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Determination of the major isosteroidal alkaloids in bulbs of *Fritillaria* by high-performance liquid chromatography coupled with evaporative light scattering detection

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Abstract

A new direct HPLC analytical method using evaporative light scattering detection coupled with a low-temperature adapter for the simultaneous determination of the major biologically active isosteroidal alkaloids in Bulbus Fritillariae, a commonly used antitussive traditional Chinese medicinal (TCM) herb, has been developed. The simultaneous separation of eight *Fritillaria* alkaloids was achieved on a reversed-phase C_8 column with an isocratic mobile phase system consisting of acetonitrile–methanol–water (66.5:3.5:30, v/v) containing 0.006% triethylamine. This method provides good reproducibility and sensitivity for the quantification of six major isosteroidal alkaloids, namely peimissine, verticine, verticinone, imperialine, isoverticine and ebeiedine in different *Fritillaria* species with overall intra- and inter-day precision and accuracy of less than 11% and higher than 90%, respectively. The assay was successfully utilized to quantify the major biologically active alkaloids in five *Fritillaria* species. The results demonstrate that this method is simple, selective, and suitable for the quality control of this commonly used antitussive TCM herb, Bulbus Fritillariae.

Keywords: *Fritillaria* spp.; Isosteroidal alkaloids

1. Introduction

Bulbus Fritillariae (Beimu in Chinese), which is derived from the bulbs of various *Fritillaria* species, has been used as one of the most important antitussive and expectorant drugs in traditional Chinese medicine (TCM) for thousands of years [1,2]. Various chemical and pharmacological studies on Beimu have demonstrated that the major biologically active ingredients present in this TCM herb are isosteroidal alkaloids [3–5] with their types and contents varying in different *Fritillaria* species [6–11]. Therefore both quality and quantity controls of the major active alkaloids in this herb have always been an important issue to ensure its effective and safe clinical usefulness [7–13].

Most of the *Fritillaria* alkaloids (Fig. 1) are non-chromophoric, which make the use of direct UV detection without pre- or post-column derivatization impossible. Therefore, only a few methods have been

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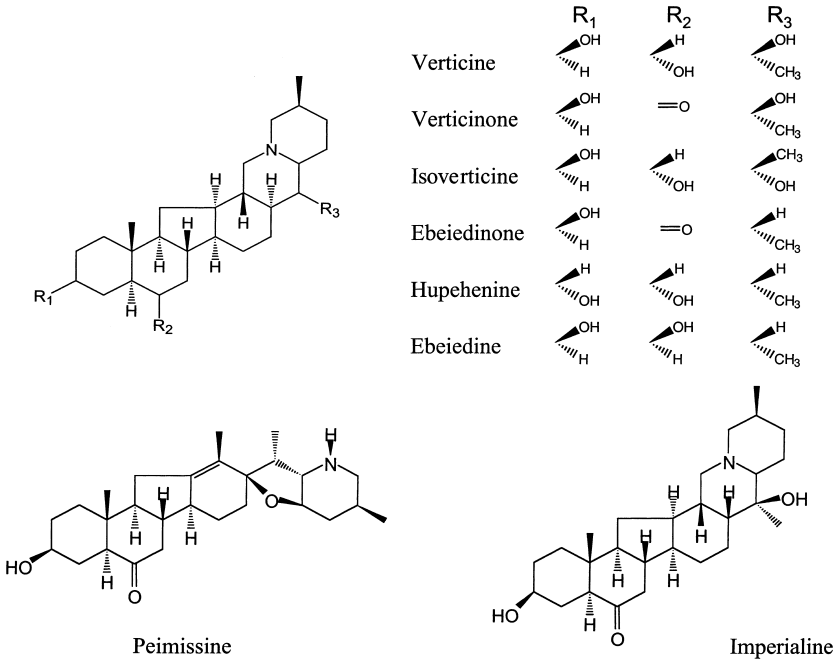

Fig. 1. Structures of peimissine, verticine, verticinone, imperialine, isoverticine, ebeiedinone, hupehenine and ebeiedine.

previously reported to analyze the major active *Fritillaria* alkaloids in Beimu. One reported high-performance liquid chromatography (HPLC)–UV method could only determine peimissine (Fig. 1), a *Fritillaria* alkaloid containing one double bond, at a very low wavelength at 205 nm [9]. Our research team has previously developed both HPLC–UV [11] and gas chromatography (GC) [12] methods with pre-column derivatization. However, the pre-column derivatization methods require time-consuming sample preparation and a complication of derivatizing reaction. Recently, a direct GC method has been established in our laboratory for a simultaneous analysis of seven *Fritillaria* alkaloids, however, verticine and verticinone, the two major biologically active alkaloids in Beimu, could not be determined separately by this method [13].

Recently, publications on the use of HPLC coupled with evaporative light scattering detection (ELSD) have markedly increased, and the published results demonstrated that ELSD is an excellent detection method for the analysis of non-chromophoric compounds [14–19]. Since the response of ELSD depends on the size, shape and number of eluate particles rather than the structure and/or chromophore of analytes, ELSD should also be a suitable detection method for HPLC analysis of *Fritillaria* alkaloids. The aim of the present study is to develop a simple and sensitive direct HPLC analytical method using ELSD for the simultaneous determination of the major biologically active *Fritillaria* alkaloids in various *Fritillaria* species.

2. Experimental

2.1. Chemicals and materials

Various *Fritillaria* species were purchased in local TCM shops in China. For each species, at least 0.5 kg of herbal samples was obtained and authenticated by Professor Li Ping. The voucher specimens were deposited in the Department of Pharmacognosy, China Pharmaceutical University, Nanjing, China. Peimissine, verticine, verticinone, imperialine, isoverticine, ebeiedinone, hupehenine and ebeiedine were isolated from several *Fritillaria* species in our laboratories [5,20–23], and their identities were confirmed by IR, ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR), and MS analyses. Solanidine and

all solvents of analytical-reagent grade were purchased from Sigma (St. Louis, MO, USA).

2.2. Apparatus and chromatographic conditions

HPLC analysis was performed using a HP1100 system (Hewlett-Packard, Palo Alto, CA, USA) equipped with an Alltech 500 ELSD system coupled with an Alltech lower temperature adaptor (LTA) (Alltech, Deerfield, IL, USA) and a Nitrox nitrogen generator. A Supelco reversed-phase C_8 analytical column (150×4.6 mm I.D., 3 μm) coupled with a C_8 guard column (20×4.0 mm, 5 μm) was utilized at a column temperature of 28°C. The mobile phase consisted of acetonitrile–methanol–water (66.5:3.5:30, v/v) containing 0.006% triethylamine with a flow-rate of 1.0 ml/min. Temperatures for the detector drift tube and LTA were set at 65°C. The nitrogen flow was 2.64 SLPM (standard liters per minute) with the pressure of a nebulizing gas of 5 bar. A HP1100 auto-sampler was utilized for the sample injection with an injection volume of 20 μl.

2.3. Calibration curves

Methanol stock solutions containing six *Fritillaria* alkaloids: peimissine, verticine, verticinone, imperialine, isoverticine, and ebeiedine were prepared and diluted to appropriate concentration ranges for the construction of calibration curves. Each calibration curve was performed with six different concentrations in triplicate. The concentration of the internal standard, solanidine, was 10 μg/ml for all analyses. Calibration curves were constructed by adding authentic analytes, the internal standard and powders of *F. cirrhosa* (100 mg) into the diethyl ether solution (5.0 ml) pre-saturated with ammonium hydroxide. The resultant mixture was extracted as described in Section 2.6. Aliquots (20 μl) of the extracts were analyzed by HPLC–ELSD. For the control samples, extracts of *F. cirrhosa* spiked with the internal standard only were prepared and analyzed in the same manner. The peak area ratio (analyte/internal standard) for each analyte in both spiked and control samples were determined. Consequently, calibration curves were constructed by plotting concentrations of each analyte as a function of peak area ratio differences (peak area $\text{ratio}_{\text{spiked}}$ − peak area $\text{ratio}_{\text{control}}$) between spiked and control extracts.

2.4. Accuracy and precision

Intra- and inter-day variability was utilized to determine the accuracy and precision of the developed assay. Known quantities of six analytes and the internal standard were added to the pre-alkalized diethyl ether solution (5.0 ml) containing powders of *F. cirrhosa* (100 mg) prior to extraction. Control samples spiked with internal standard only were also prepared similarly. The resultant samples were extracted and analyzed as described in Section 2.6. The peak area ratio difference between testing and control samples for each analyte was calculated, and the quantity of each analyte was subsequently obtained from the corresponding calibration curve. Each sample was analyzed with two concentrations (at lower and middle concentration in the range of the corresponding calibration curve) in triplicate to determine the intra-day variability. Similarly, the inter-day reproducibility was performed in three separate days. The relative standard deviation (RSD) was taken as a measure of precision and the percentage difference between amounts determined and spiked was considered as a measure of accuracy.

2.5. Limits of detection

Aliquots of six analytes quantified were spiked into the pre-alkalized diethyl ether solution (5.0 ml) containing powders of *F. cirrhosa* (100 mg) to provide a concentration ranges of 0.1–1.0 μg/ml. The resultant mixtures were extracted and analyzed in the same manner as described in Section 2.6. The limit of detection for each analyte was determined when the ratio of peak area of the analyte to noise was greater than five.

2.6. Analysis of the major alkaloids in Fritillaria species

To the dried powders of Beimu samples (100–200 mg, adjusted according to the alkaloid contents of each *Fritillaria* species), 5.0 ml of diethyl ether pre-alkalized with ammonium hydroxide and 50 μl of the internal standard solution (1 mg/ml) were

added. The mixtures were shaken by vortex for 2 h and then centrifuged at 1780 *g* for 10 min. The supernatants (2.5 ml) were transferred to test tubes and evaporated to dryness. The obtained residues were reconstituted into 200 μl of methanol and filtered through a syringe filter (0.45 μm). Aliquots (20 μl) of the resultant extracts were subjected to HPLC–ELSD analysis. The contents of the analytes were determined from the corresponding calibration curves.

In addition, the crude alkaloid extracts from three *Fritillaria* species: *F. cirrhosa*, *F. thunbergii* and *F. hupehensis*, were prepared using the extraction procedure previously developed by our laboratories [11]. The contents of alkaloids in these three extracts were analyzed by HPLC–ELSD and the direct GC assay previously developed by our laboratory [13]. The results obtained by HPLC and GC analyses were compared for a confirmation of the accuracy of both analytical methods.

3. Results and discussion

Optimal chromatographic condition was obtained after testing different mobile phase systems with two reversed-phase columns (C_8 and C_{18}). In the case of the C_{18} column, the two major *Fritillaria* alkaloids, verticine and verticinone, could not be resolved as a baseline separation, although all other analytes were separated. Whereas, all analytes were resolved well with a baseline separation using the C_8 column (Fig. 2A). Furthermore, amongst various mobile phases examined, the alkalized systems at pH 7.5–8.0 showed a good separation for all analytes and triethylamine was found to be the best alkalizing agent due to its relatively higher volatility thus compatible with ELSD. Subsequently, in order to produce a best overall separation, the optimal mobile phase consisting of acetonitrile–methanol–water (66.5:3.5:30, v/v) containing 0.006% triethylamine was chosen. As shown in Fig. 2A the results demonstrated that eight *Fritillaria* alkaloids eluted as well-defined peaks on the reversed-phase C_8 column using this optimal mobile phase. Based on our previous studies [11–13], solanidine was chosen as the internal standard and the results exhibited an excellent separation of the internal standard from all analytes (Fig. 2).

Blank controls are generally unavailable for the study of herbal materials, and calibrations are normally constructed without using the internal standard method. Therefore, reproducibility and extraction yield become critical for the quantification of ingredients in herbs. Recently, in order to solve this problem, our research group has developed an internal standard method, and applied this method for the study of *Fritillaria* species [13] and other herbs [24]. Briefly, both internal standard and analytes tested were spiked into the herbal samples prior to extraction, while for the control, only the internal standard was added separately to the similar herbal samples prior to extraction. Calibration curves were then constructed as a function of the concentration of analytes versus the peak area ratio differences between spiked and non-spiked (control) herbal extracts. Therefore, quantification is not significantly influenced by variations in extraction recovery and volume of injection. This internal standard method was also adopted in the present study. Among eight major active alkaloids analyzed, six of them namely peimissine, verticine, verticinone, imperialine, isoverticine and ebeiedine were quantified in the present study, whereas ebeiedinone and hupehenine were only qualitatively determined since quantities of their authentic compounds were insufficient for the construction of calibrations. The qualitative determination was conducted by a direct comparison of the retention times between the analyte and the corresponding authentic standard.

All six calibration curves exhibited good linear regressions as shown in Table 1. The ELSD responses have been reported to be linear [19] or exponential [19] or sigmoidal [14,15] with increasing sample concentrations. The chromatographic conditions and chemical structures of the samples generally affect the ELSD response [15,19]. Whereas the flow-rate and temperature of the carrier gas are very important parameters and should be adjusted to allow the mobile phase to be completely vaporized when the residual droplets reach the light scattering cell [16]. Consequently, optimal conditions are difficult to set up for the mobile phase with a high aqueous composition. In the present study, the use of ELSD combined with LTA, which has been recently de-

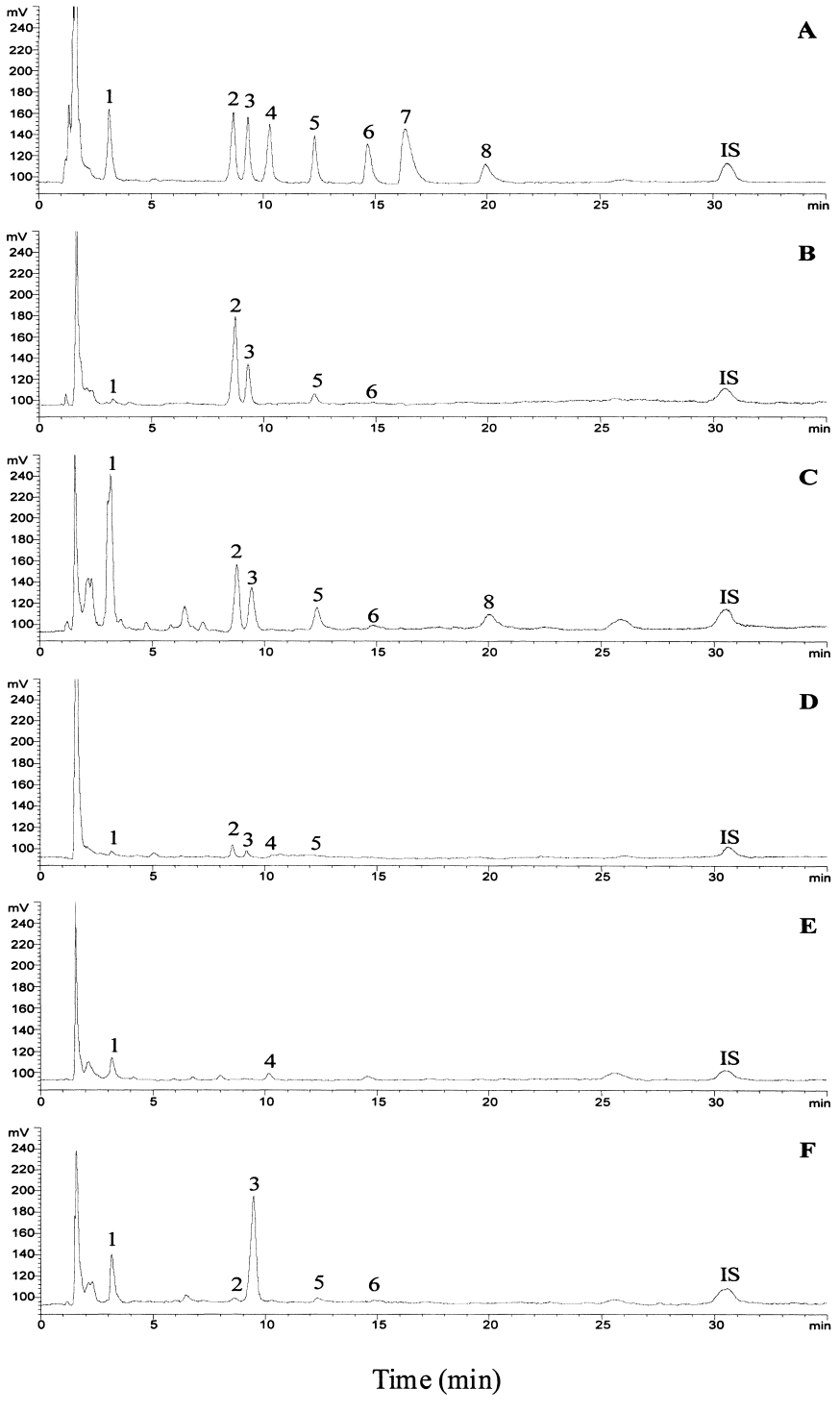

Fig. 2. Representative HPLC chromatograms of the extracts of (A) *F. cirrhosa* spiked with standards at the highest concentration in the corresponding calibration range, (B) *F. thunbergii*, (C) *F. ebeiensis* var. *purpurea*, (D) *F. cirrhosa*, (E) *F. walujewii* and (F) *F. hupehensis*. I.S., Internal standard; 1, peimissine; 2, verticine; 3, verticinone; 4, imperialine; 5, isoverticine; 6, ebeiedinone; 7, hupehenine; 8, ebeiedine.

Table 1
Calibration curves for six *Fritillaria* alkaloids

Analyte	Retention time (min)	Standard curves[a]	r^2	Test range (μg/ml)	Limit of detection (μg/ml)
Peimissine	3.3	$y=0.1232x-0.1836$	0.992	1.1–29.4	0.77
Verticine	8.1	$y=0.0965x-0.0811$	0.995	1.0–27.4	0.70
Verticinone	9.1	$y=0.1006x-0.1036$	0.996	0.9–23.7	0.63
Imperialine	10.0	$y=0.1126x-0.1834$	0.994	1.0–27.7	0.70
Isoverticine	12.1	$y=0.1080x-0.1290$	0.995	0.9–25.1	0.63
Ebeiedine	19.7	$y=0.0959x-0.0015$	0.996	1.0–25.9	0.70

[a] y: Difference of peak area ratio (peak area ratio$_{spiked}$ − peak area ratio$_{control}$); x: concentration of analyte (μg/ml). Each calibration curve was constructed with six different concentrations in triplicate.

veloped by Alltech, significantly improved the evaporation of the utilized mobile phase containing 30% water at a flow-rate of 1 ml/min, and exhibited an excellent baseline stability (Fig. 2). LTA is designed to remove large droplets in the nebulized effluents from the nebulization chamber in LTA to the waste outlet, while using ELSD alone these large droplets directly evaporate and then enter light scattering cell. Therefore, using on-line ELSD–LTA, the temperature required to evaporate the remaining droplets prior to reaching the light scattering cell is significantly reduced, and thus mobile phase containing a relatively higher water composition can be adopted [25]. The present study demonstrated good

Table 2
Intra- and inter-day variability for the assay of six *Fritillaria* alkaloids

Alkaloid spiked (μg/ml)	Intra-day variability			Inter-day variability		
	Detected ($n=3$)	RSD[a] (%)	Accuracy[b] (%)	Detected ($n=3$)	RSD[a] (%)	Accuracy[b] (%)
Peimissine						
3.67	3.43±0.05	1.3	93.5	3.70±0.23	6.1	99.2
14.68	13.77±1.25	9.1	93.8	14.38±0.66	4.6	98.0
Verticine						
3.43	3.26±0.21	6.4	95.0	3.21±0.34	10.7	93.6
13.72	13.21±0.48	3.6	96.3	13.26±0.72	5.4	96.6
Verticinone						
2.96	3.04±0.17	5.5	97.3	3.00±0.31	10.2	98.5
11.84	11.54±0.52	4.5	97.5	10.91±1.06	9.7	92.6
Imperialine						
3.46	3.32±0.05	1.5	96.0	3.16±0.19	6.1	91.3
13.84	13.15±0.38	2.9	95.0	12.87±0.62	4.8	93.0
Isoverticine						
3.14	3.13±0.28	8.8	99.7	3.10±0.30	9.6	98.7
12.56	12.46±0.37	3.0	99.2	11.71±0.93	7.9	93.2
Ebeiedine						
3.24	3.24±0.26	7.9	100.0	3.03±0.20	6.5	93.5
12.96	12.73±0.31	2.4	98.2	11.99±0.98	8.2	92.5

[a] RSD (%) (relative standard deviation)=(SD/mean)×100.
[b] Accuracy (%)=[1−(mean concentration measured−concentration spiked)/concentration spiked]×100.

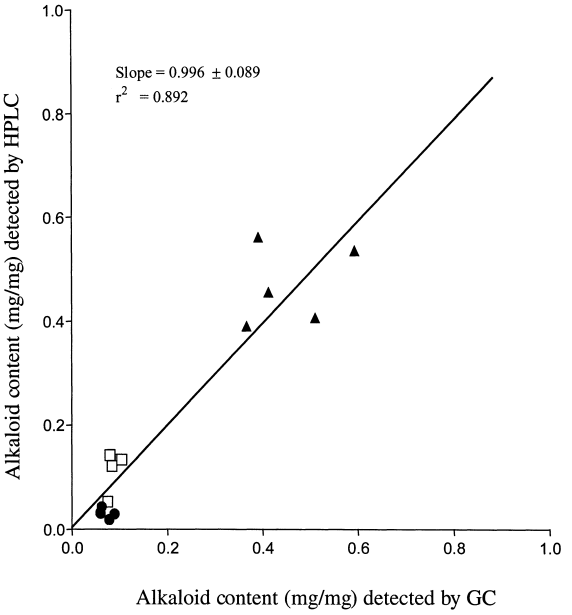

Fig. 3. Correlation between alkaloid contents in the crude alkaloid extracts, quantified by HPLC–ELSD and GC methods. ●: Imperialine in *F. cirrhosa*; □: isoverticine in *F. hupehensis*; ▲: verticine+verticinone in *F. thunbergii*.

linear responses and adequate sensitivity for all *Fritillaria* alkaloids examined (Table 1) and the advantage of on-line ELSD–LTA for high-water-containing mobile phase.

The results demonstrated that the developed analytical method is reproducible with good accuracy and sensitivity for all analytes examined. The overall intra- and inter-day variations were less than 11% for all six alkaloids, and the overall intra- and inter-day accuracies were higher than 93% and 91%, respectively (Table 2). The limits of detection were 38.5 μg/g of the dried herb for peimissine, 35.0 μg/g for verticine, imperialine and ebeiedine, 31.5 μg/g for verticinone and isoverticine, respectively. Moreover, in order to further confirm the accuracy of HPLC measurement, contents of verticine, verticinone, isoverticine and imperialine in the crude alkaloid extracts obtained from three *Fritillaria* species were determined by HPLC–ELSD and GC methods [13] in a parallel study. The results were compared and as shown in Fig. 3, a good correlation (slope=0.996, r^2=0.892) was observed for all analytes tested, indicating an adequate accuracy for both analytical methods. For the comparison of contents of verticine and verticinone, the sum of these two alkaloids was utilized since they cannot be quantified separately by GC assay [13].

The newly developed HPLC–ELSD assay was subsequently applied to a simultaneous determination of the major alkaloids in five different *Fritillaria* species. Representative chromatograms of the extracts of these Beimu samples are also shown in Fig. 2B–F, and their contents of alkaloids are summarized in Table 3. The results demonstrated a successful application of this HPLC–ELSD assay for the quantification of the major active *Fritillaria* alkaloids in different Beimu samples, and also indicated that different *Fritillaria* species may contain different types and/or different quantities of alkaloids. Therefore, although it needs to be further clarified, variations in types and quantities of the major active *Fritillaria* alkaloids present in different *Fritillaria* species may be responsible for the different usefulness and therapeutic efficacy of these TCM herbs. Investigations into the correlation between biologically active ingredients present in different Beimu herbs and their pharmacological activities are currently in progress in our research laboratories and the results will be reported separately.

Table 3
Contents of isosteroidal alkaloids in *Fritillaria* species

Fritillaria species	Content[a] (μg/g)							
	Peimissine	Verticine	Verticinone	Imperialine	Isoverticine	Ebeiedine	Ebeiedinone	Hupehenine
F. thunbergii Miq.	191.7±18.4	2075.2±213.7	802.9±78.4	−	329.3±34.6	−	+	−
F. hupehensis Hsiao et Hasi	601.7±24.0	105.2±5.5	1866.2±66.1	−	195.8±4.9	tr	+	+
F. ebeiensis var. *purpurea* Yu et Ji	716.7±25.3	629.9±19.2	2385.0±123.7	−	1119.2±8.5	110.2±3.1	+	−
F. cirrhosa Don	105.3±8.8	139.1±6.2	121.3±9.2	90.6±5.5	76.7±4.5	−	−	−
F. walujewii Rgl.	185.2±8.2	−	−	113.9±3.4	−	−	−	−

[a] tr: Content of the alkaloid was below the limit of detection; +: detected but not quantified; −: not detected.

4. Conclusion

The newly developed direct HPLC–ELSD analysis is compatible with our previously established direct GC analytical method for the determination of isosteroidal alkaloids in *Fritillaria* species. It is a simple, sensitive and selective analytical method with good accuracy and reproducibility. This HPLC assay can be readily utilized as a suitable quality control method for the determination of the major biologically active ingredients in Beimu, the most commonly used antitussive TCM herb.

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