</td>
<td>5.12</td>
<td>74.44</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>14.96</td>
<td>75.36</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>9.13</td>
<td>70.11</td>
</tr>
<tr>
<td>ABA</td>
<td>20</td>
<td>4.25</td>
<td>86.33</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>6.2</td>
<td>82.17</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>4.63</td>
<td>84.12</td>
</tr>
<tr>
<td>IAA</td>
<td>20</td>
<td>11.93</td>
<td>81.87</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>12.88</td>
<td>84.5</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>14.23</td>
<td>89.84</td>
</tr>
</tbody>
</table>

ABA, abscisic acid; GA₄, gibberellin A₄; IAA, indole-3-acetic acid; RSD, relative standard deviation; t-ZR, trans-zeatin riboside
Table 4 Matrix effect for plant hormones using pear samples spiked with 20, 40, or 80 µg kg\(^{-1}\) hormone analyte (n = 5)

<table>
<thead>
<tr>
<th>Endogenous hormone</th>
<th>Spiked concentration (µg kg(^{-1}))</th>
<th>Repeatability (RSD; %)</th>
<th>Recovery mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-ZR</td>
<td>20</td>
<td>4.34</td>
<td>87.37±2.68</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.28</td>
<td>92.56±3.46</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>2.46</td>
<td>96.81±1.68</td>
</tr>
<tr>
<td>GA4</td>
<td>20</td>
<td>4.59</td>
<td>86.30±2.80</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>7.20</td>
<td>93.51±4.76</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>5.23</td>
<td>89.09±3.29</td>
</tr>
<tr>
<td>ABA</td>
<td>20</td>
<td>2.84</td>
<td>92.74±1.86</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.41</td>
<td>87.57±3.35</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>3.04</td>
<td>94.13±2.02</td>
</tr>
<tr>
<td>IAA</td>
<td>20</td>
<td>8.74</td>
<td>87.00±5.38</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>9.17</td>
<td>85.28±5.53</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>6.40</td>
<td>90.31±4.08</td>
</tr>
</tbody>
</table>

ABA, abscisic acid; GA\(_4\), gibberellin A\(_4\); IAA, indole-3-acetic acid; ME, Matrix effect; RSD, relative standard deviation; t-ZR, \textit{trans}-zeatin riboside
Figures

A

Relative Abundance (%)

129.880

174.358

m/z

B

Relative Abundance (%)

152.858

m/z

IAA

174.4>129.9

ABA

263>152.9
Figure 1. MS/MS product ion spectrum of [M-H]^− (A) IAA at m/z 174.4 as the precursor ion, (B) ABA at m/z 263.0 as the precursor ion, (C) GA₄ at m/z 331.0 as the precursor ion, and [M+H]^+ (D) t-ZR at m/z 352.0 as the precursor ion.
Figure 2. Typical chromatograms of UPLC-MS/MS. (A) Mixed standard substances and (B) Sample collected at 1 day before anthesis.
Figure 3. Typical chromatograms of UPLC-MS/MS under different UPLC mobile phases. (A) Acetonitrile (A) - water (B) (containing 0.02% glacial acetic acid), (B) acetonitrile (A) - water (B) (containing 0.2% glacial acetic acid), (C) acetonitrile (A) - water (B) (containing 0.02% glacial acetic acid).
Figure 4. Schematic diagram to extract and analyze small amounts of plant material using UPLC/ESI-MS/MS with multiple reaction monitoring (MRM).
Figure 5. Plant hormone concentration presented in fresh pear flowers and fruits were determined by UPLC-MS/MS. Flowers were collected at 1 day before anthesis (DBA), and fruits were collected at 8 and 16 days after anthesis (DAA).
300 mg frozen plant material

- grind in liquid nitrogen
- add 3 mL solution I
- 5 min ultrasonic sonication 5 ℃
- 30 min shaker (1000 rpm, 4 ℃)

centrifugation (10000 rpm, 8 min, 4 ℃)

- add 5 mL solution II
- 5 min ultrasonic sonication 5 ℃
- 30 min shaker (1000 rpm, 4 ℃)

Collect bottom layer liquid 5 mL

- concentrated under nitrogen stream
- re-dissolved in 1.0 mL methanol
- purification by using a Sep-Pak™ C18 cartridge
- dried under a nitrogen stream
- re-dissolved in 200 µL methanol
- through a 0.22 µm PTFE filter

Schematic diagram to extract and analyze small amounts of plant material using UPLC/ESI-MS/MS with multiple reaction monitoring (MRM)