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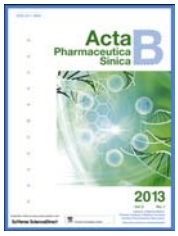

ORIGINAL ARTICLE

Analysis of the bioactive components from different growth stages of *Fritillaria taipaiensis* P. Y. Li

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KEY WORDS

Fritillaria taipaiensis
P. Y. Li;
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ELSD;
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Alkaloid;
Nucleoside;
Nucleobase;
Quality control

Abstract High-performance liquid chromatography (HPLC) coupled with an evaporative light scattering detector (ELSD) or a diode array detector (DAD) were utilized for the quantitative analysis of 4 alkaloids (peimisine, sipeimine, peimine and peiminine) and 9 nucleosides and nucleobases (uracil, uridine, adenosine, adenine, inosine, thymine, cytidine, guanosine and thymidine) from *Fritillaria taipaiensis* P. Y. Li that had been cultivated in the same field for 2–6 years. The content of peimisine, sipeimine, peimine, peiminine, uracil, thymine, adenine and inosine in plants cultivated for 2–4 years was significantly higher than that of plants cultivated for 5–6 years, while the content of cytidine, uridine, guanosine, thymidine and adenosine did not change over this period. This is the first evaluation of variation in the bioactive compounds in *F. taipaiensis* over its life cycle.

Abbreviations: DAD, diode array detector; ELSD, evaporative light scattering detection; HPLC, high pressure liquid chromatography; RSD, relative standard deviation

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1. Introduction

Taibaibeimu refers to the bulbs of *Fritillaria taipaiensis* P. Y. Li from the family Liliaceae, and it has long been used as an antitussive and anti-asthmatic herb in China. It is documented as Chuanbeimu in the China Pharmacopoeia[1]. Numerous pharmacological studies have shown that the alkaloid content of this medicinal plant is responsible for its antitussive and expectorant activity[2–4]. Additionally, many nucleosides and nucleobases present in the water extract of *Fritillaria* bulbs act as antihypertensives, platelet aggregation inhibitor and smooth muscle relaxation promoter[5–7].

The original plants used for the traditional Chinese medicine Chuanbeimu, such as *Fritillaria cirrhosa* D. Don, *Fritillaria unibracteata* Hsiao et K. C. Hsia, *Fritillaria przewalskii* Maxim. ex Bata1, *Fritillaria delavayi* Franch., *F. taipaiensis* P. Y. Li and *Fritillaria wabuensis* S. Y. Tang et S. C.Yueh, are perennial herbs. In traditional Chinese medicine, the smaller the bulb size of Chuanbeimu the better the therapeutic effect. Thus, the tradition in application of Chuanbeimu is that 50 g Songbei should contain no less than 240 bulbs and 50 g Qingbei should contain 190 bulbs. It is thought that the content and composition of the drugs during the early growth stage is more effective than that during the late growth stage. However, there is as yet no evidence that the content of alkaloids, nucleosides and nucleobases in *F. taipaiensis* P. Y. Li changes over the course of growth and maturation.

In this study, an HPLC coupled with diode array detector (DAD) and an HPLC coupled with evaporative light scattering detector (ELSD) were used to quantify 9 nucleosides and nucleobases including uracil, uridine, adenosine, adenine, inosine, thymine, cytidine, guanosine and thymidine, and 4 alkaloids including peimisine, sipeimine, peimine and peiminine in *F. taipaiensis* in a single analysis. The content of these components were compared at different growth stages of this plant to ascertain the optimal harvesting time and regulate the yield and quality of *F. taipaiensis*.

2. Materials and methods

2.1. Plant materials

Five samples of *F. taipaiensis* were collected after 2–6 years of cultivation in Lanying, Wuxi county, Chongqing municipality, China. The plants were harvested in July 2011. Plant material was authenticated at the Chongqing Academy of Chinese Materia Medica (Chongqing, China).

2.2. Chemicals and reagents

Alkaloid standards, including peimisine (MUST-11042112), sipeimine (MUST-10031103), peimine (MUST-10072201) and peiminine (MUST-11012001), were purchased from Chengmust Biotech Co., Ltd. (Chengdu, China). Nucleosides and nucleobases, such as uracil (Batch No. 100469-200702), uridine (Batch No. 887-200202), adenosine (Batch No. 110879-200202), adenine (Batch No. 886-200002), inosine (Batch No. 140669-200702), thymine (Batch No. T0376-5G), cytidine (Batch No. C4651-1G), guanosine (Batch No. G6752-1G) and thymidine (Batch No. T9250-1G), were purchased from Sigma (Shanghai, China). The purities of these compounds were determined to be more than 99.0% by HPLC. Their structures are shown in Fig. 1. Methanol for liquid chromatography was purchased from Honeywell (Burdick & Jackson, USA). Other reagents were of HPLC grade or the highest grade commercially available.

2.3. Solution preparation

2.3.1. Preparation of standard solutions

The stock solution of alkaloid standards peimisine (920 μg/mL), sipeimine (442 μg/mL), peimine (440 μg/mL) and peiminine (392 μg/mL) were prepared and diluted with methanol to obtain

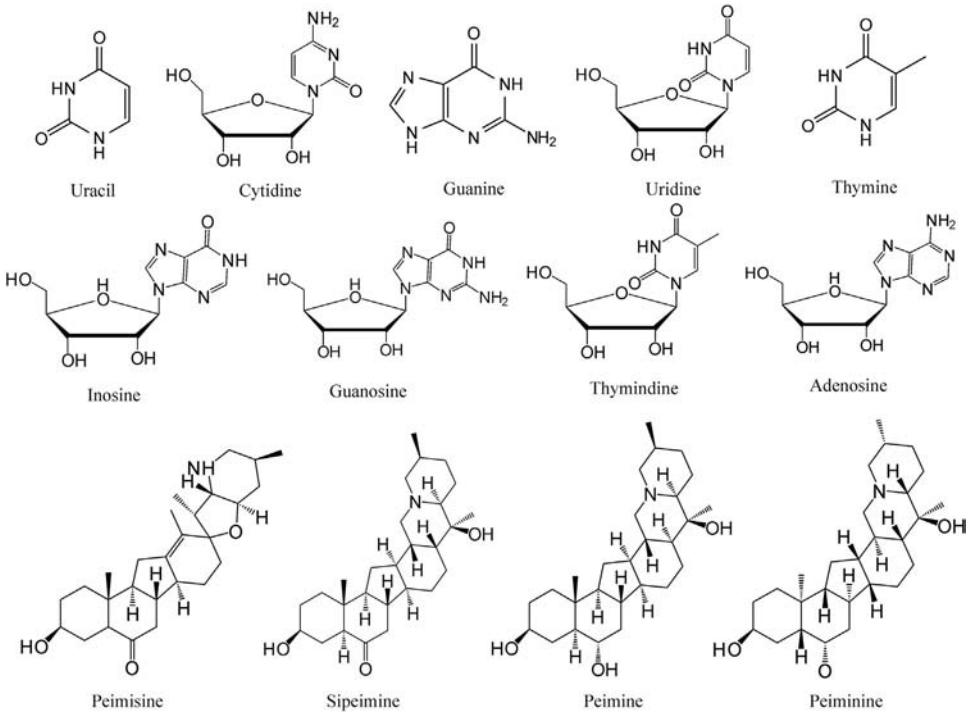

Figure 1 Chemical structures of nucleosides, nucleobases and alkaloids in *Fritillaria taipaiensis* P. Y. Li.

a series of working standard solutions for calibration curves. The nucleosides and nucleobases were dissolved in water to obtain stock solution of uracil (356 μg/mL), uridine (560 μg/mL), adenosine (432 μg/mL), adenine (432 μg/mL), inosine (1050 μg/mL), thymine (2112 μg/mL), cytidine (452 μg/mL), guanosine (2066 μg/mL) and thymidine (2112 μg/mL). All the solutions were shown to be stable when stored at 4 °C for 2 weeks.

2.3.2. *Preparation of sample solutions*

The dried bulbs of *F. taipaiensis* from 5 different growth stages of 2–6 years were powdered to a homogeneous size, sieved through a No. 4 mesh, and dried at 60 °C in an oven (DHG-9145A, China) until a constant weight was achieved. Total alkaloids were prepared as follows: the dried bulb powders (3.0 g) were extracted with 40 mL of a mixture of chloroform:methanol (80:20, *v/v*) for 2 h, during which the same mixture was added to compensate for the weight lost. After extraction, the supernatant was filtered through a 0.45 μm membrane filter. Subsequently, the extracts (30 mL) were concentrated to dryness in an evaporating dish at 50 °C. The residue was reconstituted to exactly 1 mL with methanol using a volumetric flask.

The preparation of nucleosides and nucleobases was performed as follows: the dried bulb powders (2.0 g) were extracted with 20 mL of water using ultrasonic extraction (100 W, Fisher Scientific, USA) at room temperature for 60 min. After centrifugation at 4000 × *g* for 10 min, the supernatant was filtered through a 0.45 μm membrane filter prior to injection into the HPLC system.

2.4. *Instrumentation*

Chromatographic analysis was performed on an Agilent 1200 Series liquid chromatography system (Agilent Technologies, Bobingen, Germany) equipped with a binary pump (Agilent G1312A), an autosampler, an online degasser and a G1316A column temperature controller connected to an Agilent ChemStation running on the ChemStation software.

2.4.1. *HPLC conditions for determining alkaloids in F. taipaiensis*

Chromatographic separation was achieved using an evaporative light scattering detector (Alltech 2000) on an Agilent Extend C_{18} column (250 mm × 4.0 mm, 5 μm) at 30 °C. The mobile phases were composed of methanol (A) and water contained 0.03% triethylamine (B). The gradient elution process used was 0–15 min, 35–55% A; 15–30 min, 55–72% A; 30–37 min, 72–75% A; 37–40 min, 75–80% A; 40–50 min; 80–85% A; 50–55 min, 85–90% A; and 55–60 min, 90–35% A, followed by 10 min post run for re-equilibration of the column. The flow-rate was kept constant at 1.0 mL/min and the injection volume was 10 μL. With these conditions representative HPLC chromatograms of the standards and the alkaloids were obtained (Fig. 2).

2.4.2. *HPLC conditions for quantifying nucleosides and nucleobases in F. taipaiensis*

A Gemini C_{18} column (250 mm × 4.0 mm, 5 μm) was used for chromatographic separation. Optimum separation was achieved using a mobile phase system composed of water containing 0.03% triethylamine (A) and methanol (B) with a gradient elution: 0–15 min, 65–45% A; 15–30 min, 45–28% A; 30–32 min, 28–25% A; 32–37 min, 25–20% A; 37–40 min, 20–15% A; 40–50 min, 15–10% A; 50–55 min, 10–65% A. The flow rate was kept constant at 1.0 mL/min and the injection volume was 10 μL. The DAD detector was set at 260 nm and the column temperature was maintained at 30 °C. With the above conditions a representative HPLC chromatogram of the standard and the nucleosides and nucleobases was obtained, as shown in Fig. 3.

3. Results and discussion

3.1. *Optimization of the extraction method*

3.1.1. *Alkaloid extraction*

The extraction conditions for 4 alkaloids from *Fritillaria* bulbs were determined and optimized. First, the method for the extraction was investigated, including refluxing and ultrasonication. Refluxing yielded higher amounts of all analytes than did ultrasonication. Therefore, refluxing extraction was selected for the remainder of the study. The solvent for extraction, solvent volume and time for extraction were also individually optimized. We found that methanol:chloroform (1:4, *v/v*) was more efficient than water for extracting alkaloids. The best solvent volume was 40 mL per sample. The extraction time was also optimized as follows: four duplicate samples (1.0 g per sample) were extracted by refluxing with water for 1, 2, 3 and 4 h. The extraction yield of the target analytes did not increase markedly after 2 h, thus 2 h was selected as the optimal extraction time. The residue obtained after the first extraction was further extracted with water for an additional 30 min, but few alkaloids were detected; therefore, a single refluxing

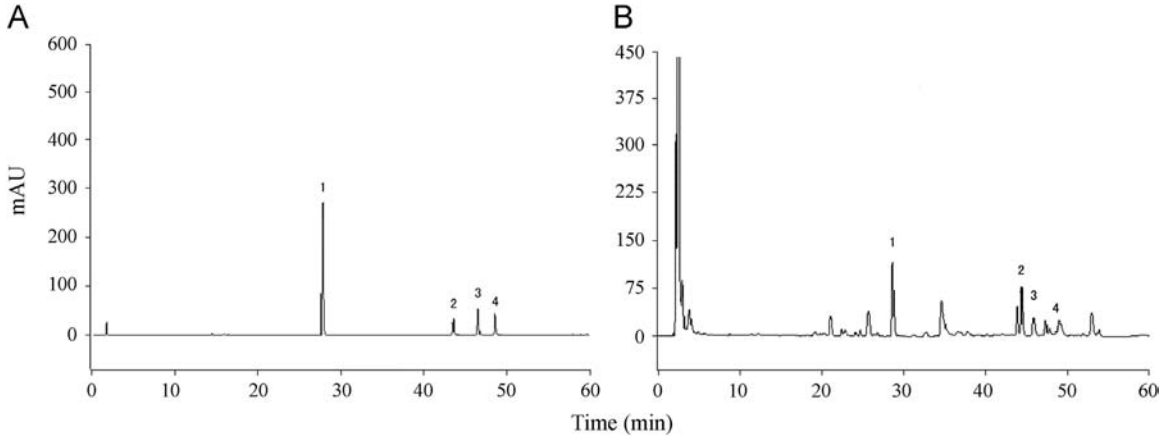

Figure 2 Representative HPLC chromatograms of the standard (A) and the alkaloids (B). 1: peiminine; 2: sipeimine; 3: peimine; 4: peiminine.

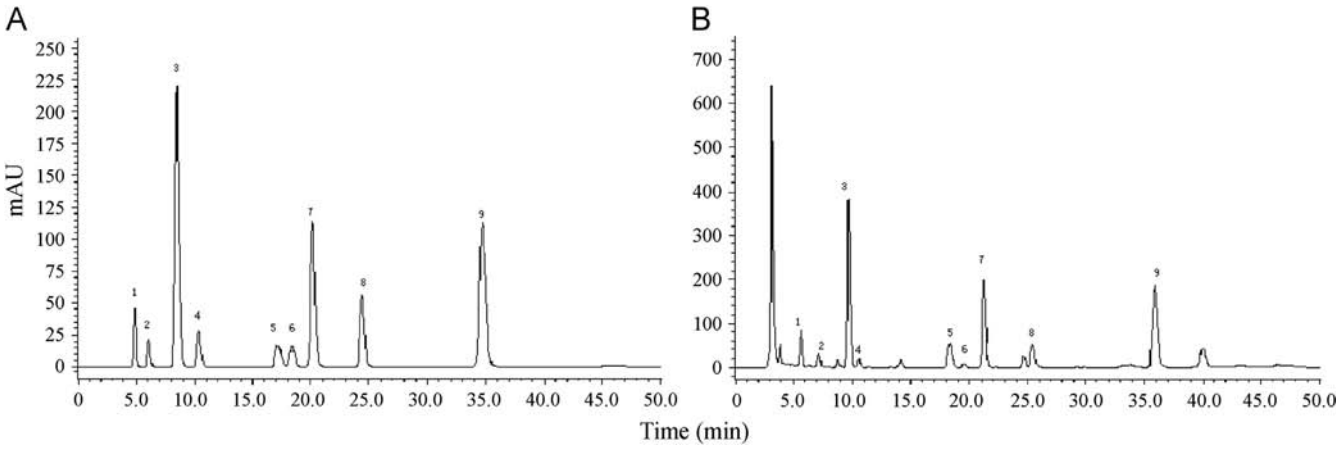

Figure 3 Representative HPLC chromatograms of the standard (A) and the nucleosides (B). 1: uracil; 2: cytidine; 3: uridine; 4: thymine; 5: adenine; 6: inosine; 7: guanosine; 8: thymidine; 9: adenosine.

extraction with a mixture of chloroform:methanol (80:20, *v/v*) for 2 h was selected to prepare the alkaloid sample solution.

3.1.2. *Optimization of the extraction method for nucleosides and nucleobases*

For quantitative extraction of nucleosides and nucleobases from *Fritillaria* bulbs, the solvent and extraction method were optimized. Due to the high polarity of the target analytes, different dilutions of aqueous methanol were evaluated for their optimal efficiency as an extraction solvent. Comparison of the extraction yields of the target constituents demonstrated that an increase in the percentage of water positively influenced the extraction yield of the 9 target analytes. Pure water gave the highest extraction yields. Different solvent volumes (10, 20, 30 and 40 mL) and extraction times (0.5, 1, 1.5 and 2 h) were also evaluated. We found that 20 mL and 1 h were the optimal extraction conditions.

3.2. *Optimization of HPLC conditions*

As the first step in developing this analytical method, an appropriate chromatographic column needed to be selected. Several columns including Hypersil BDS C_{18}, Germsil C_{18}, Synergi 4μ Hydro-RP, XB C_{18}, Gemini C_{18} and Agilent Extend C_{18} were tested to separate the 4 alkaloids and the 9 nucleosides and nucleobases. We found that the Agilent Extend C_{18} column could retain 4 alkaloids better than the other columns tested, and that the Gemini C_{18} column could retain the 9 nucleosides and nucleobases better than the other columns. Furthermore, a series of mobile phases were investigated to obtain optimal separation and analytical conditions for HPLC analysis. The most favorable resolution of the 9 nucleosides and nucleobases was achieved when methanol–ultrapure water was used as the mobile phase. Optimum separation of the 4 alkaloids was achieved using gradient elution with methanol–water (containing 0.03% triethylamine). The strength of the initial mobile phase was kept low, the flow rate was set at 1.0 mL/min, and the injection volume was 10 μL with the column temperature maintained at 30 °C.

3.3. *Method validation*

3.3.1. *Linear range and limit of detection*

Standard solutions containing the alkaloids peimisine, sipeimine, peimine and peiminine as well as uracil, uridine, adenosine, adenine, inosine, thymine, cytidine, guanosine and thymidine were prepared. Ten microlitre ($n=3$) of the series of solutions described above was injected for analysis and calibration curves constructed by plotting the peak areas *versus* the concentration of each analyte. A plot of peak area *versus* sample concentration by DAD was linear, and the plot of peak area *versus* logarithmic values of sample concentration by ELSD was also linear. The linearity of the 13 analytes is shown in Table 1. The correlation coefficients of all the analytes indicated good linearity ($r>0.999$) over the complete concentration range.

The limits of detection (LOD) and quantification (LOQ) for each analyte were determined at a signal-to-noise ratio (*S/N*) of about 3 and 10, respectively. The LODs of the 4 alkaloids were 0.5–6.8 μg/mL, the LOQs 4–50 μg/mL; and the LODs of the 9 nucleosides and nucleobases were between 0.3 and 8.0 μg/mL, and the LOQs 2 and 80 μg/mL.

3.3.2. *Precision*

A concentration series of a standard solution was analyzed on the same day, and the relative standard deviation (% RSD) values were calculated to determine the intra-day precision. The same procedure was performed on 3 consecutive days, and the inter-day precision was determined. The % RSD values varied from 0.25% to 2.39% for intra- and inter-day assays for all the analytes (Table 1).

3.3.3. *Repeatability*

To determine the repeatability of the assay, six different working solutions were prepared from the same sample. The relative standard deviation values (% RSD) for each compound are listed in Table 1. The RSD was taken as a measure of repeatability.

3.3.4. *Stability*

For the stability test, a sample solution was analyzed within 24 h. The solutions were kept at room temperature. The % RSD values varied from 0.7% to 4.59% (Table 1), indicating that the samples were sufficiently stable at room temperature for routine analysis within a day.

3.3.5. *Recovery*

Six samples (1.0 g) were accurately weighed and individually transferred to 50 mL volumetric flasks. Five microlitre of each standard stock solution was added to each sample. The mixtures

Table 1 Method validation results.

Constituent	Regression equation	Correlation coefficient (R^2)	Linearity range (μg/mL)	Precision (% RSD)		Repeatability (% RSD)	Stability (% RSD)	Recovery (% RSD)
				Intra-day	Inter-day			
Peimisine	$Y=1.732X+2.623$	0.9994	36.0–540.0	1.27	1.31	2.80	0.70	1.51
Sipeimine	$Y=1.787X+2.278$	0.9990	44.2–663.0	0.45	0.55	4.59	3.28	3.07
Peimine	$Y=1.814X+2.182$	0.9991	44.0–660.0	0.91	0.99	3.00	2.79	2.24
Peiminine	$Y=1.733X+2.318$	0.9998	39.2–588.0	2.39	2.39	2.51	2.05	1.99
Uracil	$Y=3.844\times10^4X+3.179\times10^4$	0.9990	35.6–534.0	0.32	0.51	4.73	4.59	0.56
Cytidine	$Y=1.917\times10^4X-3.842\times10^4$	0.9999	45.2–678.0	0.53	0.50	2.32	2.75	2.40
Uridine	$Y=1.858\times10^4X-2.245\times10^4$	0.9996	62.0–930.1	0.25	0.31	1.89	3.04	0.57
Thymine	$Y=3.458\times10^4X+1.367\times10^5$	0.9998	43.2–648.2	0.25	0.30	3.76	4.07	3.20
Adenine	$Y=5.174\times10^4X+9.988\times10^4$	0.9996	250.0–750.3	0.80	0.77	2.45	3.48	1.60
Inosine	$Y=1.379\times10^4X-4.221\times10^4$	0.9999	105.0–1575.1	0.35	0.44	2.02	4.02	2.40
Guanosine	$Y=5.022\times10^3X+2.428\times10^4$	0.9999	206.6–3099.0	0.35	0.39	4.75	4.06	0.87
Thymidine	$Y=1.669\times10^4X+3.679\times10^5$	0.9997	211.2–3168.1	0.56	0.62	3.26	2.01	1.30
Adenosine	$Y=2.926\times10^4X+1.153\times10^4$	0.9999	52.6–789.4	0.40	0.49	3.29	1.91	2.60

Table 2 Content of 13 components in *Fritillaria taipaiensis* P. Y. Li ($n=3$, mean ± SD).

Constituent	Content (mg/g)[a]				
	2 years	3 years	4 years	5 years	6 years
Peimisine	0.065 ± 0.0012	0.059 ± 0.0011	0.055 ± 0.0015	0.047 ± 0.0014	0.035 ± 0.0012
Sipeimine	0.059 ± 0.0019	0.051 ± 0.0013	0.051 ± 0.0015	0.050 ± 0.0014	0.039 ± 0.0012
Peimine	0.018 ± 0.0005	0.020 ± 0.0006	0.031 ± 0.0008	0.014 ± 0.0004	0.010 ± 0.0004
Peiminine	0.015 ± 0.0005	0.012 ± 0.0003	0.011 ± 0.0003	0.006 ± 0.0002	0.004 ± 0.0001
Uracil	0.173 ± 0.005	0.170 ± 0.007	0.064 ± 0.002	0.083 ± 0.003	0.037 ± 0.001
Cytidine	0.186 ± 0.005	0.251 ± 0.007	0.118 ± 0.003	0.140 ± 0.004	0.126 ± 0.004
Uridine	2.330 ± 0.072	1.406 ± 0.041	1.519 ± 0.047	1.689 ± 0.051	1.630 ± 0.054
Thymine	0.165 ± 0.005	0.158 ± 0.004	0.131 ± 0.004	tr	tr
Adenine	0.105 ± 0.003	0.122 ± 0.004	0.058 ± 0.002	0.063 ± 0.003	0.092 ± 0.004
Inosine	0.889 ± 0.028	0.662 ± 0.020	0.524 ± 0.015	0.358 ± 0.012	0.198 ± 0.006
Guanosine	3.661 ± 0.113	5.002 ± 0.140	2.983 ± 0.087	3.241 ± 0.113	2.847 ± 0.088
Thymidine	0.397 ± 0.012	0.189 ± 0.006	0.161 ± 0.005	0.149 ± 0.005	0.033 ± 0.001
Adenosine	1.038 ± 0.036	1.027 ± 0.030	1.174 ± 0.038	1.390 ± 0.040	1.343 ± 0.044

[a]Data are expressed as mean ± SD; $n=3$. tr: below the limit of quantification.

were processed according to the solution preparation procedures and the recovery was calculated. The recovery rates were between 97.6% and 103.2% and the average recovery % RSD values for each compound was less than 3.20% (Table 1). The high recovery rates illustrated the accuracy of the method.

3.4. *Sample analysis*

Using the above preparation procedure and the quantitative analysis method, the HPLC-ELSD and HPLC-DAD methods were used to quantify 4 alkaloids and 9 nucleosides and nucleobases from *F. taipaiensis* in 5 growth stages (2–6 years). Ten microlitre of each sample solution and standard solution was injected for analysis; the analyte contents are shown in Table 2. The content of alkaloids ranged 0.006–0.065 mg/g, and nucleoside and nucleobase contents ranged 0–5.002 mg/g.

The levels of the 4 alkaloids were higher in the early stages than in the later stages of *F. taipaiensis* growth, while the content of nucleosides and nucleobases varied in different stages. The content of guanosine and uridine was markedly higher than that of the other 7 compounds. The quantitative results showed that the

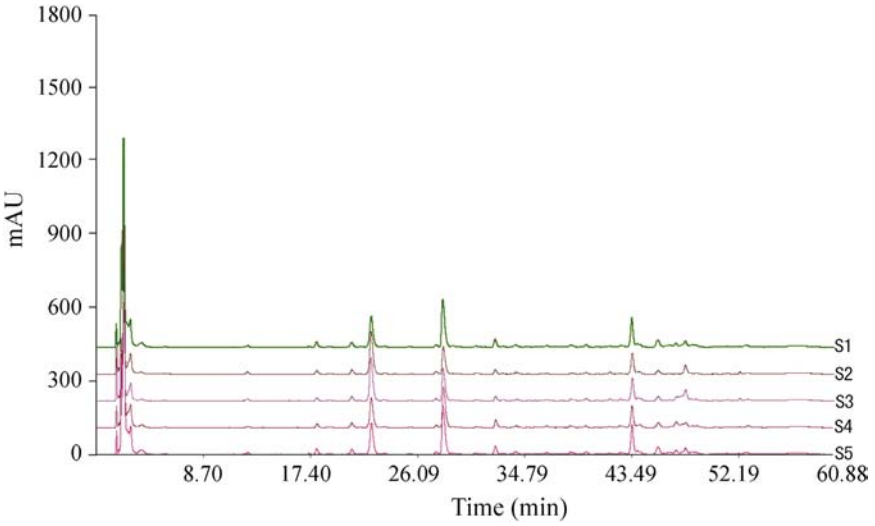

Figure 4 The HPLC profile of the alkaloids of the samples from five growth stages. S1: cultivated for 2 years; S2: cultivated for 3 years; S3: cultivated for 4 years; S4: cultivated for 5 years; S5: cultivated for 6 years.

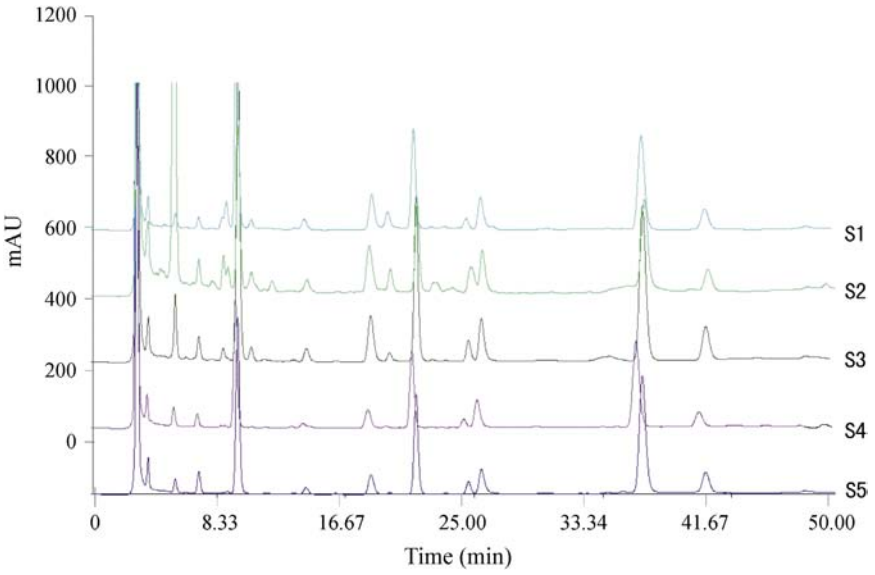

Figure 5 The HPLC profile of the nucleosides and nucleobases of the samples from the five growth stages. S1: cultivated for 2 years; S2: cultivated for 3 years; S3: cultivated for 4 years; S4: cultivated for 5 years; S5: cultivated for 6 years.

contents of peimisine, sipeimine, peimine, peiminine, uracil, thymine, adenine and inosine in plants cultivated for 2–4 years were significantly higher than that of plants cultivated for 5–6 years, while the content of cytidine, uridine, guanosine, thymidine and adenosine changed little in plants cultivated for 2–6 years. HPLC profiles of the alkaloids collected from the 5 growth stages are shown in Fig. 4, and HPLC profiles of the nucleosides and nucleobases are shown in Fig. 5.

4. Conclusions

A previous study suggested that nucleosides and nucleobases could be the major active constituents in *Fritillaria* bulbs, other than steroidal alkaloids[6]. In this study, simple, sensitive and reliable HPLC-ELSD and HPLC-DAD methods were developed and employed to quantify 4 alkaloids and 9 nucleosides and nucleobases in *F. taipaiensis* from different growth stages. Our results show that active constituents are present at higher levels in the early growth stages, rather than the late growth stage of *Fritillaria* bulbs, which is in accordance with the preferred use of smaller bulbs under the guidelines of traditional Chinese medicine.

Acknowledgments

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