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Simultaneous determination of eight components in *Radix Tinosporae* by high-performance liquid chromatography coupled with diode array detector and electrospray tandem mass spectrometry

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Abstract

High-performance liquid chromatography (HPLC) coupled with electrospray tandem mass spectrometry (ESI-MS-MS) and diode array detection (DAD) was used to identify and simultaneously determine eight major ingredients in *Radix Tinosporae*. The assay was performed on a Diamonsil C_{18} analytical column with a gradient solvent system of A (water containing 0.2% formic acid, 20 mM ammonium acetate) and B (methanol/acetonitrile = 1/1, v/v). The 217, 248, 270 and 347 nm, respectively, were chosen as the monitoring wavelengths to determine four structural types of components, say columbin, phytoecdysteroids (including 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone 3-*O*-β-D-glucopyranoside and 2-deoxy-20-hydroxyecdysone), menisperine and protoberberine alkaloids (including columbamine, jatrorrhizine and palmatine). This method was validated in respect to precision, repeatability and accuracy, and was successfully applied to quantify the eight components in 39 batches of *R. Tinosporae* for quality control purpose. The results indicated that the proposed method could be readily utilized as a quality control method for traditional Chinese medicine (TCM).
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Keywords: *Radix Tinosporae*; Quality control; Protoberberine alkaloids; Phytoecdysteroid; Traditional Chinese medicine

1. Introduction

Radix Tinosporae, a commonly used traditional Chinese medicine (TCM), is the root of *Tinospora sagittata* (Oliv.) Gagnep or *Tinospora capillipes* Gagnep (Menispermaceae family). It is widely distributed throughout the south of China, such as Guangxi, Yunnan, Sichuan, Hubei, Guizhou and Hunan provinces, and can also be found in Vietnam [1]. In the theory of TCM, *R. Tinosporae* can neutralize “poison” in the body and clear “heat”. It possesses well-documented properties of relieving sore throat, expelling superficial infection and stopping diarrhea [2]. Contemporary pharmacological studies have shown that *R. Tinosporae* also exhibited significant anti-inflammatory and antiviral effects [3,4]. As reported previously, the chemical constituents of *R. Tinosporae* mainly involve diterpenoid lactones, protoberberine alkaloids, aporphine alkaloids and phytoecdysteroids [5,6], among which, diterpenoid lactones and alkaloids are often considered as important bioactive constituents [7]. As a result of the phytochemistry study in our laboratory, columbamine, jatrorrhizine, palmatine and menisperine are isolated as the major diterpenoid lactones and alkaloids compounds in *R. Tinosporae* (to be reported elsewhere). It is reported that columbin showed potent effects of anti-inflammatory [8], antitumor [9] and affecting the hepatic drug metabolizing enzymes [10] and isoquinoline alkaloids, including palmatine and columbamine, possessed anti-inflammatory and antinociceptive activity in different potent [11].

So far, it is well known that multiple constituents are responsible for the therapeutic effects of TCM [12], and therefore, it seems necessary to determine bioactive components as much as possible to ensure the quality of *R. Tinosporae*. However, at present, the quality control of *R. Tinosporae* is mainly conducted

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according to China Pharmacopoeia, in which only two alkaloids, palmatine and jateorhizine, are identified and determined by thin layer chromatography (TLC) [2]. This method, obviously, cannot well ensure its efficacy, safety and batch-to-batch uniformity, and a novel approach for multiple components determination is urgently needed. To the best of our knowledge, there was no study on the determination of columbin and phytoecdysteroids (2-deoxy-20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone 3-*O*-β-D-glucopyranoside) yet, only on 20-hydroxyecdysone [13]. Several methods had been reported to determine some of the alkaloids (palmatine, columbamine, jatrorrhizine and menisperine), including thin-layer chromatography [2,14], micellar chromatography [15], near-infrared (NIR) diffuse reflectance spectroscopy [16], capillary electrophoresis (CE) [17–20], capillary electrophoresis/ionspray mass spectrometry (CE/MS) [21,22], high-performance liquid chromatography (HPLC) [23–27], high-performance liquid chromatography-electrospray mass spectrometry (HPLC-ESI-MS) [29–32]. To date, no study was carried out to simultaneously determine the eight compounds in one run. Moreover, these eight components thereof, which belong to four different structural types of diterpenoid lactone (columbin), protoberberine alkaloids (columbamine, jatrorrhizine, palmatine), aporphine alkaloids (menisperine) and phytoecdysteroids (2-deoxy-20-hydroxyecdysone, 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone 3-*O*-β-D-glucopyranoside), have rather different UV absorption properties, and as a result, it is difficult to simultaneously determine them by common analytical methods.

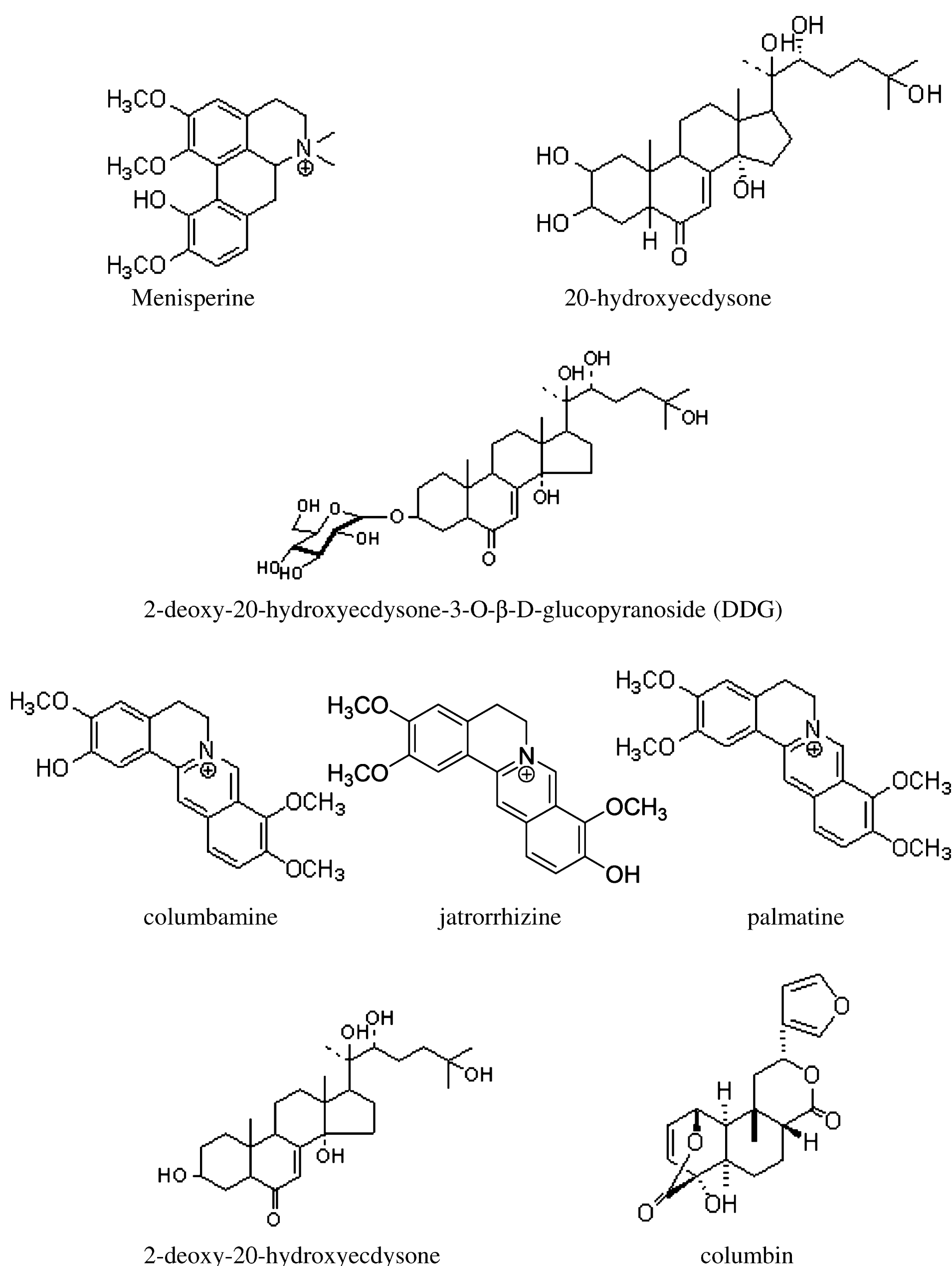

Fig. 1. Chemical structures of eight investigated compounds in *Radix Tinosporae*.

HPLC–DAD can realize detection on multiple channels and is thus expected to resolve the above problem [33]. On the other hand, HPLC-ESI-MS-MS is a powerful approach to rapidly identify and elucidate multi-ingredients in TCM for its low detection limit, with high specificity and excellent ability of structure elucidation [34]. In this study, a combinative solution of HPLC–DAD and HPLC-ESI-MS-MS is proposed, which made it possible to simultaneously identify and determine four different structural types of compounds. The proposed method could be readily utilized as a quality control method for TCM.

2. Experimental

2.1. Reagents and materials

HPLC-grade acetonitrile, methanol, ammonium acetate and formic acid were purchased from Merck Company Inc. (Merck, Darmstadt, Germany). Ultrapure water was prepared by a Milli-Q50 SP Reagent Water System (Millipore Corporation, MA, USA). Other reagents were of analytical grade.

The reference standards of 2-deoxy-20-hydroxyecdysone, menisperine, columbamine, jatrorrhizine, palmatine, 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone 3-*O*-β-D-glucopyranoside (DDG) and columbin were prepared in our laboratory (over 98% purity) and their chemical structures (shown in Fig. 1) were identified by several spectral analyses (to be reported elsewhere).

The 39 batches of roots of *T. sagittata* (Oliv.) Gagnep were gathered from 18 various provinces in China in January 2006, and their species were identified by Professor Hanchen Zheng (The School of Pharmacy, Second Military Medical University, Shanghai, China). Voucher specimens were deposited in Department of Chinese Materia Medica, Second Military Medical University.

2.2. Preparation of standard solutions

Eight references were accurately weighed, dissolved in methanol and diluted to appropriate concentration. Stock solution of the mixture of eight reference, containing 2-deoxy-20-hydroxyecdysone (14.1 μg/ml), menisperine (25.4 μg/ml), columbamine (23.6 μg/ml), jatrorrhizine (19.4 μg/ml), palmatine (22.0 μg/ml), 20-hydroxyecdysone (33.0 μg/ml), 2-deoxy-20-hydroxyecdysone 3-*O*-β-D-glucopyranoside (20.4 μg/ml) and columbin (124.2 μg/ml) was prepared in methanol. The stock solution was further diluted to make working solutions. The solutions were brought to room temperature and filtered through a 0.45 μm membrane filter before HPLC analysis.

2.3. Preparation of samples

R. Tinosporae was dried at 50 °C until constant weight. Each dried material was pulverized to 40 meshes. A 10 g pulverized powder was accurately weighed and then extracted with 100 ml 95% ethanol in solvent warm mode by a Universal Extraction System B-811 (BUCHI Labortechnik AG, Flawil, Switzerland). The material was extracted for 3 h and rinsed for 0.5 h with the lower and upper heating temperatures set at 100 and 60 °C, respectively. The solution was evaporated under vacuum until dryness. The residue was dissolved in 250 ml of methanol. The obtained solution was filtered through a syringe filter (0.45 μm) and aliquots (10 μl) were subjected to HPLC analysis.

2.4. HPLC–DAD analysis

A LC2010AHT HPLC system coupled with diode array detector (DAD) was used (Shimadzu Corporation, Kyoto, Japan) for quantitative determination. The chromatographic separation was performed on a Diamonsil C_{18} analytical column (5 μm, particle size, 100 Å, pore size, 4.6 mm i.d., 250 mm length, Dikma Corporation) with the column temperature set at 25 °C. A linear gradient elution of A (water containing 0.2% formic acid, 20 mM ammonium acetate) and B (methanol/acetonitrile = 1/1, v/v) was used (0 min, B 20% to 50 min, B 55%, v/v). The flow rate was 0.8 ml/min, and the injection volume was 10 μl. For UV detection 217, 248, 270 and 347nm were chosen to simultaneously record chromatograms, and the online UV spectra were record in the range 190–400 nm. Data acquisition was performed using CLASS-VP software (Shimadzu Corporation, Kyoto, Japan).

2.5. HPLC-ESI-MS-MS analysis

An Agilent-1100 HPLC system with diode array detector was coupled with a LC/MSD Trap XCT electrospray ion mass spectrometer, and equipped with quaternary pump, vacuum degasser, autosampler, column heater–cooler (Agilent Corporation, MA, USA). The chromatographic conditions were as described in Section 2.4. By solvent splitting, 0.3 ml/min portion of the column effluent was delivered into the ion source of mass spectrometry.

The ESI-MS spectra were acquired in positive ion mode to produce $[M+Na]^+$ or $[M+H]^+$ ions. The conditions were as follows: drying gas N_2, 10 l/min, temperature 350 °C, pressure of nebulizer 30 psi, HV voltage 3.5 kV and scan range 100–1000 nm. Data acquisition was performed using Chemstation software (Agilent Corporation).

3. Results and discussion

3.1. Optimum condition of chromatography system

In our former study, a method [27] by using the mixture of acetonitrile and 0.2% aqueous phosphoric acid (containing 20 mM potassium dihydrogen phosphate and 10 mM triethylamine) was established for the simultaneous determination of protoberberine alkaloids. However, those eluent were not suitable for HPLC-MS analysis owing to the presence of high-concentration non-volatile salt. In the improved method in this study, these and triethylamine were omitted and ammonium acetate and formic acid were used.

To optimize the separation conditions, the effect of ammonium acetate and formic acid was investigated. Without ammonium acetate, the peaks of the alkaloids were very board and

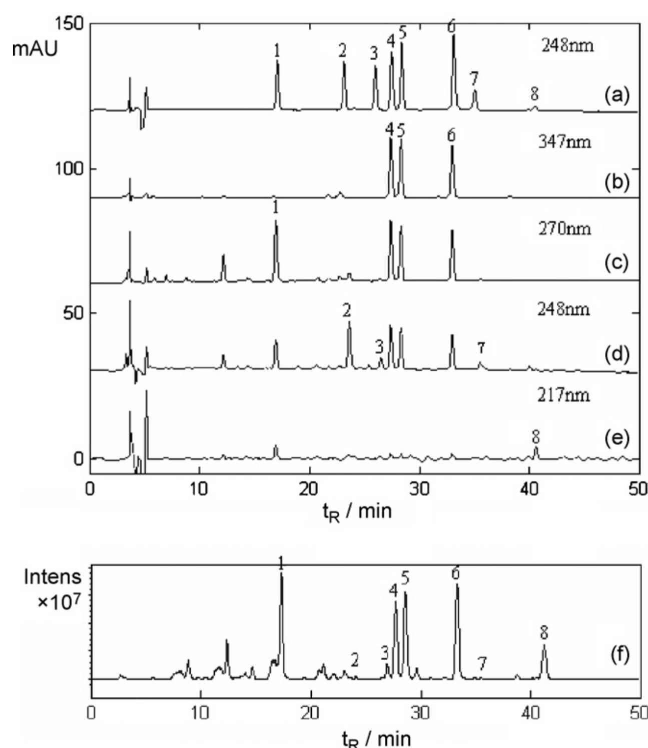

Fig. 2. Typical HPLC/DAD chromatograms of *Radix Tinosporae*.(a) Chromatogram of references (248 nm); (b–e) chromatograms of *Radix Tinosporae* at 347, 270, 248 and 217 nm, respectively (base-line subscribed in chromatogram (e)); (f) ESI-MS chromatogram of *Radix Tinosporae* (BPC). Peaks 1–8 were confirmed by standard references according to their retention times, DAD spectra and ESI-MS fragments. (1) smenisperine (17.6 min); (2) 20-hydroxyecdysone (24.4 min); (3) 2-deoxy-20-hydroxyecdysone-3-*O*-β-D-glucopyranoside (DDG) (27.1 min); (4) columbamine (28.0 min); (5) jatrorrhizine (28.8 min); (6) palmatine (33.6 min); (7) 2-deoxy-20-hydroxyecdysone (36.2 min); columbin (41.5 min).

overlapped but all alkaloids could be separated except for jatrorrhizine and columbamine with 20 mM ammonium acetate added. Formic acid was added to acquire sharp peaks, and the optimized concentration of formic acid was 0.2%.

The composition of organic phase was also investigated, and the mixture of methanol and acetonitrile (1/1, v/v) was adopted.

Two kinds of column, Diamonsil C_{18} and Zorbax Extend C_{18} were investigated. Typical chromatograms of *R. Tinosporae* on the Diamonsil C_{18} column are shown in Fig. 2 in which a better separation of the eight compounds was achieved. The 20-hydroxyecdysone, palmatine and 2-deoxy-20-hydroxyecdysone could not be well separated on the Zorbax Extend C_{18} (Figure not shown).

Table 1 shows the UV absorption maxima of each component. In the study, 217, 248, 270 and 347 nm were chosen to record chromatograms for four different structural types of components to achieve maximum sensitivity.

As reported previously [27], the isomers of jatrorrhizine and columbamine were very difficult to separate. In this study, jatrorrhizine and columbamine were baseline-separated. HPLC–DAD-ESI-MS chromatograms of *R. Tinosporae* are as shown in Fig. 2.

3.2. Method validation

3.2.1. Linearity and limit of detection (LOD)

The linearity calibration curves for the eight compounds were assessed at at least five concentration levels, and triplicate injections were applied at each concentration. Calibration curves were constructed by plotting the integrated chromatographic peak areas (Y) versus the corresponding concentration of the injected standard solutions (X μg/ml). Least square method regression was employed, and the results are presented in Table 2. High correlation coefficient values ($R^2 > 0.9996$) were achieved in relatively wide concentration ranges for all the analytes. The developed calibration curves were considered stable because all relative standard derivation (R.S.D.) values of the slope were less than 5.0%. Although the R.S.D. values of intercept were a little higher, the quantification results were still stable, due to the fact that the values of intercept were not in the same order of magnitude of the corresponding values of peak area. For most compounds, low limits of detection were obtained (LOD; S/N > 3) ranging from 0.39 to 14.10 ng, while for columbin, a high LOD over 124.2 ng was achieved which is due to the weak UV activity.

3.2.2. Precision and repeatability

The intra- and inter-day precisions were determined by analyzing calibration samples during a single day and on 3 different days, respectively. To confirm the repeatability, five different working solutions prepared from the same sample were analyzed. Table 3 shows the results of the tests of precision and repeatability. It indicates that most of the R.S.D.s are less than 5%, and the method is thus acceptable.

Table 1
UV maxima of the components determined

Components	Retention time (min)	Structural types	Wavelength of maximal absorption (nm)
Menisperine	17.6	Aporphine alkaloids	270
Columbamine	28.0		
Jatrorrhizine	28.8	Protoberberine alkaloids	347
Palmatine	33.6		
Columbin	41.5	Clerodane type diterpene	217
2-Deoxy-20-hydroxyecdysone	36.2		
20-Hydroxyecdysone	24.4	Phytoecdysteroid	248
DDG	27.1		

Table 2
Result of regression analysis on calibration curves and LODs for the components determined

Peak no.	Components	Monitoring wavelength (nm)	Regression equation[a]	Correlation coefficient (R^2)	Linear range (ng)	Limit of detection (ng)
1	Menisperine	270	$Y = (2355.1 \pm 10.7)\ X - (3184.0 \pm 329.5)$	0.9999	5.08–254	0.51
2	20-Hydroxyecdysone	248	$Y = (746.6 \pm 4.8)\ X + (980.9 \pm 87.3)$	0.9999	16.5–825	6.60
3	DDG	248	$Y = (1260.0 \pm 12.3)\ X + (2316.3 \pm 260.1)$	0.9998	10.2–510	4.10
4	Columbamine	347	$Y = (1551.1 \pm 10.6)\ X + (1117.3 \pm 46.8)$	0.9999	4.72–590	0.47
5	Jatrorrhizine	347	$Y = (5307.5 \pm 15.2)\ X + (635.7 \pm 30.6)$	0.9999	3.88–485	0.39
6	Palmatine	347	$Y = (5248.4 \pm 19.1)\ X - (1482.6 \pm 59.0)$	0.9999	4.40–550	0.44
7	2-Deoxy-20-hydroxyecdysone	248	$Y = (186.6 \pm 1.6)\ X - (545.7 \pm 48.8)$	0.9999	141–2820	14.10
8	Columbin	217	$Y = (958.5 \pm 33.7)\ X + (11636.0 \pm 3123.2)$	0.9996	248–4968	124.20

[a] *Y* is the peak area, *X* the concentration.

Table 3
Precision and repeatability of the proposed HPLC/DAD method (mg/g)

Peak no.	Compounds	Precision				Repeatability ($n = 5$)	
		Intra-day ($n = 5$)		Inter-day ($n = 3$)		Mean	R.S.D. (%)
		Mean	R.S.D. (%)	Mean	R.S.D. (%)		
1	Menisperine	1.27	2.35	1.23	2.42	1.25	2.79
2	20-Hydroxyecdysone	3.30	0.58	3.30	1.24	3.27	2.70
3	DDG	0.49	4.18	0.48	5.27	0.45	5.49
4	Columbamine	1.73	0.51	1.75	1.35	1.77	2.97
5	Jatrorrhizine	0.50	0.53	0.51	1.02	0.51	3.75
6	Palmatine	0.48	0.59	0.48	0.95	0.49	1.94
7	2-Deoxy-20-hydroxyecdysone	2.55	2.79	2.63	4.26	2.24	5.72
8	Columbin	7.12	4.11	6.97	4.67	6.80	3.79

3.2.3. Accuracy

The recoveries of the compounds were determined by the method of standard addition. Suitable amounts (about 50% of the content) of the eight references were spiked into a sample of *R. Tinosporae*, which were analyzed previously. The mixture was extracted and analyzed by using the proposed procedure. For comparison, an unspiked sample was concurrently prepared and analyzed simultaneously. Table 4 shows the results of the tests of accuracy. It indicates that most of the R.S.D.s are less than 5%, and the method is thus acceptable.

3.3. Identification of 8 components in R. Tinosporae by DAD spectrum and MS-MS

The MS spectra of major components in *R. Tinosporae* were acquired. Fig. 2f lists their base peak chromatograms (BPC). Table 5 shows their retention times (t_R), MS and MS^2 fragment ions. In MS spectra, $[M+Na]^+$ or $[M+H]^+$ of the eight components can be easily observed, and in MS^2 spectra, fragment ions of losing H_2O, CH_3OH and CH_3 are observable. Their fragmentation patterns were well matched with their chemical structures (listed in Fig. 1). The fragmentation patterns of the eight compounds were described in detail in our earlier publication [32]. The proberberine alkaloids showed typical fragmentation patterns as reported previously [28].

From the *m/z* value, DAD spectrum, retention feature and comparison with authentic standards, the eight compounds were identified from 95% methanol extract in 39 batches of *R. Tinosporae*.

3.4. Application

The developed HPLC/DAD analytical method was subsequently applied to simultaneously determine the eight ingredi-

Table 4
Accuracy tests for the proposed HPLC/DAD method (mg/g)

Compounds	Added amount (μg)	Recorded amount (μg)	Recovery (%)	Mean recovery (%)	R.S.D. (%)
Menisperine	101.6	90.9, 86.8, 92.6, 92.1, 92.8	89.5, 85.4, 91.1, 90.6, 91.3	89.6	2.71
20-Hydroxyecdysone	165.0	159.1, 171.1, 169.2, 172.8, 153.1	96.4, 103.7, 102.5, 104.7, 92.8	100.0	5.18
DDG	40.8	41.5, 41.7, 43.2, 42.9, 42.1	101.7, 102.2, 105.9, 105.1, 103.2	103.6	1.80
Columbamine	70.8	68.3, 66.6, 65.5, 66.8, 66.9	96.5, 94.1, 92.5, 94.4, 94.5	94.4	1.49
Jatrorrhizine	38.8	38.3, 35.9, 36.1, 35.9, 36.8	98.7, 92.5, 93.0, 92.5, 94.8	94.3	2.80
Palmatine	44.0	41.5, 41.1, 41.8, 41.5, 42.7	94.3, 93.4, 95.0, 94.3, 97.0	94.8	1.36
2-Deoxy-20-hydroxyecdysone	56.4	58.5, 59.0, 55.9, 59.1, 55.7	103.7, 104.6, 99.1, 104.8, 98.8	102.2	3.99
Columbin	372.6	336.7, 342.8, 347.9, 356.0, 357.2	90.4, 92.0, 93.4, 95.5, 95.9	93.4	2.51

Table 5
HPLC-ESI-MS-MS data

Peak	Compounds	t_R (min)	MS (*m/z*)	MS^n fragmention (*m/z*)
1	Menisperine	17.6	356 $[M]^+$	311 $[M-C_2H_7N]^+$; 279 $[M-C_2H_7N-CH_3OH]^+$
2	20-Hydroxyecdysone	24.4	481 $[M+H]^+$	463 $[M+H-H_2O]^+$; 445 $[M+H-2H_2O]^+$427 $[M+H-3H_2O]^+$
3	DDG	27.1	649 $[M+Na]^+$	465 $[M-C_6H_{10}O_5+H]^+$;447 $[M-C_6H_{10}O_5-H_2O+H]^+$, $[M-C_6H_{10}O_5-2H_2O+H]$
4	Columbamine	28.0	338 $[M+H]^+$	323 $[M-CH_3]^+$
5	Jatrorrhizine	28.8	338 $[M+H]^+$	323 $[M-CH_3]^+$
6	Palmatine	33.6	352 $[M+H]^+$	337 $[M-CH_3]^+$
7	2-Deoxy-20-hydroxyecdysone	36.2	487 $[M+Na]^+$	429 $[M-2H_2O]^+$
8	Columbin	41.5	381 $[M+Na]^+$	341 $[M-H_2O+H]^+$; 323 $[M-2H_2O+H]^+$

ents in *R. Tinosporae* samples. The quantitative analyses were performed by means of the external standard methods (data not shown). The content of each constituent is quite different in various batches of *R. Tinosporae*, which is due to the variation of habitat, climate, circumstances and soil condition. Therefore, it is necessary to establish a quality control method to ensure the batch-to-batch uniformity of *R. Tinosporae*. Palmatine and jatrorrhizine were the target components for the quality control of *R. Tinosporae* by the current method accepted by Pharmacopoeia of China [2], and actually, columbin, 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone are the most abundant which were not considered in quality control.

4. Conclusion

The proposed method makes it possible to simultaneously determine different structural multi-components in one run with acceptable levels of linearity, precision, repeatability and accuracy. The method has been applied successfully to analyze eight ingredients in 39 batches of *R. Tinosporae* from different habitats. Compared with the currently used quality control method, this assay permits a much more reasonable and efficient manner to ensure the efficacy, safety, and batch-to-batch uniformity of *R. Tinosporae*. The proposed method, therefore, could be readily utilized as a quality control method for traditional Chinese medicine.

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