



An ultrahigh-performance liquid chromatography method with electrospray ionization tandem mass spectrometry for simultaneous quantification of five phytohormones in medicinal plant *Glycyrrhiza uralensis* under abscisic acid stress

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Abstract An efficient simplified method was developed to determine multiple classes of phytohormones simultaneously in the medicinal plant *Glycyrrhiza uralensis*. Ultrahigh-performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC/ESI-MS/MS) with multiple reaction monitoring (MRM) in negative mode was used for quantification. The five studied phytohormones are gibberellic acid (GA₃), abscisic acid (ABA), jasmonic acid (JA), indole-3-acetic acid, and salicylic acid (SA). Only 100 mg of fresh leaves was needed, with one purification step based on C₁₈ solid-phase extraction. Cinnamic acid was chosen as the internal standard instead of isotope-labeled internal standards. Under the optimized conditions, the five phytohormones with internal standard were separated within 4 min, with good linearities and high sensitivity. The validated method was applied to monitor the spatial and temporal changes of the five phytohormones in *G. uralensis* under ABA stress. The levels of GA₃, ABA, JA, and SA in leaves of *G. uralensis* were increased at different times and with different tendencies in the reported stress mode. These changes in phytohormone levels are discussed in the context of a possible feedback regulation mechanism. Understanding this mechanism will provide a good chance of revealing the mutual interplay between different biosynthetic routes, which could further help

elucidate the mechanisms of effective composition accumulation in medicinal plants.

Keywords Medicinal plant · *Glycyrrhiza uralensis* · Phytohormones · UPLC/ESI-MS/MS · Abscisic acid stress · Spatial and temporal changes

Introduction

Glycyrrhiza uralensis [1] has been recognized as one of the most famous medicinal plants in traditional Chinese medicine for thousands of years. Owing to its diverse pharmacological properties including anti-allergic [2], anti-inflammatory [3], and antitumor effects [4, 5], it is widely used for the treatment of respiratory ailments, inflammatory problems, and liver diseases [6]. Although much research has been conducted on the mechanisms of how *G. uralensis* is used to cure disease, the accumulation of active constituents in medicinal plants remains a big problem. It is therefore important to find other ways to study the mechanism of effective composition accumulation in medicinal plants.

Phytohormones, which play a central role at nanomolar levels in most physiological processes in plants, are becoming increasingly important in the area of plant functional genes and their molecular mechanisms [7, 8]. These structurally diverse compounds regulate processes: e.g., cytokinins (CKs), brassinosteroids, and auxins regulate cell division and enlargement [9–11]; gibberellins (GAs), CKs, and ethylene regulate seed germination and dormancy as well as bud formation [12, 13]; abscisic acid (ABA) and jasmonic acid (JA) regulate senescence. Furthermore, phytohormones often mediate stress responses by synergistic or antagonistic actions, especially for ABA [14], JA

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[15, 16], and salicylic acid (SA) [17]. Extensive studies [14, 18] have been carried out to explain how ABA plays a role in the regulation of stress responses under drought and salinity stress. ABA is the essential regulatory phytohormone that controls the stomatal opening and closing under drought stress. Increases in the concentration of ABA in the apoplast of guard cells will induce stomatal closing when the plants come across drought [19]. Recently a paper reported that drought stress of *G. uralensis* could increase the effective composition accumulation [20], which could provide the theoretical basis for the correlation of endogenous ABA and the effective composition in *G. uralensis*. So if one uses exogenous ABA to stimulate *G. uralensis* (i.e., induce ABA stress) and observe the change of levels other phytohormones, the result may reveal the influence between different secondary metabolites.

Research on the function and metabolism of phytohormones depends on the ideal analytical method for simultaneous quantification of these structurally diverse compounds. Gas chromatography coupled to mass spectrometry (GC–MS) is a useful tool for hormone analysis owing to its sensitivity [21–23]. However, GC–MS is used with volatile compounds and requires the derivatization of hormones before analysis. The process of derivatization may degrade thermolabile compounds at high temperatures [24]. An alternative tool is liquid chromatography coupled to mass spectrometry (LC–MS). The approach is carried out by LC–MS/MS with multiple reaction monitoring (MRM) in a triple quadrupole mass spectrometer; this method has been applied to simultaneously quantify specific classes of phytohormones, including auxins, ABA, JA, SA, GAs, and cytokinins [25–28]. Furthermore, ultra-high-performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC/ESI–MS/MS)

has been used to analyze several phytohormones in crops of economic value [29–31]; however, there is no report about the simultaneous quantification of phytohormones with UPLC/ESI–MS/MS in medicinal plants, and how to monitor changes of phytohormones has become a big problem, which may be a barrier to the development of studies on the mechanisms of effective composition accumulation in medicinal plants.

Here we present a new efficient simplified UPLC/ESI–MS/MS method for the simultaneous analysis of GA₃, ABA, JA, indole-3-acetic acid (IAA), and SA in *G. uralensis* leaves. In our study, only 100 mg of fresh plant tissues is needed to quantify the phytohormones by using C₁₈ solid-phase extraction (SPE) for concentration and purification. Five phytohormones with internal standard cinnamic acid (CA) were separated within 4 min, which was superior to the HPLC–MS/MS analyses taking 30 min. In addition, CA was used instead of isotope-labeled internal standards. The whole procedure proved to be simpler and faster. Finally, the method was successfully applied to monitor levels of phytohormones in *G. uralensis* under ABA stress.

Experimental

Chemicals and reagents

GA₃, ABA, JA, IAA, and SA were purchased from Sigma Chemical Co. (St Louis, MO, USA). CA was purchased from the National Institutes for Food and Drug Control (Beijing, China). The structures of all the phytohormones and CA are illustrated in Fig. 1. Formic acid, methanol, and acetonitrile of LC/MS grade were obtained from Fisher

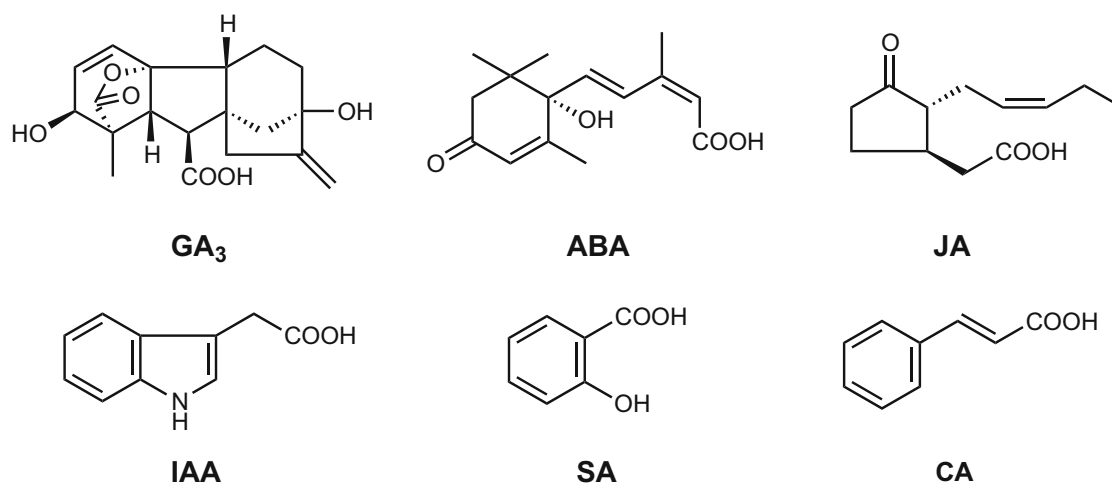


Fig. 1 The structures of the five phytohormones and CA

Scientific (Pittsburgh, PA, USA). Concentrated hydrochloric acid, dichloromethane, and *n*-propanol of analytical grade were obtained from Chemical Reagent Company (Beijing, China). C₁₈ SPE cartridges (3 mL, 500 mg) were purchased from Dikma Technologies Inc. (Lake Forest, CA, USA). Water was produced in-house with a Milli-Q water system (Millipore, Billerica, MA, USA). Each stock solution of GA₃, ABA, JA, IAA, SA, and CA was prepared in methanol, then diluted with methanol/0.05 % formic acid (1:1, v/v) when needed.

Plant materials

The 1-year-old seedlings of *G. uralensis* (Chifeng, Inner Mongolia, China) were moved to grow in an experimental field, and were authenticated as *G. uralensis* by Prof. Chunsheng Liu from Beijing University of Chinese Medicine. The upper sides of leaves were used for the experiments. Two levels of ABA (3.96 and 1.32 mg/L) were applied to the *G. uralensis* by using a small spray to inoculate the whole plant until the leaves were completely wet but water droplets did not drop. The whole experiment used a randomized block design. The treated leaves were collected at day 0 (before inoculation) and another four times after inoculation of ABA (at 7, 15, 30, and 45 days, four times). ABA was sprayed onto the leaves four times during the whole experiment and samples were collected 7 days after each spraying. The leaves from water-inoculated plants were used as blank samples and collected at the same time. The experiment took eight samples for each treatment group at each time period, providing a total of 120 samples. The collected leaves were immediately frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$ for further use.

Sample extraction and purification procedures

Optimized sample preparation: Samples (approximately 100 mg) of fresh leaves were ground to powder in the presence of liquid nitrogen. Subsequently, 400 ng CA was added to each tube. With the addition of 0.5 mL *n*-propanol/H₂O/concentrated HCl (2:1:0.002, v/v/v), the tube was vigorously shaken at $4\text{ }^{\circ}\text{C}$ for 30 min. Afterward, 1 mL dichloromethane was added and the solution was shaken immediately for another 30 min. The mixture was centrifuged at $4\text{ }^{\circ}\text{C}$ for 10 min at 13,000 rpm. The lower layer was then concentrated and transferred to a clean tube. Subsequently, 0.5 mL dichloromethane was added to the plant debris and centrifuged for another 10 min, then the solution was transferred. The solution was collected together (around 1.5 mL), dried under nitrogen, and then dissolved in 1 mL 80 % methanol. The crude extracts were further purified with C₁₈ SPE cartridges, which had been sequentially preconditioned with 6 mL methanol and 6 mL

80 % methanol. After the samples were loaded, 1 mL 80 % methanol was used to wash the SPE cartridge. The eluent (2 mL) was dried under nitrogen gas and finally dissolved in 2 mL methanol/0.05 % formic acid (1:1, v/v), then centrifuged at $4\text{ }^{\circ}\text{C}$ for 1 min at 13,000 rpm to afford a clarified supernatant that was collected prior to UPLC–MS/MS analysis.

In the optimization processes, two different organic extraction solvents, two eluents, and three sample dissolution solvents were tested. In the test of different organic extraction solvents, one was extracted with cold 80 % methanol, the mixture was shaken overnight at $4\text{ }^{\circ}\text{C}$ and centrifuged at 13,000 rpm for 10 min, then prepurified with a C₁₈ SPE cartridge as per the optimized sample preparation. The other extraction solvent was *n*-propanol/H₂O/concentrated HCl (2:1:0.002, v/v/v) and dichloromethane, which was used according to the optimized sample preparation. In the test of different eluents, 70 % methanol and 80 % methanol were used to wash the SPE cartridge. In the test of different sample dissolution solvents, 100 % methanol, methanol/0.025 % formic acid (1:1, v/v), and methanol/0.05 % formic acid (1:1, v/v) were tested. After the purification procedure using the C₁₈ SPE column, the eluate of the five standard compounds was evaporated to dryness and finally dissolved in 2 mL 100 % methanol, methanol/0.025 % formic acid (1:1, v/v), or methanol/0.05 % formic acid (1:1, v/v).

Liquid chromatography and mass spectrometry

A Waters ACQUITY UPLC system (Waters, Milford, MA, USA) was used with a Waters ACQUITY UPLC BEH C₁₈ column (2.1 × 100 mm, 1.7 μm). The mobile phases consisting of mobile phase A (acetonitrile) and mobile phase B (water with 0.05 % formic acid) were used with a gradient elution of A/B from 20:80 (0–0.5 min, hold for 0.5 min), 20:80 to 30:70 (0.5–1 min), 30:70 to 38:62 (1–3 min), 38:62 to 50:50 (3–3.5 min), 50:50 to 70:30 (3.5–3.7 min), 70:30 to 20:80 (3.7–3.8 min), and 20:80 (3.8–4 min, hold for 0.2 min) at a flow rate of 0.40 mL/min. The injection volume for all samples was 10 μL. The column was maintained at $30\text{ }^{\circ}\text{C}$ during analysis, and the samples were kept at $4\text{ }^{\circ}\text{C}$.

The UPLC system was coupled to a Xevo TQ-S mass spectrometer (Waters; Etten-Leur, the Netherlands) equipped with an ESI source. The MS was operated in MRM mode with four time-segmented scannings by using electrospray ionization in negative ion mode with a capillary voltage of 2.5 kV, a source temperature of $150\text{ }^{\circ}\text{C}$, a desolvation temperature of $400\text{ }^{\circ}\text{C}$, a desolvation gas flow of 800 L/h, and a cone gas flow of 150 L/h. Cone voltage and collision energy were optimized for each compound individually. Peak identification and quantification was

performed using MassLynx software version 4.1. Peak identification was according to the retention time and precursor and product ions that both corresponded to the reference substance; peak quantification was according to peak area.

Results and discussion

Optimization of extraction and purification procedures

In order to determine the extraction efficiency of different organic solvents, two simultaneous extractions were tested. Figure 2 shows the extraction efficiencies of the target phytohormones obtained by using different organic solvents. The recovery yields of the five phytohormones ranged from 68.5 % for JA to 113.74 % for GA₃ when extracted with 80 % methanol, while they ranged from 87.76 % for JA to 107.88 % for IAA when extracted with *n*-propanol/H₂O/concentrated HCl (2:1:0.002, v/v/v) and eluted with 80 % methanol. The result showed that the recoveries of the five phytohormones extracted with *n*-propanol/H₂O/concentrated HCl (2:1:0.002, v/v/v) and dichloromethane were better and had higher reproducibilities than those extracted by using 80 % methanol. Thus, *n*-propanol/H₂O/concentrated HCl (2:1:0.002, v/v/v) and dichloromethane were chosen as the extraction solution.

The quantification of acidic phytohormones can be easily affected by lipids, chlorophyll, and other neutral and basic substances in plant extracts. It was reported that reversed-phase SPE columns such as C₁₈ cartridges could concentrate acidic phytohormones and 70–80 % methanol could dissolve the vast majority of phytohormones [32]. However, the matrix effects and recoveries of phytohormones were unsatisfactory. In the present study, 70 % methanol and 80 % methanol were tested as eluent in order to obtain accurate data. As shown in Fig. 2b, c,

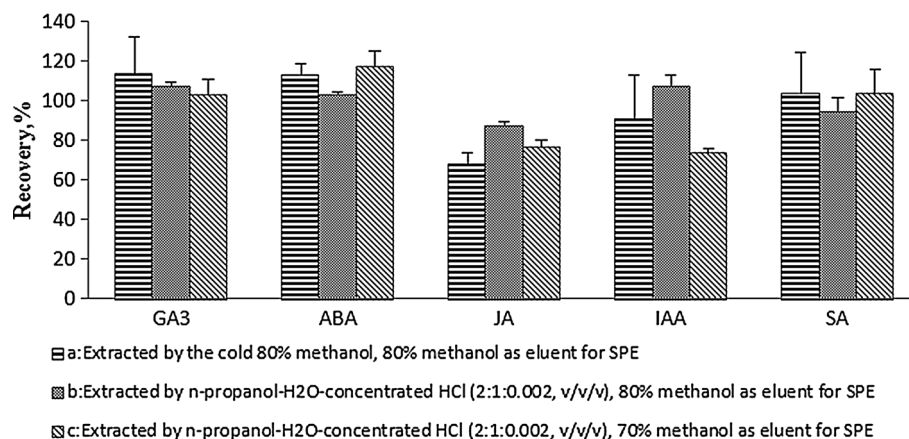
phytohormones eluted with 80 % methanol showed good recoveries for all five phytohormones (87.76–107.88 %), while those eluted with 70 % methanol were only good for GA₃ (103.56 %), ABA (117.18 %), and SA (104.28 %), but not good for JA (76.60 %) and IAA (73.66 %). Comparatively, 80 % methanol was more suitable for the targets, and the five phytohormones could be eluted completely with 1 mL 80 % methanol. Thus, 80 % methanol was selected as the eluent for the purification procedure in this study.

In order to obtain a high signal, the sample dissolution solvent was examined. Although the carboxyl group could be ionized in the negative mode, higher pH values resulted in undesirable peak shape and signal. Taking SA for example, it can be seen in Fig. 3 that compared with the other solvents, the peak heights were significantly enhanced by using methanol/0.05 % formic acid (1:1, v/v) as the solvent and afforded sharper peak shape and better peak symmetry. The peak height corresponding to 100 % relative intensity was 4.55e4 cps for 100 % methanol (Fig. 3a), 5.44e4 cps for methanol/0.025 % formic acid (1:1, v/v) (Fig. 3b), and 5.90e4 cps for methanol/0.05 % formic acid (1:1, v/v) (Fig. 3c). Therefore, methanol/0.05 % formic acid (1:1, v/v) was chosen for the subsequent analysis.

UPLC–MS/MS optimization and MRM transition selection

The mass spectrometer parameters were optimized for the phytohormone quantitation. GA₃, ABA, IAA, JA, SA, and CA were all analyzed in negative scan mode as [M–H][–] ions. The different phytohormones were specifically quantified using precursor and product ions which were almost the same as in a previous report [28]. It should be noted that the transition *m/z* 345 → 239 for GA₃ was previously reported to have the highest intensity [32]. In our full-scan product ion experiments, we found that the intensity of the transition *m/z* 345 → 143 was two times

Fig. 2 The extraction efficiencies of the five phytohormones by using different organic solvents



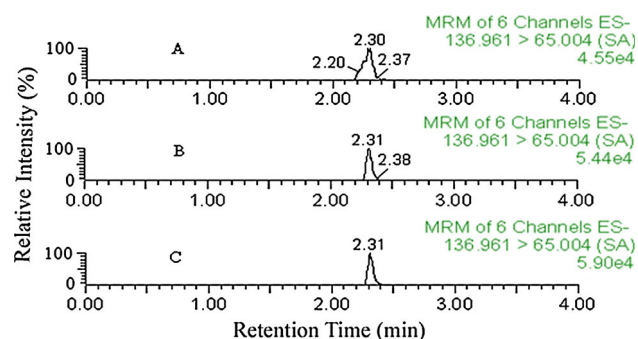


Fig. 3 Effect of the sample dissolution solvent on the signal peak height (taking SA for example): **a** 100 % methanol, 4.55e4 cps; **b** methanol/0.025 % formic acid (1:1, v/v), 5.44e4 cps; **c** methanol/0.05 % formic acid (1:1, v/v), 5.90e4 cps

higher than that of m/z 345 \rightarrow 239 in the ESI-MS/MS spectra, so m/z 345 \rightarrow 143 was chosen for the quantitation. Table 1 summarizes the optimum parameters for the five phytohormones and CA.

The five phytohormones were separated using a C_{18} column followed by analysis via ESI-MS/MS. A UPLC gradient was needed to enhance the sensitivity and to lower ionization suppression. Methanol, acetonitrile, and water with variable pH values were tested for these acidic targets. Finally, acetonitrile and water containing 0.05 % formic acid was found to separate the six targets within 4 min with sharper peak shape and better peak symmetry, which was superior to the HPLC-MS/MS analyses taking 30 min. Total ion current chromatograms of the five phytohormones and CA are shown in Fig. 4. In addition, CA was

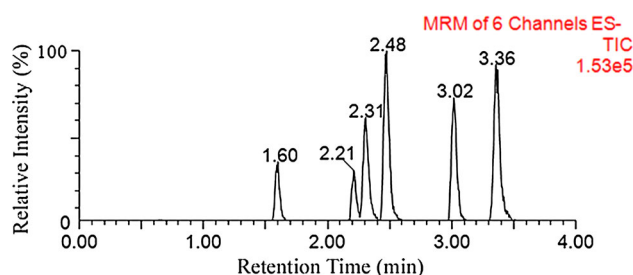


Fig. 4 Total ion current (TIC) of the five phytohormones and CA. MRM transitions: RT 1.60 min, GA_3 (345.15 \rightarrow 143.01); RT 2.21 min, IAA (174.02 \rightarrow 130.45); RT 2.31 min, SA (136.96 \rightarrow 65.00); RT 2.48 min, ABA (263.03 \rightarrow 153.02); RT 3.02 min, CA (147.02 \rightarrow 76.99); RT 3.36 min, JA (209.05 \rightarrow 59.00)

used as the internal standard instead of isotope-labeled compound. Isotope-labeled internal standards such as d_5 -IAA, d_4 -SA, d_6 -ABA, d_5 -JA, and d_2 - GA_3 were expensive and not readily available. In any event, CA has a similar structure to the target phytohormones; not only did it eliminate the matrix effect and improve the recovery but it also made the experiment simpler.

Method performance

The calibration curves were constructed on the basis of the peak area ratios of the analytes to internal standard versus concentrations of the analyte standards. Table 2 provides a summary of the calibration curves, correlation coefficients (R^2), linear ranges, quantification limits (LOQs), and detection limits (LODs) of the five phytohormones. All five

Table 1 Optimized MS/MS conditions for the quantitation of the five phytohormones and CA

Analytes	Scan mode	Retention time (min)	Parent ion (m/z)	Daughter ion (m/z)	Cone voltage (V)	Collision energy (eV)
GA_3	Negative	1.60	345.15	143.01	42	30
ABA	Negative	2.48	263.03	153.02	10	12
JA	Negative	3.36	209.05	59.00	36	12
IAA	Negative	2.21	174.02	130.45	14	8
SA	Negative	2.31	136.96	65.00	38	26
CA	Negative	3.02	147.02	76.99	32	22

Table 2 Quantitation results of the five phytohormones under optimized UPLC-MS/MS conditions

Phytohormone standard	Calibration curve	Correlation coefficient (R^2)	Linear range (ng/mL)	LOQ (ng/mL)	LOD (ng/mL)
GA_3	$y = 864.108x + 5.66910$	0.9947	0.02–19.2	0.02	0.01
ABA	$y = 872.291x + 3.66448$	0.9955	0.08–76.8	0.02	0.005
JA	$y = 549.646x - 2.30486$	0.9955	0.08–80	0.02	0.01
IAA	$y = 2.00351x + 9.72435$	0.9912	8.75–4480	8.75	4.38
SA	$y = 2.50811x + 3.69553$	0.9919	2.88–736	2.88	0.18

y peak area ratio of the standard and the internal standard, x concentration ratio of the standard (ng/mL), LOQ limits of quantification, LOD limits of detection

correlation coefficients were higher than 0.99. The limits of detection for the different phytohormones were in the range 0.005–4.38 ng/mL, and the limits of quantification ranged from 0.02 to 8.75 ng/mL. The reproducibility was evaluated by analyzing the intraday ($n = 6$) and interday ($n = 3$) variations for three concentrations. The RSDs of the retention times and the peak areas of the intraday variation were below 0.43 and 5.85 %, respectively, while for the interday variation, the RSDs were below 0.72 and 9.04 %, which illustrates that the stability of the sample solutions and the method’s repeatability were all acceptable.

The extraction recoveries and matrix effects of the phytohormones were investigated with three different concentrations. Phytohormone standards with CA were added, then extracted and analyzed by UPLC/ESI–MS/MS. When it comes to matrix effect, solution A was the standard and CA in methanol/0.05 % formic acid (1:1, v/v), solution B was the extract and the standards and CA were added after extraction; matrix effect (%) = $B/A \times 100$ [33]. The averages and the RSDs ($n = 6$) of the recoveries and matrix effects are reported in Table 3. For all analytes, the mean analytical recovery was in the range of 80.33 to 114.99 %, with a matrix effect ranging from 84.89 to 119.20 %; these values are superior to those in previous studies [28, 30]. The results showed that this method was versatile for the analysis of the five phytohormones, and the chosen internal standard was good for the analysis.

Analysis of leaf tissue of *G. uralensis*

This method was applied to the determination of phytohormones in leaf tissue of *G. uralensis* stimulated by ABA.

The levels of GA₃, ABA, JA, and SA in the plant extracts were successfully quantified in MRM mode, but this was not possible for IAA because of the low level of this phytohormone in mature leaves. The content of the aforementioned phytohormones among eight different individuals was about GA₃ 0.48 ng/g, ABA 162.26 ng/g, JA 80.37 ng/g, and SA 7040.69 ng/g fresh weight (FW, $n = 8$) in natural conditions, respectively. After stimulation of the leaf tissue of *G. uralensis* with ABA, the levels of GA₃, ABA, JA, and SA increased at different times (Fig. 5). There was a large increase in the level of ABA 7 days after treatment with 3.96 mg/L ABA, and the ABA concentration remained higher than control leaves until 45 days after treatment. Rapid growth of JA was seen at 7 days after treatment with 1.32 mg/L ABA, and then JA showed a decline at 15 days. But the level of JA was always higher than control leaves until 45 days after treatment with 3.96 mg/L ABA. GA₃ also performed like JA; rapid growth of GA₃ was seen at 7 days after treatment with 1.32 mg/L ABA, and then it showed a decline at 15 days. GA₃ and JA both showed an increase in concentration after the levels reached their lowest point after treatment with 1.32 mg/L ABA. When it came to SA, the control leaves and treated leaves showed almost the same tendencies (except for at 45 days), but the means were all higher than reported elsewhere [28, 31]. Usually, when plants are confronted with an external stimulus, SA and JA in the plant can induce resistance [14–17]. The concentrations of JA and SA usually increase in the short term and then show a tendency to decrease in the long term. In the present study, the change in SA concentration in treated leaves was very similar to that in the control leaves. The concentration of JA in leaves treated with 3.96 mg/L ABA was higher

Table 3 Recoveries and matrix effects (IS-MF) of the five phytohormones for extraction

Analyte	Added (ng/g)	Recovery (%) ($n = 6$)		IS-MF (%) ($n = 6$)	
		Average of recovery	RSD (%)	Average of IS-MF	RSD (%)
GA ₃	1.8	85.19	8.32	89.68	9.46
	4.5	88.74	5.62	97.04	9.13
	9	107.26	2.04	116.69	7.83
ABA	150	97.43	6.13	86.53	2.35
	375	99.76	3.24	107.54	9.72
	750	103.40	1.36	119.20	4.90
JA	30	80.33	2.64	85.03	5.40
	75	80.56	4.25	97.84	8.61
	150	87.76	1.91	114.85	6.68
IAA	900	92.07	8.79	84.89	5.18
	2250	108.60	6.09	118.31	6.71
	4500	107.88	4.73	95.88	9.16
SA	1000	109.50	9.03	85.24	7.02
	2500	114.99	8.35	94.53	8.54
	5000	94.40	7.77	100.27	7.23

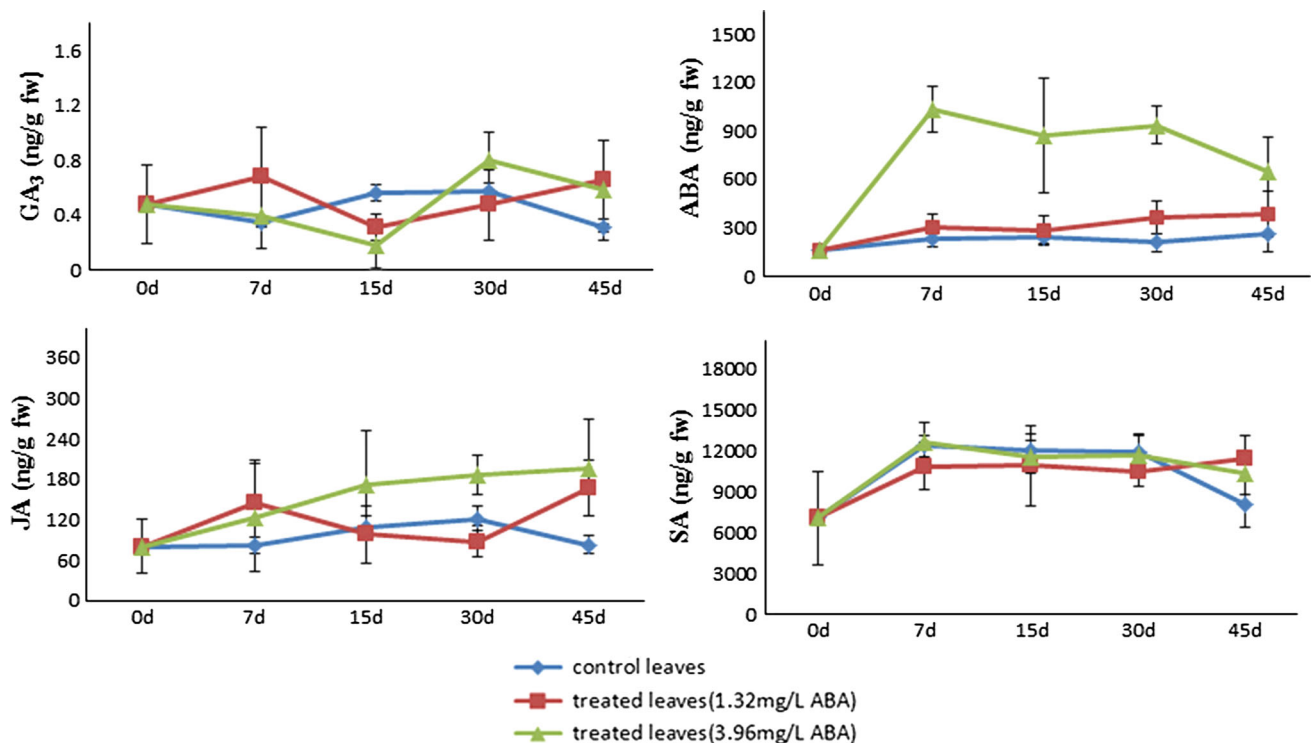


Fig. 5 Concentration changes of phytohormones in control leaves and leaves stimulated by treatment with ABA (3.96 or 1.32 mg/L) of *Glycyrrhiza uralensis*. All leaves were collected at the indicated time

after treatment, providing a total of 120 dependent samples from different individuals. Values are expressed as mean \pm SD ($n = 8$)

than that in control leaves until 45 days. It did not show any tendency to decrease. However, there has been no previous report about *G. uralensis* stimulation by ABA. The result in this paper may suggest that the regulation mechanism of plants under ABA stress was not the same as it was following stimulation by oxalic acid [24] or insect pests and wounding [31]. Plants develop a wide range of adaptive mechanisms to meet these adverse conditions, including adjustment of growth and development brought about by changes in stomatal activity, especially for the level of ABA changes under drought stress [34]. Glycyrrhizic acid is an effective component for quality control of *G. uralensis* according to the Chinese pharmacopoeia. Recent studies showed that there was a positive correlation of endogenous ABA and glycyrrhizic acid under drought stress [35]. As we know, ABA biosynthesis occurs via the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, while glycyrrhizic acid biosynthesis occurs via the mevalonate (MVA) pathway. The two pathways intersect at isopentenyl diphosphate (IPP) or its isomer dimethylallyl diphosphate (DMAPP). The change of glycyrrhizic acid content may be suggested as a possible feedback regulation mechanism in plant growth control. When ABA increases in a short period under drought stress or exogenous stimulus, it may lead to the increase of the content of IPP or DMAPP, and then it may lead to the increase of

glycyrrhizic acid via the other synthetic route. This type of mechanism was verified in grapes such that when the level of ABA increased, the content of anthocyanin in another synthetic route was increased [36]. The change of JA and SA content could also possibly be explained by the feedback regulation mechanism. It may suggest that the mutual influence between different synthetic routes determines the influence of secondary metabolites.

G. uralensis usually grows in arid and semiarid desert grassland. Because of this wild environment, *G. uralensis* may suffer stresses such as drought, salt, and high or low temperature. According to recent studies, there is a negative correlation between output and efficacy in *G. uralensis* [37]. So if we could confirm that ABA definitely increased the content of glycyrrhizic acid, it could be used to control cultivated *G. uralensis* growth in suitable and controllable conditions. This method could provide a new way to solve the lack of wild resources and guarantee the sustainable development of *G. uralensis*.

Conclusion

GA₃, ABA, JA, IAA, and SA are the essential players in the regulation of plant growth. Knowledge about their spatial and temporal distribution in medicinal plant tissues

is necessary for understanding the mechanisms of plant responses to external stimuli, and thereafter for further understanding the mechanisms of effective composition accumulation. In the present study, an efficient simplified UPLC/ESI–MS/MS method for the simultaneous determination of GA₃, ABA, JA, IAA, and SA in the medicinal plant *G. uralensis* was developed. A C₁₈ SPE method was used in the extraction step, and the analytical recovery was improved by using *n*-propanol/H₂O/concentrated HCl (2:1:0.002, v/v/v) for extraction and 80 % methanol for elution. As a result of the presence of a carboxyl group in the five phytohormones, finding balance between the retention and the negative ionization was essential to the analysis. The use of methanol/0.05 % formic acid (1:1, v/v) as the dissolution solvent and mobile phase afforded sharper peak shape and better peak symmetry. CA was chosen as the internal standard instead of isotope-labeled internal standards owing to its lower price and easier availability; CA also has the advantage on eliminating the matrix effect and improving the recovery. Finally, five phytohormones with internal standard were separated within 4 min, with good linearities and high sensitivity. For all analytes, the mean analytical recovery was in the range of 80.33–114.99 %, with a matrix effect ranging from 84.89 to 119.20 %; these values are superior to those in previous studies.

This method was successfully applied to the phytohormone analysis of *G. uralensis* stimulated by ABA. The levels of GA₃, ABA, JA, and SA were increased at different times (IAA was not determined because of its low level in mature leaves). The changes of these phytohormone levels were not the same as those reported in plants stimulated with oxalic acid, insect pests, and wounding, which may indicate that the mutual influence between different synthetic routes determines the influence of secondary metabolites. Further experiments are being conducted to verify the crosstalk effect between specific phytohormones and effective composition in *G. uralensis* in the stress model.

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