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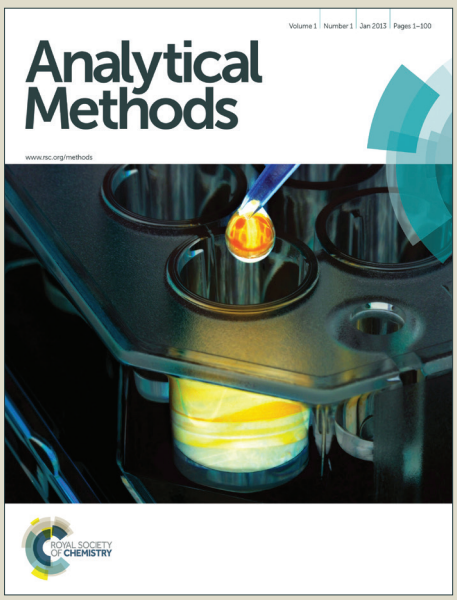

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Simultaneous determination of three alkaloids in *Huperzia serrata* by UPLC-PDA and UPLC-Q/TOF-MS

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Abstract

In the current study, a reliable method has been developed and validated for quantification of three alkaloids (Huperzine A, Huperzine B and Huperzine C) from *Huperzia serrata* based on ultra performance liquid chromatography (UPLC) with photodiode array detector (PDA) and confirmed by tandem quadrupole time-of-flight mass spectrometry (Q/TOF-MS). Separation was performed on Waters BEH shield RP 18 column with a gradient elution. The detection wavelength was set at 310 nm. Evaluation of the method showed good linearity, repeatability accuracy and precision. The limits of quantification varied from 0.11 to 0.4 μg/g depending on the analytes. The proposed method was successfully applied to determine the three alkaloids in 18 batches samples of different origins from China. The results indicated that significant variation in the amount of quantitative ingredients was observed in different parts of samples from different sources. The contents of Huperzine A and Huperzine B in Hainan samples were significantly lower than other areas. The method developed could be helpful for quality control of *Huperzia serrata*. The present study can provide necessary information for the rational utilization of *Huperzia serrata* resources in Hainan province.

Keywords: *Huperzia serrata*; alkaloids; UPLC-PDA; quality control

1 Introduction

Huperzia serrata Thunb. ex Murray (*H. serrata*, Qian Ceng Ta in Chinese), a perennial herb, has been used as a traditional Chinese folk medicine for centuries for the treatment of contusion, strain, swelling and schizophrenia.[1] In the 1980s, Huperzine A (Hup A) and Huperzine B (Hup B) were isolated from this plant.[2] Since Hups A and Hup B have proved to be potent, reversible and selective inhibitors for acetylcholinesterase (AchE) activity, *H. serrata* has been payed more attention from all over the world.[2-4] Huperzine C (Hup C) has also been found to be a strong AChE inhibitor.[5] Furthermore, it has been found that Hup A and Hup B were effective for other cholinesterase-activity-related diseases, such as myasthenia gravis and vascular dementia.[6] For these reasons, many researchers have mainly focused on the alkaloids in the *H. serrata.* Therefore, it is necessary to develop a quick, accurate and selective analytical method for the analysis of alkaloids in *H. serrata*.

H. serrata is distributed mainly in the areas along the Yangtze River and throughout the southern parts of China.[7] Previous researches indicated that the content of Hup A is very low in *H. serrata* and are higher in leaves than other parts. However, the distribution of Hup A, Hup B and Hup C in *H. serrata* from Hainan is not under investigation.

Currently, various analytical methods for Hup A in *H. serrata* can be found,[8-11] however, there are few methods to simultaneously determine Hup A, Hup B and Hup C.[12] Additionally, these procedures are not convenient for analysis, i.e. time-consuming, lack of reproducibility. UPLC analysis has the advantage of accelerating speed, improving sensitivity, selectivity and specificity compared to HPLC analysis.[13] Besides, UPLC coupled with mass spectrometry could provide adequate structural information of multiple compounds.[14]

In this study, an UPLC-PDA method was developed for simultaneous analysis of Hup A, Hup B and Hup C in *H. serrata*. The validated method was applied to the assay of 18 samples and investigated the difference on the contents of the three alkaloids in *H. serrate* from different sources. Furthermore, comparative analysis of

alkaloids in different parts of *H. serrate* collected from Hainan, Hunan and Guangxi province was carried out. The contents of Hup A and Hup B were significantly lower than other origins. Due to the complex matrix of *H. serrata*, an UPLC-Q/TOF-MS method was established to confirm the results. These results give some useful insights into the rational utilization of *H. serrata* resources in Hainan province.

2 Experimental

2.1 Plant material

Eighteen batches of *H. serrata* were collected from Chongqing City, Guangxi province, Guizhou province, Jiangxi province, Fujian province, Hainan province, Hubei province and Hunan province, China, respectively. All samples were authenticated by Professor Yulin Lin and Dr. Yaodong Qi, Institute of Medicinal Plant Development, Peking Union Medical College, Chinese Academy of Medical Sciences.

2.2 Chemicals and reagents

Chemical standards of Hup A (NO: 13051711), Hup B (NO: 12091901) and Hup C (NO: 13022816) were purchased from Shanghai Tauto Bio-Technology Co., LTD. (Shanghai, China). The purity of each reference compound was over 98%.HPLC grade acetonitrile (ACN) and methanol (MeOH) were obtained from Fisher Co. Ltd. (Emerson, IA, USA). Trichloromethane ($CHCl_3$), triethylamine (TEA), acetic acid (AcOH) and ammonium acetate of analytical grade were purchased from Beijing Chemical Works (Beijing, China). Other chemicals were of analytical grade. Ultra-pure water was prepared using a Milli-Q academic water purification system (Milford, MA, USA). All the reagents were passed through a 0.22 μm PTFE membrane (Agela Technology, Tianjin, China) before injection into the UPLC system.

2.3 Instruments and conditions

Chromatographic analysis was performed on an Acquity^{TM} UPLC H-Class system (Waters Corp., Milford, MA, USA) including quaternary solvent manager, sampler

manager, column compartment and Photo-Diode Array detector (PDA), connected to Waters Empower 2 data station. Separation was carried out on an Acquity BEH shield RP18 column (100 × 2.1 mm i.d., 1.7 μm; Waters Corp., Milford, MA, USA). The mobile phase consisted of 8 mM ammonium acetate in purified water (solvent A) and ACN (solvent B). The solvent A was adjusted to pH 5.8 by acetic acid and filtered through 0.22 μm membrane before used. The gradient program was as follows: 0-4min, 17% B; 4-6min, 17%-19% B; 6-7min, 19% B; 7-8min，19% -31% B; 11-12min, 31%-47% B; 12-16min, 47% B；16-17min, 47%-100% B; 17-18min 10% B. The flow rate of the mobile phase was kept constant at 0.2 ml/min and the volume of each injection was 5.0 μL. The column temperature was maintained at 35℃ and the detection was recorded at 310 nm. The total run time was 18 min.

2.4 Mass spectrometry confirmation

MS data were recorded using the above UPLC condition on the waters Acquits UPLC system (Waters, USA) coupled with a quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Waters Q/TOF Premier^{TM}). MS analysis was carried out by ESI source in positive ion mode. The optimized condition was desolvation gas at 600L/h, at a temperature of 450℃, cone gas at 30L/h and source temperature at 120℃, capillary and cone voltages at 3.0kv and 40v， respectively. All analyses were performed using the lock spray to ensure accuracy and reproducibility. Leucine-enkephalin was utilized as the lock mass (mass-to-charge ratio m/z 556.2771 for positive mode).The MS data were collected in centroid mode from *m/z* 50 to 1000. Centroid and integrated MS data were processed to generate a multivariate data matrix using MassLynx (Waters Crop.).

2.5 Preparation of standard solutions

A mixed standard stock solution containing Hup A, Hup B and Hup C were prepared in MeOH. The working standard solutions were prepared by diluting the mix standard solution with MeOH to a series of proper concentrations within the ranges: Hup A,

0.11-440 μg/mL; Hup B, 0.40-320 μg/mL; Hup C, 0.30-240 μg/mL. All the solutions were stored at 4°C and filtered through a 0.22 μm nylon membrane prior to injection into the UPLC system.

2.6 Preparation of sample solutions

The *H. serrata* samples were dried at 60°C until constant weight was attained. The extraction of alkaloids was mainly based upon previously described.[11] Briefly, approximately 1.5g dried powder (40 mesh) of each sample was accurately weighed and extracted twice with 60mL of 95% ethanol under refluxing for 2 hours. After filtering off the insoluble material, the ethanol solutions were evaporated to dryness under reduced pressure. The residues were taken up in 0.8% HCL (50mL). Then the aqueous solutions were adjusted to pH 9.0-10.0 with NH_4OH, and extracted at least three times with $CHCL_3$. The combined organic solvent was evaporated and the residues were redissolved in 1.5 mL MeOH, filtered using 0.22 μm microporous film and transferred to an UPLC vial. All samples were determined in triplicate.

2.7 Method validation

The newly developed UPLC method was validated in terms of linearity, precision, accuracy, stability and repeatability according to ICH guidelines.[15] The limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting a series of dilute solutions with known concentrations.

Linearity, LOD and LOQ

Working solutions containing three reference compounds were prepared for construction of calibration curves. At least six levels of the solution concentration were analyzed in duplicates and then calibration curves were constructed by plotting the peak area against the concentration of each analyte. The acquired regression equation was calculated in the form of $Y = aX + b$, where Y and X were the peak area and concentration of the reference compound, respectively. LOD and LOQ were defined as the signal-to-noise ratio equal to 3 and 10, respectively.

Precision, repeatability, stability and accuracy

The intra- and inter-day precisions were investigated by analyzing a mixed standard solution in six replicates during a single day and by duplicating the experiments on three consecutive days. *H. serrata* was analyzed in six replicates with the proposed method to confirm the repeatability of the developed assay. The relative standard deviation (RSD) was calculated as a measurement of precision and repeatability. The analysis of six time period within a day (0, 2, 4, 8, 16, 24h) was used to evaluate the stability of sample solution in 24h. A recovery experiment was carried out to investigate the accuracy of the method. Three different quantities (low, medium and high) of the standards were spiked to a sample (0.75g) which was previously analyzed and whose concentrations of the compounds of interest were known. Then the resultant sample was extracted and analyzed with the above method in triplets at each level. The recovery percentage for the 3 compounds was calculated according to the following equation: (detected amount – original amount)/ spiked amount ×100.

3 Results and discussion

3.1 Analytical method validation

The proposed method for quantitative analysis was validated by determination of the linearity, r^2, LOD, LOQ, intra-day and inter-day precisions, stability, and accuracy. As shown in Table 1, the calibration curves of Hup A, Hup B, Hup C showed good linearity ($r^2 \geq 0.9993$) within the test ranges. The LOD and LOQ were in the range of 0.035-0.08 μg/mL and 0.11-0.40 μg/mL, respectively. What's more, the proposed method showed good precision, repeatability and stability with the RSD less than 2.85%. The overall recoveries of the analytes laid between 95.17% and 99.23% with RSD less than 3.11% (Table 2). All the results mentioned above showed that the proposed method was accurate for the determination of the target analytes in the *H. serrata.*

3.2 Optimization of sample pre-treatment

Sample preparation is an important step for accurate and reliable assay. In order to obtain efficient extraction, multiple related extraction conditions were designed and evaluated, which involved the following factors and corresponding levels: extraction method (ultrasonication and refluxing), extraction repetitions (1 and 2 times), solvent volume (20, 40 and 60 mL), extraction time (1, 2, and 3h) and the concentration of HCL (0.2, 0.4, 0.6, 0.8 and 1.0%). When one of the factors was determined, the others were set at the default (extraction repetitions, 2 times; solvent volume, 60mL; extraction time, 2h; the concentration of HCL, 0.8%). Firstly, comparing refluxing with ultrasonic extraction, the results indicated that the refluxing extraction was more effective than ultrasonic extraction for the tested alkaloids analyzed (Figure 1). Hence, refluxing was selected as the technique of choice for the extraction of analytes in *H. serrata.* As for the volume of 95% ethanol-water solution, 60 mL was found to be more effective for the samples because it provided the highest values in the contents of the three markers. Furthermore, the duration and times of extraction method were also screened to optimize the extraction procedure. In addition, the concentration of HCL was optimized. It was observed that the concentration of HCL did not have significant impact on the extraction efficiency of the method. According to the results, 0.8% was chosen as the optimum concentration of HCL for extraction. The results (Figure 1) showed that the established extraction method was adequate and appropriate for analysis.

3.3 Optimization of UPLC conditions

To achieve the resolution and separation of the studied alkaloids in the shortest possible time and using the least amount of organic solvent, chromatographic conditions were optimized, including columns, mobile phase, flow rate of mobile phase and column temperature. In an initial experiment, a Waters Acquity BEH C18 column (100 × 2.1 mm, 1.7 μm) was tested for the separation of the alkaloids. However, it showed a poor separation for the target compounds in this study. Therefore, other analytical columns, such as shield RP 18 column (100 × 2.1 mm, 1.7 μm) and Hilic C18 (100 × 2.1 mm, 1.7 μm), were compared to separate the

compounds. The BEH Shield RP18 Column contains an embedded-polar group that combines the hydrophobicity of a straight-chain-alkyl ligand (C_{18}) with the hydrophobicity of an embedded polar group (carbamate). This unique bonding chemistry provides complementary selectivity to a C_{18} column. It improves peak shape and separation efficiently for analytes, especially for alkaline compounds. Therefore, the use of shield RP 18 column provided a better resolution of the three analytes. As for the mobile phase, different kinds of the solvent system (ACN - H_2O, ACN-0.1% TEA, ACN- ammonium acetate,) mentioned in the previous literature were tested. It was found that the mixture of ACN and 8 mM ammonium acetate (pH 5.8) was a suitable solvent system, which not only can simultaneously separate the three compounds in the samples, but also be propitious to MS detector. The column oven temperature was also varied from 25 to 40°C to improve peak resolution. In addition, flow rate of mobile phase (0.1, 0.2 and 0.3 mL/min) was probed. The optimized results were: the column temperature was 35°C, flow rate was 0.2 mL/min. The final optimal UPLC condition was defined as above description and a representative chromatogram obtained under the new UPLC-PDA method is shown in Figure 2.

3.4 Comparison of different origins of *H. serrata*

The development UPLC-PDA analytical method was subsequently applied for simultaneous determination of HupA, HupB and HupC in the whole plant of *H. serrata* from different regions in China. Each sample was determination in triplicate. Quantification of each analyte in the samples was calculated with the external standard using the calibration curves. Representative chromatograms of these samples are shown in Figure 2 and the results are summarized in Table 3.

As shown in Table 3, all the constituents investigated coexist in all samples. However, the colors of extracts of the samples from different areas were different (Figure 3). Moreover, the contents of the three alkaloids were varied dramatically among different origins, which may result in difference of quality and efficacy. The amounts of Hup A ranged from 17.00 to 411.32 μg/g, Hup B from 3.13 to 302.56 μg/g

and Hup C from less than LOQ to 73.46 μg/g, which were 24.20-, 96.66-, and 19.03-fold variation, respectively. Also, the sum amount of three analyzed alkaloids in the tested samples varied widely from 57.01 to 712.70 μg/g. It was worth noting that the contents of Hup A and Hup B in Hainan samples were obviously lower than that in the samples collected from other locations. In addition, Hup C contents in Hainan samples were higher or equivalent with the others regions. The results also showed that in all samples (except Hainan), Hup A was the highest component, followed by Hup B and Hup C. On the contrary, the content of Hup C in Hainan samples was highest of the quantitative compositions. In summary, the analyzed ingredients were present in all *H. serrata*, and significant differences in their content.

3.5 Comparison of different parts of *H. serrata*

Although the literature contains numerous reports on the alkaloids contents in *H. serrata*, little researches attention have been developed to compare the active constitutes in different parts of this plant. It was found that the content of Hup A and Hup B in leaves is the highest and that is the lowest in roots.[9,12] For Hup C, the content in roots is higher than it in leaves and stems. However, the three alkaloids in different parts of Hainan sample were not studied. In this work, the proposed method was applied to determine the three alkaloids in different parts of *H. serrata* collected from Hainan, Hunan and Guangxi province. The data (Figure 4) indicated that roots, stems, and leaves (except Guangxi) all consist of the three alkaloids, but the alkaloid contents in roots, stems, and leaves were dramatically different. The content of Hup A and Hup B in aerial parts are much higher than those in root from Hunan and Guangxi. However, the Hainan sample provided the contrary results. The content of Hup C in the root is higher than in other parts. The total alkaloids in leaves was highest, the next was stems. From this point, aerial parts seem to be the optimum medicinal materials.

Up to now, no direct biosynthetic studies have reported to identify the route to Hup A. Besides, no enzymes have been identified in the Huperziaceae plants that might be involved in the production of the Hup A.[1] Only one enzyme, lysine

decarboxylase (LD), has been proposed as the entry point enzyme into the pathways to the Lycopodium alkaloids.[16] Du *et al*[17] analyzed the relationship between the distribution of Hup A and the expression of LD gene in different parts of *H. serrata*. The results showed that LD gene had nearly identical expression among the roots, stems and leaves of *H. serrata*. They speculated that lysine decarboxylase might be not one key enzyme to regulate the biosynthesis of Hup A. Our results showed that Hup A and Hup B had the similar distribution rule (Leaves>stems>roots) in different parts of *H. serrata* (except Hainan samples). This result was consistent with the literature.[9,12,18] We speculated that the biosynthetic pathway of Hup A and Hup B might be same, in which Hup A and Hup B were firstly synthesized in the leaves and then transported from stems to roots.[12,18] Surprisingly, the study found that the distribution of Hup C in roots were significantly higher than the stems and leaves. This result couldn't justified that Hup C and Hup A and B were different secondary metabolic pathways, which required further study. Hainan island (18°10′–20°10′ N latitude and 108°37′–111°03′ E longitude) is the only tropical zone of China, the climate is different from the other regions. Distribution of the three alkaloids in Hainan samples may correlated with the climate.

In China, *Huperzia* plants are widely distributed in tropical, subtropical, and temperate zones and *H. serrata* is the only relatively common species. Nevertheless, these plants are not abundant, growing very slowly and are found only in very specialized habitats. The differences in alkaloids contents of *H. serrata* from different regions may be related to the local ecological environment. Shi *et al*.[19] found that the key environmental factors influencing Hup A content of *H. serrata* in Jiuhua Mountain (Anhui province, China) were the contents of organic matter, TN and TP in the rhizosphere soil, and temperature and rainfall showed the least correlation with Hup A content. However, Wang *et al*.[20] found that Hup A levels were negatively correlated with annual rainfall, and no significant correlations with mean annual temperature or altitude of plots. *H. serrata* normally requiring 15–20 years of growth from spore germination to maturity. The sporophytes of *H. serrata* only reach a height of 5–15 cm and the whole plant body is harvested for Hup A collection.Our previous

studies found that the content of Hup A in different ages of *H. serrata* were of significant difference (seeding or young plants >adult plants). Furthermore, the contents of Hup A and Hup B in *H. serrata* collected at different seasons were evidently different.[10] Therefore, harvest time is also a major factor affecting the content of lycopodium alkaloids in *H. serrata*. Cultivation and regeneration of *H. serrata* is very difficult. In fact, no successful report on the cultivation or propagation of this herb has been published. This presents a very difficult problem for research on the factors influencing biosynthesis and accumulation of lycopodium alkaloids in *H. serrata*. These results did not indicate whether these differences in accumulation are due to environmental, harvesting time or genetic factors. Nevertheless, it is obvious that *H. serrata* possessed a very low content of Hup A, Hup B and Hup C. Fortunately, the developed UPLC-PDA method is suitable for determination of three alkaloids in *H. serrata*.

3.6 MS Confirmation

For a component to be positively confirmed, its retention time has to match that of a standard to within 5%. Firstly，the identification of Hup A, Hup B and Hup C in extracts from the samples was achieved by comparing retention times with standards. Due to the content of the analytes are low and the complex matrix of the *H. serrata*, an UPLC-Q/TOF-MS method was developed to further confirm the identification result. As the negative ionization mode did not give significant signals for analytes, the positive ionization mode was chosen in the study. Secondly, structural confirmation was obtained by ESI-Q/TOF-MS (Table 4). Figure 5 showed that protonated molecular ions $[M+H]^+$ and $[M+Na]^+$ were present as major peaks for the three constitutes within 5.0 ppm. Small amounts of $[2M+Na]^+$ were also detected. Product ion spectra of $[M+H]^+$ showed fragment ions at 240, 226, 198, 184 matching values published in literature and reference standards.[21,22]

4 Conclusion

In the present study, we have established a suitable UPLC-PDA method for

simultaneous quantification of Hup A, Hup B and Hup C in *H. serrata*. This method has already been successfully applied to determine the three alkaloids in different parts and places of *H. serrata*. The results were confirmed by the UPLC-Q/TOF-MS. The data showed that there were remarkable differences in the content of the alkaloids in different parts and places. The results mentioned above showed that the established method is helpful for the quality control of *H. serrata,* and also provide useful information for rational utilization of this resources.

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Figure captions:

Figure 1. .Effects of extraction method, solvent volume, time, repetitions, concentration of HCL of the extraction efficiency of the target analytes in *H. serrata* collected from Chongqing (NO.2), China.

Figure 2. Typical UPLC-PDA chromatograms of mixed standards and samples.

Figure 3. The colors of liquid extracted alkaloids in *H. serrata* from different regions.

Figure 4. Comparative analysis of analytes in differents parts of *H. Serrata*.

Figure 5. MS spectra of Hup A (A), HupB (B) and HupC (C) from sample 16.

Table captions:

Table 1. Calibration curves, test range, LOD, LOQ, precision and repeatability for the three analytes.

Table 2. Recoveries of the target analytes (n=3).

Table 3. Contents of three compounds in 18 tested samples

Table 4. Mass data of the three analytes from *H. serrata* by UPLC-Q/TOF-MS

Table 1 Calibration curves, test range, LOD, LOQ, precision and repeatability for the three analytes

Analytes	Calibration curves	R^2	Linear range (μg/mL)	Precision (RSD, %)		Stability	Repeatability (RSD,%, n=6)	LOQ (μg/g)	LOD (μg/g)
				Intraday	Interday				
Hup A	y = 54729x +144484	0.9995	0.11-440	1.82	1.32	1.23	2.02	0.11	0.035
Hup B	y = 27211x +118341	0.9997	0.40-320	1.56	1.18	1.23	1.72	0.40	0.08
Hup C	y = 25926x -40579	0.9993	0.30-240	2.17	2.56	2.85	2.63	0.30	0.06

Table 2 Recoveries of the target analytes (n=3).

Analyte	Sample (g)	Original (μg)	Added (μg)	Found (μg)	Recovery(%)	RSD（%）
Hup A	0.75	105.47	84.00	188.11	98.37	1.58
			105.00	208.35	97.98	2.04
			126.50	231.00	99.23	1.77
Hup B	0.75	70.71	56.50	126.48	98.71	2.52
			70.00	138.84	97.34	1.49
			85.00	153.88	97.85	1.83
Hup C	0.75	15.19	12.00	26.65	96.46	2.96
			15.00	29.42	95.82	2.24
			18.00	32.14	95.17	3.11

Table 3 Contents of three compounds in 18 tested samples

NO.	Origin	Content (μg/g, n=3)			
		Hup. B	Hup. C	Hup. A	Total alkaloids
1	Chongqing	64.40	16.50	92.32	173.21
2	Chongqing	94.27	20.26	140.63	255.17
3	Guizhou	158.51	24.88	276.68	460.08
4	Guizhou	34.31	<LOQ	99.92	134.23
5	Guangxi	278.60	57.80	290.45	626.85
6	Guangxi	238.69	40.49	249.60	528.78
7	Guangxi	133.47	35.36	229.25	398.08
8	Fujian	132.96	48.98	294.92	476.87
9	Fujian	174.45	63.07	300.84	538.35
10	Jiangxi	34.18	5.24	61.85	101.27
11	Hubei	219.76	3.86	411.32	634.93
12	Hubei	135.81	8.68	232.12	376.61
13	Hunan	302.56	16.23	373.91	692.70
14	Hainan	13.93	68.67	27.90	110.50
15	Hainan	6.88	46.78	22.02	75.67
16	Hainan	10.29	73.46	29.38	113.13
17	Hainan	3.13	36.88	17.00	57.01
18	Hainan	6.21	51.37	17.46	75.04

Table 4 Mass data of the three analytes from *H. serrata* by UPLC-Q/TOF-MS

Analyte	Rt (min)	Molecular formula	Theoretical Mass (Da)	Measured Mass (Da)	Error (ppm)	Fragment ions (ESI+, *m/z*)
Hup B	2.387	$C_{16}H_{20}N_2O$	257.1654$[M+H]^+$	257.1656$[M+H]^+$	0.77	240.1397;198.0926
			279.1473$[M+Na]^+$	279.1468$[M+Na]^+$	1.79	184.0753
Hup C	2.511	$C_{15}H_{18}N_2O$	243.1497$[M+H]^+$	243.1490$[M+H]^+$	1.23	240.1395;226.1233;
			265.1317$[M+Na]^+$	265.1317$[M+Na]^+$	0	198.0941;184.0778
Hup A	2.821	$C_{15}H_{18}N_2O$	243.1497$[M+H]^+$	243.1502$[M+H]^+$	2.05	226.1235;197.0844
			265.1317$[M+Na]^+$	265.1318$[M+Na]^+$	0.37	184.0788

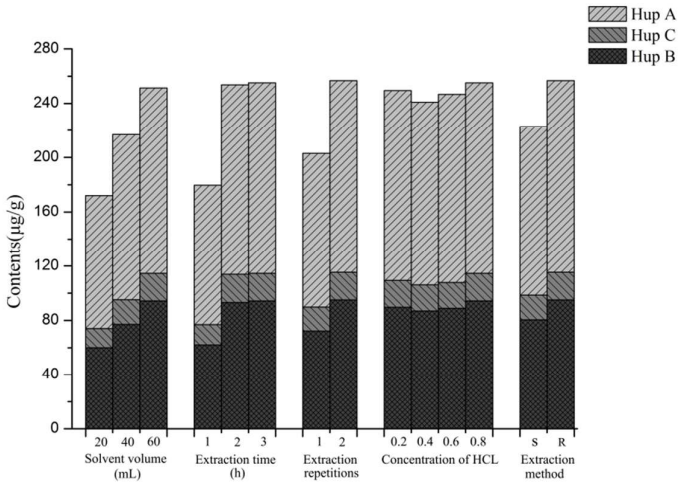

Fig. 1.Effects of extraction method (S, sonication; R, refluxing), solvent volume, time, repetitions, concentration of HCL of the extraction efficiency of the target analytes in *H. serrata* collected from Chongqing (NO.2), China.

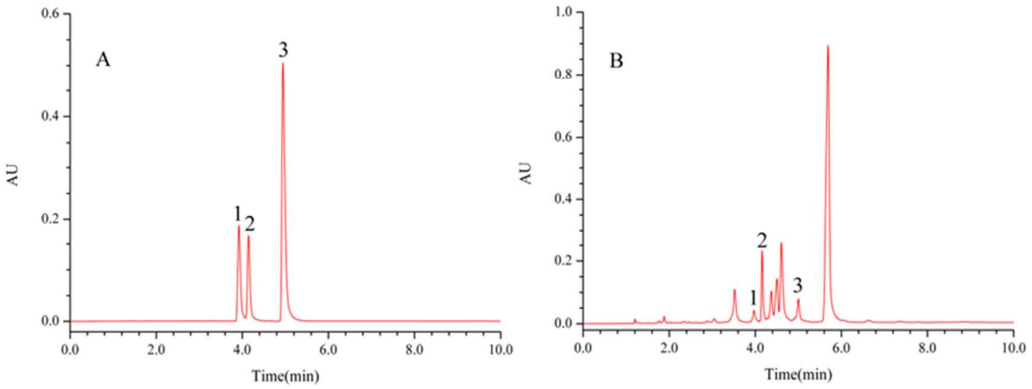

Fig. 2 Typical UPLC-PDA chromatograms of mixed standards and samples. (A) Mixed standards, (B) Sample16 (Hainan); 1, Huperzine B; 2, Huperzine C; 3, Huperzine A

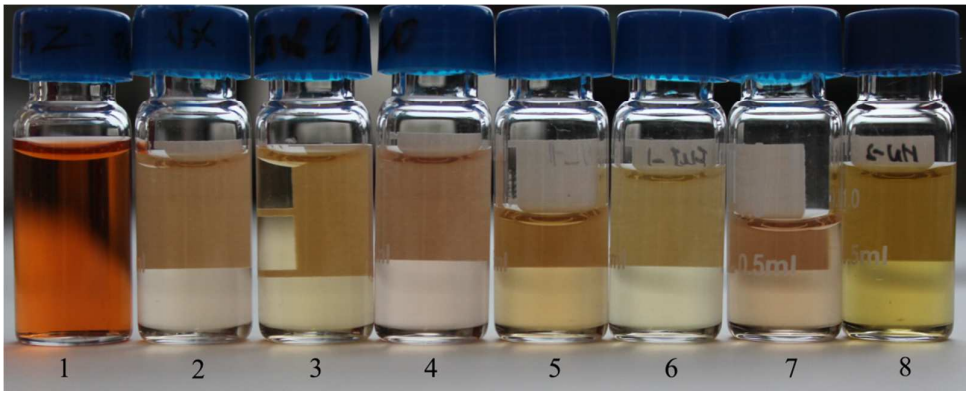

Fig. 3 The colors of liquid extracted alkaloids in *H. serrata* from different regions.
1.Guizhou;2.Jiangxi;3.Guangxi;4.Chongqing;5.Hubei;6.Fujian;7.Hunan;8.Hainan

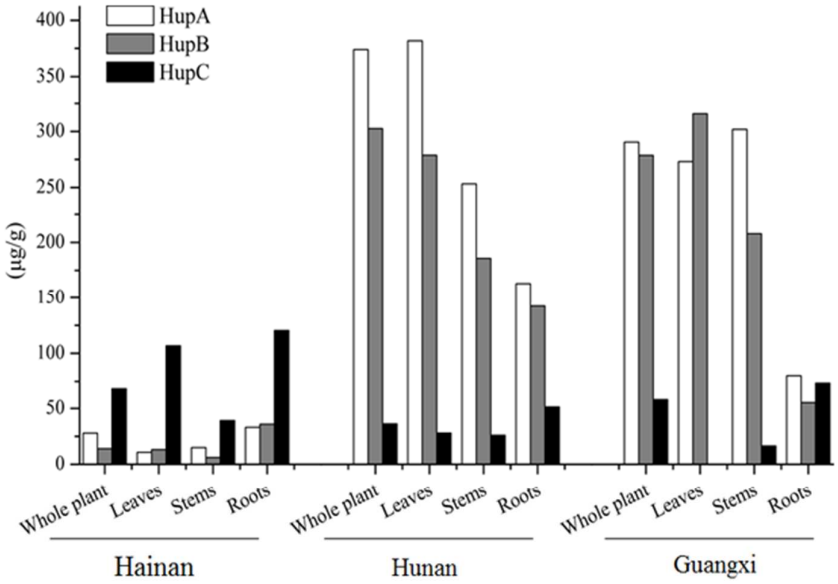

Fig. 4 Comparative analysis of analytes in differents parts of *H. Serrata*.

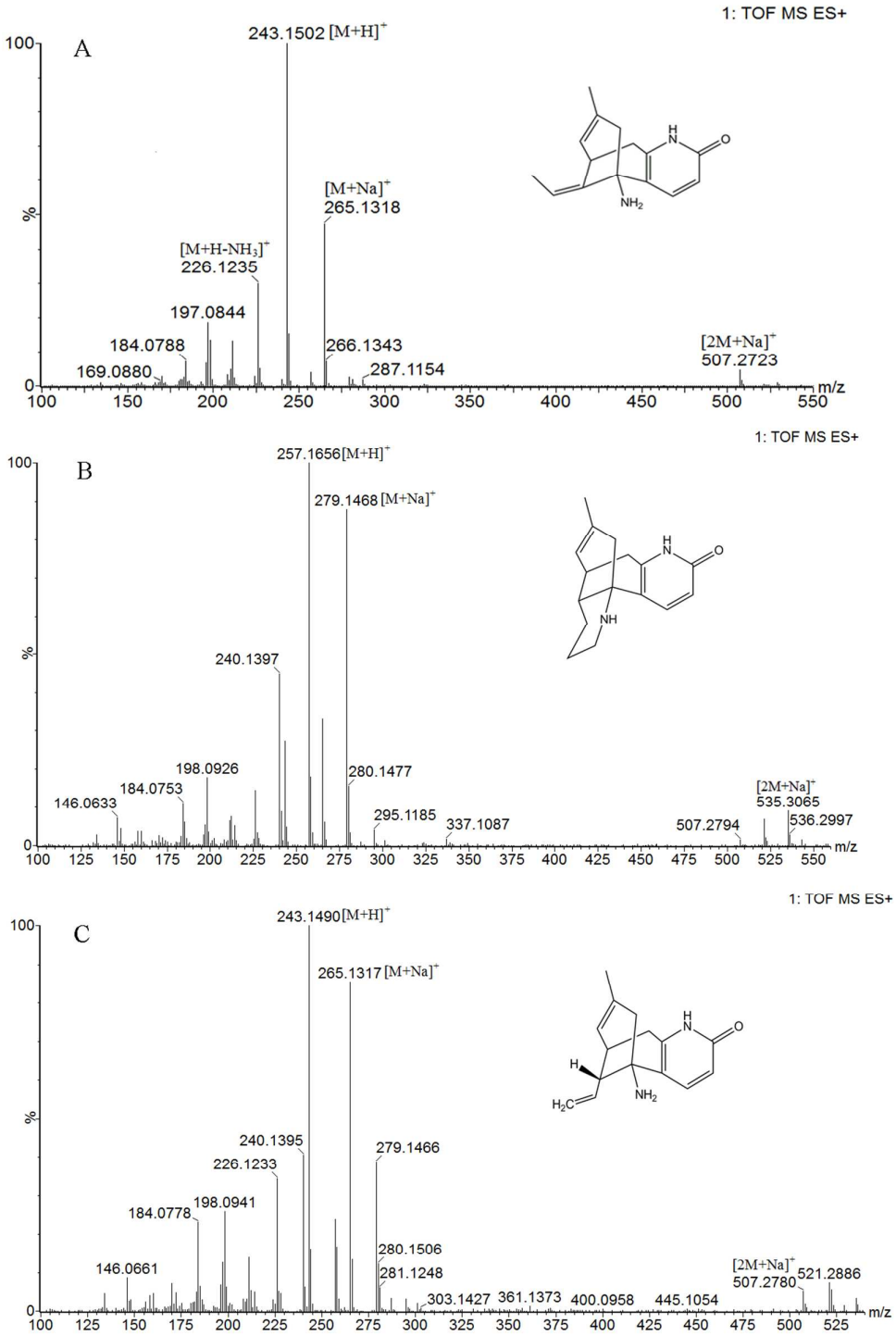

Fig. 5. MS spectra of Hup A (A), HupB (B) and HupC (C) from sample 16.