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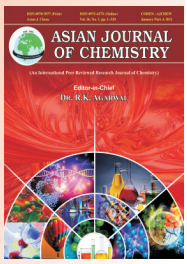

Simultaneous Determination of Four Classes of *Acanthopanax giraldii* Hormone by HPLC Method

SHUN-LIN ZHENG[1], JIN-LIANG LIU[1], RUI GU[2,*], MENG-LIANG TIAN[1], SHI-HONG ZHONG[3] and YUN CAO[2]

[1]College of Agronomy, Sichuan Agricultural University, Wenjiang 611130, P.R. China
[2]National Medical College, Chengdu University of Traditional Chinese Medicine, Chengdu 611131, P.R. China
[3]Pharmaceutical School, Chengdu Medical College, Chengdu 610083, P.R. China

*Corresponding author: Fax: +86 28 86290870; Tel: +86 15108237310; E-mail: 664893924@qq.com

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A simple and quick method is described for the confirmation of hormones in annual branches of *Acanthopanax giraldii*. The hormones in the samples were extracted by using methanol and analyzed by HPLC. Chromatography separation was performed by using Agilent 1100 series HPLC system with symmetry C_{18} column and gradient elution with a mixture of two solvents: methanol and 1 % aqueous acetic acid. The effluent was monitored by a VWD detector set at 254 nm. The average recoveries of zeatin, gibberellins, indole acetic acid and abscisic acid were 99.43, 97.64, 93.16 and 95.28, respectively (n = 3). The limits of detection and quantification were 0.044 and 0.133 μg mL^{-1}, respectively. The method has been successfully applied to analysis of 64 samples, zeatin, gibberellins, indole acetic acid and abscisic acid content of leaves ranged from 3.57-84.83, 0.00-43.09, 0.00-92.51 and 0.00-53.78 ng g^{-1}, the zeatin, gibberellins, indole acetic acid and abscisic acid content of rinds ranged from 3.71-38.91, 0.00-34.36, 0.00-6.57 and 0.00-22.07 ng g^{-1}.

Keywords: Hormones, HPLC, *Acanthopanax giraldii*, Quantitative determination, Method validation.

INTRODUCTION

Acanthopanax giraldii Harms is a Araliaceae deciduous shrub plant and mainly locates in Sichuan, Gansu, Ningxia provinces, China. The stem bark of *A. giralidii* is used as a material for traditional Chinese medicinal (Tibetan medicine) and the essential biological active ingredients are polysaccharide, adenosine, hyperoside, chlorogenic acid and isofraxidin[1-5], which has long been used in the treatment of rheumatism, immunomodulation, antitumor, antiinflammation and antivirus[6-9].

Every year, the annual branch barks were harvested in May and the medicinal herbs production depends on the length and numbers of annual branches. Interestingly, our recent study has discovered that the stronger germination ability at the branches, the more branches of each stubbles grow. Averagely, 2.04 more branches will grow. This phenomenon is known as 'the more cutting, the more lush'. It is believed that this is the result of plant hormone regulation. The plant hormones are important natural mediate substances which regulate the processes in plant's life cycle, for instance, differentiation, morphogenesis, growth and metabolism[10-14]. On the basis of the structure and physiological function, hormones are categorized into several major classes including cytokinins, abscisic acid (ABA), auxins, gibberellins (GA), jasmonates (JA), ethylene and salicylic acid[15].

Finding out the content of different groups of plant hormones can explain the different stage of plants such as growth, morphogenesis and differentiation. Therefore, selecting or developing a highly efficient and sensitive determination method is very important. Traditionally, enzyme-linked immunosorbent assay (ELISA) was utilized in field of the plant hormone analysis. Immunoassay techniques, which are excellent in specificity due to the unique ligand-antibody binding, can be used as a sensitive for estimation of hormones levels[16]. However, the antibody preparation required for this technique is rather laborious and cross-reactivity of the antibody with other plant compounds including hormone analogues is inevitable. Due to the gradual development of new technology, mass spectrometry has been widely applied for analysis of the plant hormones. However, the gas chromatography-mass spectrometry (GC/MS) requires considerable sample purification in prior to GC analysis and break down the labile compounds in analysis progress[17]. Due to the high performance liquid chromatography (HPLC) does not require tedious derivatization steps, selectivity and high sensitivity which have been adopted for the quantitative analysis of plant hormones, including indole acetic acid, abscisic acid and cytokinins[18-20]. Various reported detection methods have been used, but all of which are not suitable for determination of *A. giralidii* hormones.This result may be due to the sample of different ingredients, or extraction

and purification methods are also important for the quantification of plant hormones.

Therefore, the aim of this study is to develop the simultaneous analysis of different groups of zeatin, indole acetic acid, gibberellins and abscisic acid by HPLC[18-20]. Further, development method for analysis of different parts samples is applied, for the future study of the hormones change rule of *A. giralidii* and helping better production.

EXPERIMENTAL

Indole acetic acid was purchased from Phytotechnology Laboratories (Shawnee mission, USA); zeatin, gibberellins and abscisic acid were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Methanol (HPLC grade) was purchased from Fisher Scientific, Inc. Acetic acid, petroleum ether, ethyl acetate and *n*-butyl alcohol (all are AC grade) was obtained from Chengdu Kelong Chemical Factory (Chengdu, China). Water (HPLC grade) was purified with Milli-Q Plus system (Millipore, Bedford, MA, USA).

A. giralidii of annual branches on the top, middle and base of the rind and leaves were used in the experiment. Sixty four samples were collected from the experimental field during June, July and August.The above samples were weighted, placed in valve bag (10#) and then immediately frozen in liquid nitrogen and stored at -80 °C.

Sample extraction and purification: The extraction method was employed as described previously with minor modifications[21-23]. 10 g weighed sample was put into the mortar and added the precooling methanol to100 mL, then after the weak light grinding into homogenate and put in 4 °C refrigerator inner extracting overnight, the filtered residue extracted with methanol repeat three times, each time at 4 °C, 4000 rpm, centrifugal 10 min and combined with the supernatant, then added to 1/2 volume of petroleum ether (boiling range of 60-90 °C) extraction of 3 times and removing the organic phase. The aqueous phase is through the C_{18} column and the pH value was adjusted to 3, then added to 0.5 volume of ethyl acetate extraction of 3 times, removing the aqueous phase and combined with the organic phase, nitrogen gas dried and dissolved with 1 mL methanol, the solutionswere filtered through 0.45 µm syringe filter and collected into 1.5 mL vial as sample.

HPLC instrumental setup and procedure: HPLC analysis were carried outusing Agilent 1100 LC system (Agilent, USA), which included a quaternary pump, an ALS auto injector, column oven and a VWD detector, connected to LC Chem. Station. A Symmetry C_{18} column (250 mm × 4.6 mm, 5 µm) from Waters (USA) was used. The column temperature was maintained at 35 °C. The standards and samples were separated using gradient mobile phase consisting of methanol and 1 % aqueous acetic acid (Table-1) and post time 10 min. The flow rate was set at 1 mL min^{-1} and the injection volume was 10 µL. The detection wavelength was set at 254 nm.

RESULTS AND DISCUSSION

Different gradient elution programs were tested to find the optimum separation conditions for the four hormones (indole acetic acid, abscisic acid, gibberellins and zeatin). With respect to separation efficiency and shorter analysis time, the following gradient elution approach was eventually used as the preferred method: the mobile phase was methanol-1 % acetic acid, 1-10 min: 30-40 % methanol, 10-20 min: 40-60 % methanol, then a linear gradient to methanol-1 % acetic acid (60:40, v/v) and methanol-1 % acetic acid (30: 70, v/v) maintained for isocratically 10 min, respectively (Table-1 for the gradient elution program). The sample was injected while a weaker mobile phase was being applied to the analysis system and the strength of the mobile phase was later increased by raising the organic solvent fraction, which subsequently resulted in elution of the retained components. Under the optimum separation conditions, four ingredients were successfully separated within 0.5 h.

TABLE-1
GRADIENT CONDITIONS FOR THE ANALYSIS

Time (min)	Methanol (%)	1 % Acetic acid (%)
0	30	70
10	40	60
20	60	40
30	60	40

Considering the difference absorbance wavelength of different hormones, 254 nm was chosen as the compromised optimum detecting condition and the previous reported literatures[22,23]. Fig. 1 showed that better separation of 4 hormones and this method also allowed for the identification through the comparison of their retention times and of their UV spectra with those of the standards.

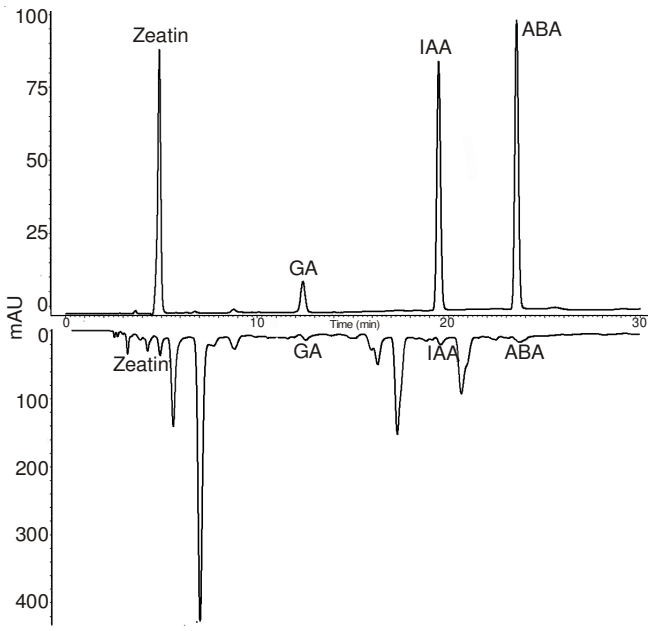

Fig. 1. Typical HPLC chromatograms of hormone standards and actual samples

HPLC method evaluation: The developed method was validated for its linearity, precision, accuracy, LOD and LOQ. Linearity of the method was studied by injecting four known concentrations of the each standard in the range of 0-40 ng mL^{-1} in triplicate. The calibration curves were obtained by plotting the peak area *versus* the amount of the standards. Calibration plots for 4 classes hormones the linear relationship

Y = aX + b, where Y and X are the peak area (mAU) and concentration of the standard solution 10, 40, 20 and 40 ng mL^{-1}, respectively. Linear regression showed good linearity in the range of 0.10-10 ng mL^{-1} (zeatin), 0.40-40 ng mL^{-1} (gibberellins), 0.20-20 ng mL^{-1} (indole acetic acid) and 0.40-40 ng mL^{-1} (abscisic acid) with a correlation co-efficient of 0.991-0.997. This allows the determination of hormones over a wide range of concentrations. This method results similar to that previous study, in which hormones range from 0.5 to 5000 µmol and 10 to 125 µmol[18,24]. Determination of signal-to-noise ratio was calculated under the proposed chromatographic condition. LOD was considered as 3:1 and LOQ as 10:1[25]. The reproducibility of the retention time of four hormones under optimum HPLC conditions was investigated by doing repeated injections (n = 6) of a mixture of the standards. The LOD and LOQ for hormones were found to be 0.03-0.62 and 0.10-2.07 ng mL^{-1}, indicating high sensitivity of the method. All the response characteristics of the hormone standards using HPLC analysis are summarized in Table-2.

The measurement of intra-day and inter-day precision was done by analyzing five samples extracted solution to determine the precision of the method. The intra-day precision (repeatability) was examined by analyzing three times within 1 day, while the inter-day precision (reproducibility) was examined for 3 consecutive days by the proposed method. The results showed acceptable precision of the method with percent retention time and peak area relative standard deviation (% RSD) values lower than 0.4 and 1.54 % (Table-3).

Recovery study of hormones was performed using the method of standard addition for measuring accuracy of method. Known amount of hormones were added to 5 raw materials extraction solution and carried out the extraction procedure, the concentration of standard reached 0.2, 1, 2 and 0.1 ng mL^{-1}. Spiked samples were prepared in triplicate. The recovery was calculated as follows: recovery (%) = (amount found-original amount)/amount spiked × 100. The mean recovery of hormones, representing accuracy of the method, zeatin, gibberellins, indole acetic acid and abscisic acid mean recovery are 99.43, 97.64, 93.16 and 95.28, respectively. These results show that the method is accurate and precise as evidenced by the high recovery.

Application of the method: The proposed HPLC method was used for the quantification of the contents of the hormones in 64 samples (*A. giralidii* of annual branche son the top, middle and base of the rind and leaves). The zeatin, gibberellins, indole acetic acid and abscisic acid content of leaves ranged from 3.57-84.83, 0.00-43.09, 0.00-92.51 and 0.00-53.78 ng g^{-1}, the zeatin, gibberellins, indole acetic acid and abscisic acid content of rinds ranged from 3.71-38.91, 0.00-34.36, 0.00-6.57 and 0.00-22.07 ng g^{-1}. The details are summarized in Table-4. According to the results, some *A. giralidii* samples were rich in hormones, but there was a large variation amongst the samples, because the organ and collection time of samples were different.

Plant hormones are present at low concentrations of 0-1000 ng × g^{-1} WG[21,23], the linear, accuracy, precision, LOD and LOQ were investigated in this method, it is showed that the plant hormones content range from 0.00-92.51 ng g^{-1} WG in our study, which is similar to previous literatures and suitable for detection hormones in *A. giralidii*. Due to the samples, ingredients and quantity different and development the suitable detection method is major. This method is fist developed and analysis hormones in *A. giralidii*.

Conclusion

This study reported the development and evaluation of relatively simple HPLC method for the simultaneous analysis of various hormones (zeatin, gibberellins, indole acetic acid and abscisic acid) with high detection sensitivity. The proposed method is simple and quick with high precision, sensitivity, accuracy and reliability and is appropriate for the detection work. The separation of the four hormone standards, were completed within 0.5 h with well resolved peaks. Run time was shortened and this method developed in this project will definitely be useful for future studies. Sixty four *A. giralidii* samples were investigated for their content of hormones. By comparing the results, a substantial variation among different samples is found. It will be interesting to investigate the factors which lead to the variations in the hormone contents of different *A. giralidii* samples (collection time and organs) in the future.

TABLE-2
RESPONSE CHARACTERISTICS OF HORMONE STANDARDS USING HPLC

Hormone standards	Retention times (min)	RSD (%, n = 6)		Equation of calibration curve	R^2	Linear range (ng mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
		Time	Area					
Zeatin	4.94	0.48	0.35	y = 3079.5x + 110.48	0.995	0.1-10	0.05	0.18
Gibberellins	12.38	0.30	0.27	y = 9.1099x – 7.9028	0.991	0.4-40	0.35	1.51
Indole acetic acid	19.50	0.30	0.13	y = 198.56x – 41.52	0.997	0.2-20	0.62	2.07
Abscisic acid	23.58	0.22	0.09	y = 7141.1x – 76.975	0.994	0.4-40	0.03	0.10

TABLE-3
INTRA-DAY AND INTER-DAY PRECISION OF HORMONES; RESULTS ARE SHOWN AS RSD %

Hormones	Intra-day variability		Inter-day variability					
	Retention time	Area	Day 1 time	Day 1 area	Day 2 time	Day 2 area	Day 3 time	Day 3 area
Zeatin	0.01	1.05	0.22	0.23	0.12	0.32	0.09	0.65
Gibberellins	0.40	1.54	0.18	0.45	0.17	0.45	0.17	0.41
Indole acetic acid	0.08	0.83	0.28	0.31	0.24	0.76	0.18	0.47
Abscisic acid	0.34	0.82	0.18	1.23	0.21	0.53	0.32	0.52

TABLE-4
CONTENT OF HORMONES IN ANNUAL BRANCHES OF *Acanthopanax giraldii* SAMPLES (ng g^{-1}, n = 3)

Samples	Zeatin	Gibberellins	Indole acetic acid	Abscisic acid	Samples	Zeatin	Gibberellins	Indole acetic acid	Abscisic acid
Leaf-1	29.61	6.97	45.45	25.06	Leaf-33	84.83	43.09	7.62	13.52
Leaf-2	29.46	7.05	45.58	27.03	Leaf-34	83.63	41.80	7.54	12.92
Leaf-3	26.94	8.29	89.90	45.90	Leaf-35	13.26	7.86	8.83	5.57
Leaf-4	27.21	8.18	81.64	44.43	Leaf-36	13.19	7.74	9.06	5.52
Leaf-5	46.28	6.86	79.12	47.35	Leaf-37	17.94	9.48	12.92	15.89
Leaf-6	54.74	8.35	92.51	53.78	Leaf-38	17.69	9.08	12.63	15.72
Leaf-7	6.63	0.00	13.18	5.47	Leaf-39	6.38	4.87	5.25	13.82
Leaf-8	8.41	0.00	10.57	5.24	Leaf-40	6.78	4.57	5.04	13.60
Leaf-9	9.17	0.00	12.49	6.81	Leaf-41	14.22	8.20	10.23	15.46
Leaf-10	10.04	0.00	12.67	6.32	Leaf-42	14.22	7.93	9.93	15.53
Leaf-11	29.87	5.77	27.52	13.16	Leaf-43	9.70	6.16	5.33	7.29
Leaf-12	37.66	5.25	23.18	11.71	Leaf-44	9.38	6.08	6.34	7.05
Leaf-13	28.48	0.00	9.09	11.96	Leaf-45	9.65	7.74	5.64	5.54
Leaf-14	29.29	0.00	11.70	14.20	Leaf-46	9.61	7.67	5.50	5.65
Leaf-15	14.12	28.40	18.63	30.65	Leaf-47	9.37	5.42	4.21	4.40
Leaf-16	12.61	27.53	17.75	27.52	Leaf-48	9.23	5.26	3.96	4.36
Leaf-17	11.97	7.39	10.76	14.91	Rind-1	4.02	12.56	2.56	20.99
Leaf-18	11.25	7.98	8.69	15.39	Rind-2	4.77	14.30	3.57	22.07
Leaf-19	16.45	8.06	7.33	9.26	Rind-3	38.61	34.36	6.57	0.00
Leaf-20	16.81	8.21	7.70	8.43	Rind-4	38.91	34.18	6.35	0.00
Leaf-21	3.57	7.72	39.94	30.21	Rind-5	3.71	0.00	5.43	0.00
Leaf-22	4.18	7.33	40.31	29.89	Rind-6	4.05	0.00	5.18	0.00
Leaf-23	8.71	5.41	4.53	4.57	Rind-7	4.14	0.00	0.00	0.00
Leaf-24	9.07	5.65	3.66	5.30	Rind-8	4.04	0.00	0.00	0.00
Leaf-25	10.80	7.92	9.90	24.80	Rind-9	7.62	6.00	5.72	0.00
Leaf-26	10.43	8.22	10.68	26.00	Rind-10	7.47	5.89	5.85	0.00
Leaf-27	10.41	7.06	5.03	5.89	Rind-11	22.07	10.68	0.00	0.00
Leaf-28	11.00	6.98	5.64	5.86	Rind-12	21.68	10.57	0.00	0.00
Leaf-29	41.88	15.55	3.32	0.00	Rind-13	34.88	14.47	0.00	0.00
Leaf-30	40.04	15.62	3.16	0.00	Rind-14	34.50	14.19	0.00	0.00
Leaf-31	11.03	9.71	14.42	8.54	Rind-15	33.85	15.94	0.00	0.00
Leaf-32	10.81	9.89	14.02	8.35	Rind-16	33.04	16.08	0.00	0.00

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