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LC–MS/MS Coupled with Chemometric Analysis as an Approach for the Differentiation of *Fritillariae cirrhosae* Bulbus and *Fritillariae pallidiflorae* Bulbus

Xiaomu Zhu [1], Ting Zhou [2,3], Shu Wang [2], Bengui Ye [2], Rajeev K. Singla [4], Devesh Tewari [5], Atanas G. Atanasov [6,7], Dongdong Wang [8] and Simei Liu [2,9,*]

1 Department of Pharmacy, Sichuan University, Chengdu 610041, China
2 Key Laboratory of Drug-Targeting and Drug Delivery System of the Education Ministry and Sichuan Province, Sichuan Engineering Laboratory for Plant-Sourced Drug and Sichuan Research Center for Drug Precision Industrial Technology, West China School of Pharmacy, Sichuan University, Chengdu 610041, China
3 Department of Public Service Management, Chongqing Vocational College of Transportation, Chongqing 402247, China
4 School of Pharmaceutical Sciences, Lovely Professional University, Phagwara, Punjab 144411, India
5 Department of Pharmacognosy and Phytochemistry, School of Pharmaceutical Sciences, Delhi Pharmaceutical Sciences and Research University, New Delhi 110017, India
6 Ludwig Boltzmann Institute for Digital Health and Patient Safety, Medical University of Vienna, Spitalgasse 23, 1090 Vienna, Austria
7 Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, Jastrzebiec, 05-552 Magdalenka, Poland
8 Centre for Metabolism, Obesity, and Diabetes Research, Department of Medicine, McMaster University, HSC 4N71, 1280 Main Street West, Hamilton, ON L8S 4K1, Canada
9 Institute of Pharmacology and Toxicology, Chongqing Academy of Chinese Materia Medica, Chongqing 400065, China
* Correspondence: 2019324050029@stu.scu.edu.cn; Tel./Fax: +86-28-8550-3950

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Abstract: *Fritillariae cirrhosae* bulbus (FCB) is one of the most important traditional Chinese medicines (TCM) for the treatment of cough and phlegm. Due to increasing demand and the complexity of FCB's botanical origin, various substitutes have appeared in the market, resulting in a major challenge to distinguish FCB and its substitutes (*F. pallidiflorae* bulbus, FPB). Therefore, discriminating FCB from FPB has becoming an urgent necessity. In this study, an ultra-high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (UPLC–ESI–MS/MS) method was developed for the simultaneous quantification of nine steroidal alkaloids (imperialine-3-β-D-glucoside, imperialine, verticine, verticinone, peimisine, yibeinoside A, delavine, delavinone, ebeidinone) within 8 min. According to the composition and content of the above nine compounds, multivariate chemometric analyses were applied for the classification of FCB and FPB. The quantitative results showed that there were both similarities and differences in the content of nine steroidal alkaloids between FCB and FPB, and it was difficult to directly distinguish these two species. Fortunately, with the aid of chemometric analyses, FCB and FPB were successfully differentiated by partial least squares discrimination analysis (PLS-DA) and orthogonal partial least squares discrimination analysis (OPLS-DA) models based on the nine alkaloids' content. Moreover, four compounds (yibeinoside A, ebeiedinone, delavinone and imperialine) were discovered as potential markers for the identification and differentiation of FCB and FPB. Additionally, compared to other studies, this work collected a large number of samples (49 batches of FCB and 17 batches of FPB) to ensure the reliability of the results. In conclusion, this work established a new approach for the authentication of FCB based on its active components, which provides a good reference for the quality control of FCB and will help us to understand the chemical composition differences between FCB and its adulterants further.

Keywords: *Fritillariae cirrhosae* bulbus; *Fritillariae pallidiflorae* bulbus; steroidal alkaloids; UPLC-ESI-MS/MS; chemometric analysis; authentication

1. Introduction

The genus *Fritillariae*, family *Liliaceae*, is made up of over 131 species worldwide, and of these, *Fritillariae cirrhosae* bulbus (FCB, Chuan-Beimu in Chinese) is one of the most well-known herbs, which has been used as an antitussive, expectorant and anti-asthma drug in traditional Chinese medicine (TCM) for over 2000 years [1]. Since the 2010 edition of the Chinese Pharmacopoeia, six species of FCB, namely *F. cirrhosa* D. Don, *F. unibracteata* Hsiao et K.C. Hsia, *F. przewalskii* Maxim., *F. delavayi* Franch., *F. taipaiensis* P. Y. Li and *F. unibracteata* Hsiao et K.C. Hsia var. wabuensis (S.Y. Tang et S.C. Yue) Z.D. Liu, S. Wang et S.C. Chen, have been officially recorded. In fact, with the continuous discovery of related species of FCB, there have been more than 30 species of botanical origin of FCB [2], which makes the authentication of FCB difficult. Moreover, due to its good therapeutic efficacy and high safety, the market demand for FCB has overwhelmed its production, resulting in a rise in FCB adulterants in herbal markets, which has caused a major challenge in the authentication of FCB.

In China, FCB has been further divided into different commercial specifications, namely Song-Beimu (SB), Qing-Beimu (QB) and Lu-Beimu (LB), according to their morphological features. Additionally, due to the limited wild resources of FCB, cultivated FCB has gradually become predominant in the herbal market, which also could be divided into cultivated SB (ZSB) and cultivated QB (ZQB) depending upon their appearance characteristics [3]. In these commercial specifications, QB and ZQB almost occupy 40% of the market share of FCB, which means that their differentiation and that of their adulterants is important for the quality control of FCB. *Fritillariae pallidiflorae* bulbus (FPB, Yi-Beimu in Chinese) is one of the most common adulterants of FCB, especially for QB and ZQB. For a long time, it has been difficult to differentiate FCB (QB and ZQB) and FPB, because they have similar morphological characteristics, chemical compositions and bioactive properties. Based on this, some researchers suggest that FPB may be replaced with FCB. FCB and FPB belong to two different species because their origins, sources and plant morphological characteristics are very different, so we should exercise caution in the replacement of FCB with FPB without sufficient evidence to prove that these two species are similar in terms of many different aspects, especially their pharmacological activity and toxicology. In addition, it is also not acceptable to use FPB as a replacement for FCB in the commercial market. At present, FPB is still recognized as an adulterant of FCB by the authority of the Chinese government. Thus, more efforts are still needed to find a solution to authenticate FCB and FPB. Although some previously reported molecular biological methods and near-infrared spectroscopy can be useful to classify FCB and its adulterants [4–7], these methods are still limited because they cannot reflect the internal quality of FCB and its adulterants, particularly in terms of specialized metabolites, which is one reason that the quality control of FCB still faces huge challenges. Thus, it is highly necessary to find an effective method that can distinguish the adulterants of this important plant based on the bioactive components to differentiate FCB and FPB.

Phytochemical studies showed that FCB and FPB both contain steroidal alkaloids, saponins, terpenoids and fatty acids [8]. Among these components, steroidal alkaloids have been recognized as the most valuable compounds in the *Fritillariae* species because they are mainly responsible for their therapeutic effects [9]. Therefore, studying steroidal alkaloids is quite necessary for the differentiation of FCB and FPB, as well as the quality control of FCB. In the past few decades, several analytical methods focused on steroidal alkaloids in *Fritillariae* bulbus were developed, including thin-layer chromatography (TLC) [10], high-performance liquid chromatography (HPLC) coupled with UV detection or evaporative light scattering detection (ELSD) [11–13], gas chromatography (GC) [14], liquid chromatography coupled with tandem mass spectrometry (LC–MS) [15–18] and so on. Among them, the LC–MS method successfully resolved the problem wherein steroidal alkaloids have no UV absorption, and it provided a powerful approach for multiple chemical composition analysis due to its high resolution and sensitivity, as well as the advantages of low separation requirements [19,20]. In previous studies, the distribution of various

steroidal alkaloids in *Fritillariae* species was qualitatively or quantitatively investigated by LC–MS [16–18,21–23], and different *Fritillariae* species were discriminated through chemometric analysis based on the differences in steroidal alkaloids. However, to the best of our knowledge, there are no studies systematically reporting the differences in chemical composition between FCB (QB and ZQB) and FPB, especially regarding the alkaloids, including imperialine-3-β-D-glucoside, imperialine, verticine, verticinone, peimisine, yibeinoside A, delavine, delavinone and ebeidinone. Most of the previous studies use a few alkaloids as the quantitative indexes and lack sufficient samples. Thus far, studies on the simultaneous quantification of the above nine alkaloids in sufficient batches of FCB and FPB have not been reported.

Therefore, in the present study, we aim to develop a new UPLC–ESI–MS/MS method to analyze these nine alkaloids in FCB and FPB and compare the differences in chemical composition between these two species. Furthermore, we attempt to distinguish these *Fritillariae* bulbus species by employing chemometric analysis based on the content of the above nine alkaloids. We also determine alkaloid markers for the discrimination of FCB and FPB, which will provide a further reference for the quality control of FCB. In addition, to overcome the flaws of previous studies, we provide full, detailed information on the origins and sources of samples and use sufficient batches of samples (49 batches of FCB and 17 batches of FPB) as possible to ensure reliable results.

2. Materials and Methods

2.1. Chemicals and Reagents

Imperialine was isolated and elucidated from *F. cirrhosae* bulbus in our laboratory [24]. Verticinone, verticine and peimisine were purchased from Push Bio-Technology (Chengdu, Sichuan, China). Imperialine-3-β-D-glucoside and delavine were purchased from Chengdu Herbpurify Co., Ltd. (Chengdu, Sichuan, China). Yibeinoside A, delavinone and ebeidinone were purchased from Chengdu Mansite Pharmaceutical Co. Ltd. (Chengdu, Sichuan, China). The purity of all the standard substances was above 99.8%. Their chemical structures are shown in Figure 1.

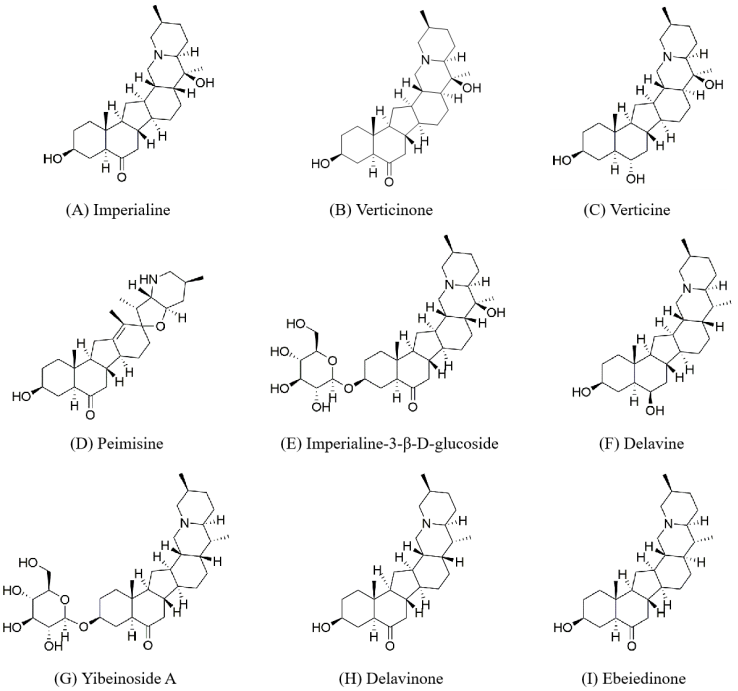

Figure 1. The chemical structures of nine alkaloids: (**A**) Imperialine; (**B**) Verticinone; (**C**) Verticine; (**D**) Peimisine; (**E**) Imperialine-3-β-D-glucoside; (**F**) Delavine; (**G**) Yibeinoside A; (**H**) Delavinone; (**I**) Ebeiedinone.

Acetonitrile, methanol and formic acid (98%) were of LC–MS grade, from Sigma-Aldrich (St. Louis, MO, USA). Water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Other solvents were of analytical grade and purchased from Chengdu Chron Chemicals Co., Ltd. (Chengdu, Sichuan, China).

2.2. Plant Materials

A total of 49 batches of FCB (QB and ZQB) and 17 batches of FPB were collected from wild or cultivated sources in different areas of China and authenticated by the authors. Detailed information for all samples is shown in Table 1.

Table 1. Sources and origins of analyzed bulbus of Fritillariae.

Species	Sample Code	Source	Collection Date
F. cirrhosa	ZQB-1	Maoxian, Sichuan; cultivated	15 September 2017
	ZQB-2	Qinghai; cultivated	30 July 2018
	ZQB-3	Qinghai; cultivated	30 July 2018
	ZQB-4	Qinghai; cultivated	22 January 2016
	ZQB-5	Kangding, Sichuan; cultivated	22 January 2016
	ZQB-6	Qinghai; cultivated	22 January 2016
	ZQB-7	Kangding, Sichuan; cultivated	22 January 2016
	ZQB-8	Maoxian, Sichuan; cultivated	28 March 2016
	ZQB-9	Maoxian, Sichuan; cultivated	1 November 2016
	ZQB-10	Maoxian, Sichuan; cultivated	21 August 2018
	ZQB-11	Qinghai; cultivated	22 August 2017
	ZQB-12	Tibet; cultivated	August 2017
	ZQB-13	Qinghai; cultivated	20 March 2015
	ZQB-14	Qinghai; cultivated	25 October 2014
	ZQB-15	Maoxian, Sichuan; cultivated	1 November 2016
	ZQB-16	Qinghai; cultivated	20 March 2015
	ZQB-17	Maoxian, Sichuan; cultivated	28 March 2016
	ZQB-18	Maoxian, Sichuan; cultivated	27 July 2016
	ZQB-19	Maoxian, Sichuan; cultivated	27 July 2016
	ZQB-20	Maoxian, Sichuan; cultivated	27 July 2016
	ZQB-21	Qinghai; cultivated	20 June 2018
	ZQB-22	Qinghai; cultivated	10 July 2018
	ZQB-23	Qinghai; cultivated	10 August 2018
	ZQB-24	Qinghai; cultivated	20 June 2018
	ZQB-25	Qinghai; cultivated	20 August 2018
	ZQB-26	Qinghai; cultivated	20 June 2018
	ZQB-27	Qinghai; cultivated	August 2017
F. unibracteata	ZQB-28	Qinghai; cultivated	10 September 2015
	ZQB-29	Songpan, Sichuan; cultivated	October 2014
	ZQB-30	Songpan, Sichuan; cultivated	May 2013
	ZQB-31	Qinghai; cultivated	30 June 2018
	ZQB-32	Qinghai; cultivated	21 July 2018
F. unibracteata var. *wabuensis*	ZQB-33	Maoxian, Sichuan; cultivated	6 April 2018
	ZQB-34	Maoxian, Sichuan; cultivated	28 September 2015
	ZQB-35	Songpan, Sichuan; cultivated	21 August 2018
	ZQB-36	Songpan, Sichuan; cultivated	21 August 2018
	ZQB-37	Songpan, Sichuan; cultivated	August 2014
F. cirrhosa	QB-1	Xiangcheng, Sichuan; wild	July 2012
	QB-2	Yajiang, Sichuan; wild	1 July 2012
	QB-3	Kangding, Sichuan; wild	13 July 2015
	QB-4	Kangding, Sichuan; wild	June 2018
	QB-5	Qinghai; wild	21 August 2018
	QB-6	Tibet; wild	July 2017
	QB-7	Changdu, Tibet; wild	August 2018
	QB-8	Ganzi, Sichuan; wild	June 2018
	QB-9	Yushu, Qinghai; wild	June 2018
	QB-10	Songpan, Sichuan; wild	5 January 2019
F. unibracteata	QB-11	Danba, Sichuan; wild	September 2016
	QB-12	Songpan, Sichuan; wild	June 2017
F. pallidiflora	YB-1	Yili, Xinjiang; cultivated	June 2015
	YB-2	Yili, Xinjiang; cultivated	June 2015
	YB-3	Yili, Xinjiang; cultivated	15 May 2014
	YB-4	Yili, Xinjiang; cultivated	15 May 2014
	YB-5	Yili, Xinjiang; cultivated	6 October 2015
	YB-6	Yili, Xinjiang; cultivated	June 2015
	YB-7	Yili, Xinjiang; cultivated	June 2015
	YB-8	Yili, Xinjiang; cultivated	June 2015
	YB-9	Yili, Xinjiang; cultivated	June 2015
	YB-10	Yili, Xinjiang; cultivated	6 October 2015
	YB-11	Yili, Xinjiang; cultivated	15 May 2014
	YB-12	Yili, Xinjiang; cultivated	10 June 2016
F. walujewii	YB-13	Tuoli, Xinjiang; cultivated	16 June 2016
	YB-14	Tuoli, Xinjiang; cultivated	6 June 2016
	YB-15	Tacheng, Xinjiang; cultivated	6 June 2016
	YB-16	Xinjiang; cultivated	6 June 2016
	YB-17	Yili, Xinjiang; cultivated	6 June 2016

Note: A missing date indicates an incomplete record when collected.

2.3. Standard Solution Preparation

The stock standard solution of each analyte was prepared by accurately weighing imperialine (5.970 mg), imperialine-3-β-D-glucoside (5.550 mg), verticine (6.280 mg), verticinone (4.940 mg), yibeinoside A (5.470 mg), peimisine (4.710 mg), delavine (5.500 mg), delavinone (4.930 mg) and ebeidinone (5.140 mg) and dissolving them in 5 mL of methanol, respectively.

A mixed stock standard solution of each of the nine alkaloids was prepared in 50% methanol at concentrations of 20 $\mu g \cdot mL^{-1}$ for each analyte. Then, the mixed stock solution was diluted to appropriate concentrations for calibration curves.

2.4. Sample Solution Preparation

The bulbus were grounded into powder, sieved through a No. 20 mesh and dried at 45 °C for 8 h. The dried powder (0.3–0.5 g) was alkalized with an ammonia solution (25%) for 1 h and refluxed with 10 mL chloroform–methanol (4:1, *v/v*) at 80 °C for 2 h. After being filtered, the extracts were concentrated to dryness at 65 °C. The residue was dissolved in 2 mL methanol. The solution was centrifuged at 10,000 rpm for 5 min and the supernatant was transferred to a new tube as a sample stock solution. Then, we pipetted 0.1 mL sample stock solution into a tube, diluted it at 1:10 with 50% methanol and centrifuged it at 20,000 rpm for 10 min. The supernatant was filtered through a membrane filter (0.22 μm) for LC–MS/MS analysis.

2.5. LC–MS/MS Conditions

Analyses were performed on the Shimadzu Nexera UHPLC system equipped with a binary pump, an online degasser, a well-plate autosampler and a column oven (Shimadzu, Tokyo, Japan) coupled to an AB Sciex 5500 Triple Quadrupole Mass Spectrometer (AB Sciex, CA, USA) with an electrospray ionization (ESI) source. Chromatographic separation was performed on a Shim-Pack XR-ODS column (2.2 μm, 100 mm × 2.00 mm I.D.) (Shimadzu, Japan) at 40 °C. The mobile phase was a gradient of 0.1% (*v/v*) aqueous formic acid (A) and acetonitrile (B): 0.0–15.0% B for 0.00–1.00 min, 15.0–25.0% B for 1.00–3.00 min, 25.0% B for 3.00–4.00 min, 25.0–95.0% B for 4.00–5.00 min, 95.0% B for 5.00–5.50 min, 95.0–15.0% B for 5.50–5.60 min, 15.0% B for 5.60–8.00 min. The flow rate was set at 0.4 $mL \cdot min^{-1}$ and the injection volume of the sample was 1 μL.

The ESI source was operated in positive ionization mode, using multiple reaction monitoring (MRM). The optimal MS parameters were as follows: capillary voltage, 5500 V; ion source temperature, 500 °C; nebulizer gas, 50 psi; heater gas, 50 psi; curtain gas, 40 psi. Nitrogen was used in all cases. The monitoring ion pair, declustering potential (DP) and collision energy (CE) for each analyte are shown in Table 2. Analyst 2.0 software (AB Sciex, Foster City, CA, USA) was used for all the operations, data acquisition and data analysis. The secondary mass spectra of each analyte are shown in the Supplementary Materials (Figure S1).

Table 2. MS detection parameters of nine alkaloids.

Standard Reference	Retention Time (min)	Q1 Mass (Da)	Q3 Mass (Da)	Dwell time (ms)	DP (Volts)	CE (Volts)
Imperialine-3-*β*-D-glucoside	1.911	592.4	574.4	15	40	75
Imperialine	2.874	430.4	138.1	15	100	60
Peimisine	3.217	428.3	114.1	20	230	40
Verticine	3.385	432.4	414.3	15	130	75
Verticinone	3.604	430.3	412.3	15	120	65
Yibeinoside A	3.715	576.4	414.4	15	150	80
Delavine	4.068	416.4	98.1	15	180	65
Delavinone	4.310	414.4	98.1	15	40	65
Ebeiedinone	4.679	414.4	91.1	15	130	110

2.6. Method Validation

A series of diluted standard stock solutions were injected to obtain calibration curves. Linearity was evaluated by correlation coefficients. The limit of quantification (LOQ) and limit of detection (LOD) were determined as the concentration for each analyte with a signal-to-noise ratio of 10 and 3, respectively. Precision was evaluated by intra- and inter-day variations. Intra-day precision was determined by analyzing six replicates of the same standard solution on the same day. Inter-day precision was tested on the same standard solution in triplicate on three consecutive days. Repeatability was examined in six independent sample solutions from the same batch. Stability was evaluated by analyzing the same standard solution and sample solution at 0, 12, 24, 36, 48 and 72 h at room temperature. Accuracy was evaluated in a recovery experiment, in which known amounts of the standards were spiked at low, medium and high concentration levels into the samples and then analyzed according to the procedure in Section 2.5. The recovery of each analyte was calculated as follows: recovery (%) = (detection amount—original amount)/amount added $\times$ 100.

2.7. Sample Measurement

Quantitative analyses were performed in triplicate for each sample. The identification and assignation of each compound were performed by comparing the retention time and monitoring pair ions to a pure standard. Calibration curves were used for quantification. Results were given as micrograms analyte per gram herb (μg/g).

2.8. Statistical Analysis

Data were expressed as mean $\pm$ standard deviation (SD). Heatmap and hierarchical cluster analysis (HCA) were performed on MeV 4.9.0 software (https://sourceforge.net/projects/mev-tm4/files/mev-tm4/; accessed on 11 August 2019). Principal component analysis (PCA), partial least squares discrimination analysis (PLS-DA) and orthogonal partial least squares discrimination analysis (OPLS-DA) were performed using Simca 13.0 software (Umetrics, Umea, Sweden).

3. Results and Discussion

3.1. LC–MS/MS Method

In the present study, LC–MS/MS was performed with small adjustments according to the previously described methods [21], and the method was validated according to the European Medicines Agency (EMEA) guidelines relating to the validation of analytical methods (Quality Guidelines: Validation of Analytical Procedures—Text and Methodology (ICH Q2)). The results showed that under modified chromatographic conditions, we successfully achieved good separation within 8 min for nine steroidal alkaloids, and the method was proven to be sensitive, precise, accurate, reliable and reproducible for the simultaneous quantification of the nine steroid alkaloids in *Fritillariae* samples. Compared with previous studies [16–18,23], our method firstly was able to simultaneously quantify the content of imperialine-3-β-D-glucoside, imperialine, verticine, verticinone, peimisine, yibeinoside A, delavine, delavinone and ebeidinone. Additionally, this method greatly shortened the analysis time and improved the sensitivity of analytes. The specific data and figures are shown in the Supplementary Materials (Figure S2 and Table S1).

3.2. Sample Analysis

The above LC–MS/MS method was applied to analyze the content of nine steroidal alkaloids in all samples. Representative total ion chromatograms (TIC) of the nine steroidal alkaloids in FCB and FPB are shown in the Supplementary Materials (Figure S3). The concentrations of analytes in samples were calculated from their corresponding calibration curves. The content of the nine steroidal alkaloids in 67 batches of samples was displayed on a heatmap intuitively (Figure 2). The specific data of each alkaloid's content are shown in the Supplementary Materials (Table S2). In addition, these nine alkaloids

were selected in this study because (1) they are very common in FCB and FPB; (2) there is no study that uses them simultaneously; and (3) these alkaloids are readily available in the commercial market.

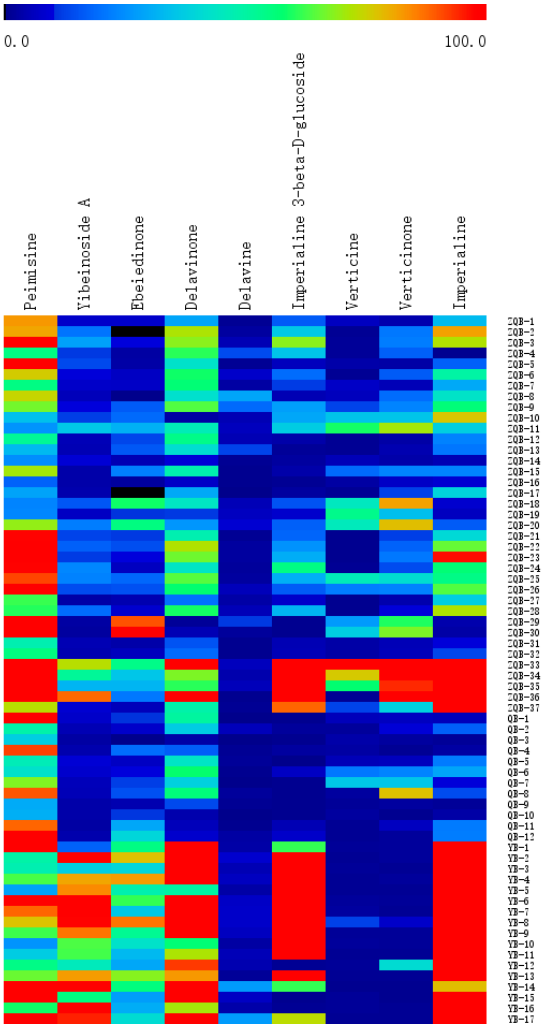

Figure 2. The heatmap of nine alkaloids in 58 batches of FCB and FPB samples.

In FCB (QB), the content of peimisine was generally higher than that of the other alkaloids, ranging from 30.49 to 248.58 $\mu g \cdot g^{-1}$. Delavine and imperialine-3-β-D-glucoside had a lower level in general, and their content was less than 7.50 $\mu g \cdot g^{-1}$. Yibeinoside A was detected in all QB samples with a level below 9.50 $\mu g \cdot g^{-1}$, but the fluctuation was small. The content of verticine was also low in most QB samples. By contrast, the levels of ebeiedinone, delavinone, imperialine and verticinone varied widely, at 0.68~39.83, 3.50~58.18, 1.00~77.87 and 1.57~29.27 $\mu g \cdot g^{-1}$, respectively. In FCB (ZQB), the levels of the nine steroidal alkaloids all fluctuated greatly. Among the nine alkaloids, peimisine, delavinone, imperialine-3-β-D-glucoside, verticinone and imperialine were generally higher (especially in ZQB-33~ZQB-37), which were 17.92~208.26, 1.93~196.53, 0.00~619.73, 3.24~123.70, 3.25~680.29 $\mu g \cdot g^{-1}$, respectively.

In FPB, we could easily see that the content of verticine, verticinone and delavine was lower than that of the other six steroidal alkaloids, and the levels of verticine and verticinone in most FPB samples were below 2.50 $\mu g \cdot g^{-1}$, while the level of delavine was almost less than 8.30 $\mu g \cdot g^{-1}$. In comparison, FPB contained a high level of peimisine, yibeinoside A, ebeiedinone, delavinone, imperialine-3-β-D-glucoside and imperialine, which amounted to 26.09~234.59, 17.78~382.00, 27.77~86.68, 50.29~279.53, 1.02~1180.07 and

78.05~344.09 $\mu g \cdot g^{-1}$, respectively. According to the above results, it was not difficult to observe that most FCB samples were different from FPB in the content of the nine steroidal alkaloids, except for some ZQB samples derived from the cultivated bulbus of *F. unibracteata* Hsiao et K.C. Hsia var. wabuensis (S.Y. Tang et S.C. Yue) Z.D. Liu, S. Wang et S.C. Chen. These findings were fully coincident with our previous expectations.

3.3. Chemometric Analysis

3.3.1. HCA

HCA is an unsupervised classification procedure that reveals the inherent connections of objects according to the intrinsic characteristics of experimental data without previous information on the objects [18]. HCA produces a dendrogram with samples grouped into branches to show the hierarchy of the clusters or the similarity of objects. In this study, 67 batches of samples were grouped in two main clusters according to the composition and content of the nine steroidal alkaloids (Figure 3). One cluster contained almost all batches of FPB (except YB-15) and six batches of FCB (ZQB-10, ZQB-33~ZQB-37). The other cluster was mainly composed of most of the batches of FCB (all QB and remaining ZQB) and one batch of FPB (YB-15). As shown in Figure 3, it was clear that QB and most ZQB samples could be distinguished from FPB by HCA, which demonstrated that these samples had a low degree of similarity in the content of the nine alkaloids. However, some ZQB samples (especially those that originated from the cultivated bulbus of *F. unibracteata* Hsiao et K.C. Hsia var. wabuensis (S.Y. Tang et S.C. Yue) Z.D. Liu, S. Wang et S.C. Chen) still could not be differentiated from FPB by HCA. These results were coincident with the above analysis of the alkaloid content.

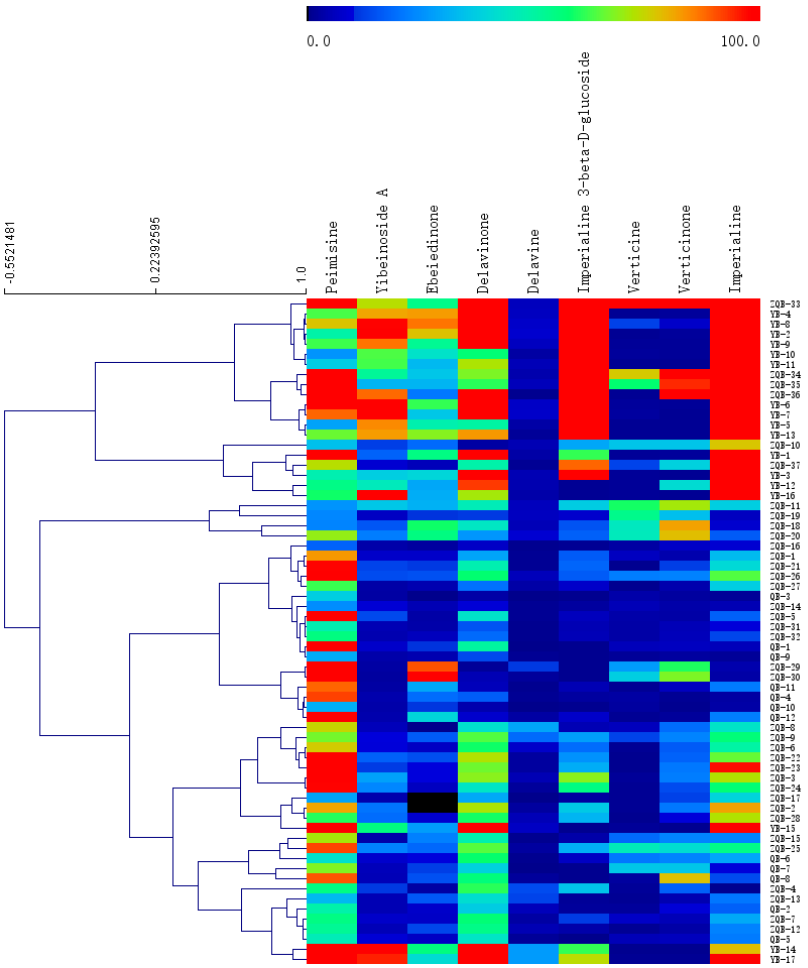

Figure 3. The HCA of FCB and FPB based on the content of nine steroidal alkaloids.

3.3.2. PCA

To compensate for the disadvantages of HCA, PCA was applied for the identification of FCB and FPB. As with HCA, PCA is also an unsupervised mathematical method that reduces the dimensionality of the data, with a small loss of variability explained by the majority of variables in the original data, so as to identify new, meaningful underlying variables and visualize the clustering, trends and outliers among the observations [25–27]. As shown in Figure 4A, most FCB (ZQB) and FPB could be entirely separated, but a few FCB (ZQB) samples were still clustered in the FPB group. The first principle component (PC1) and the second principle component could explain 91.46% of the variance (PC1 = 81.70% and PC2 = 9.76%, respectively). Moreover, PC1 was highly and positively correlated with imperialine-3-β-D-glucoside and imperialine, and PC2 was mainly correlated with imperialine (Figure 4B). As shown in Figure 4C, FCB (QB) could be discriminated from FPB, and the first two components explained 81.50% of the total variance (PC1 = 66.00% and PC2 = 15.50%, respectively). As shown in the loading plot in Figure 4D, imperialine-3-β-D-glucoside contributed strongly to PC1 and delavinone highly contributed to PC2. The above classification was quite consistent with the results of HCA, and the PCA results also demonstrated important steroidal alkaloids that contributed strongly to the clusters of FCB and FPB.

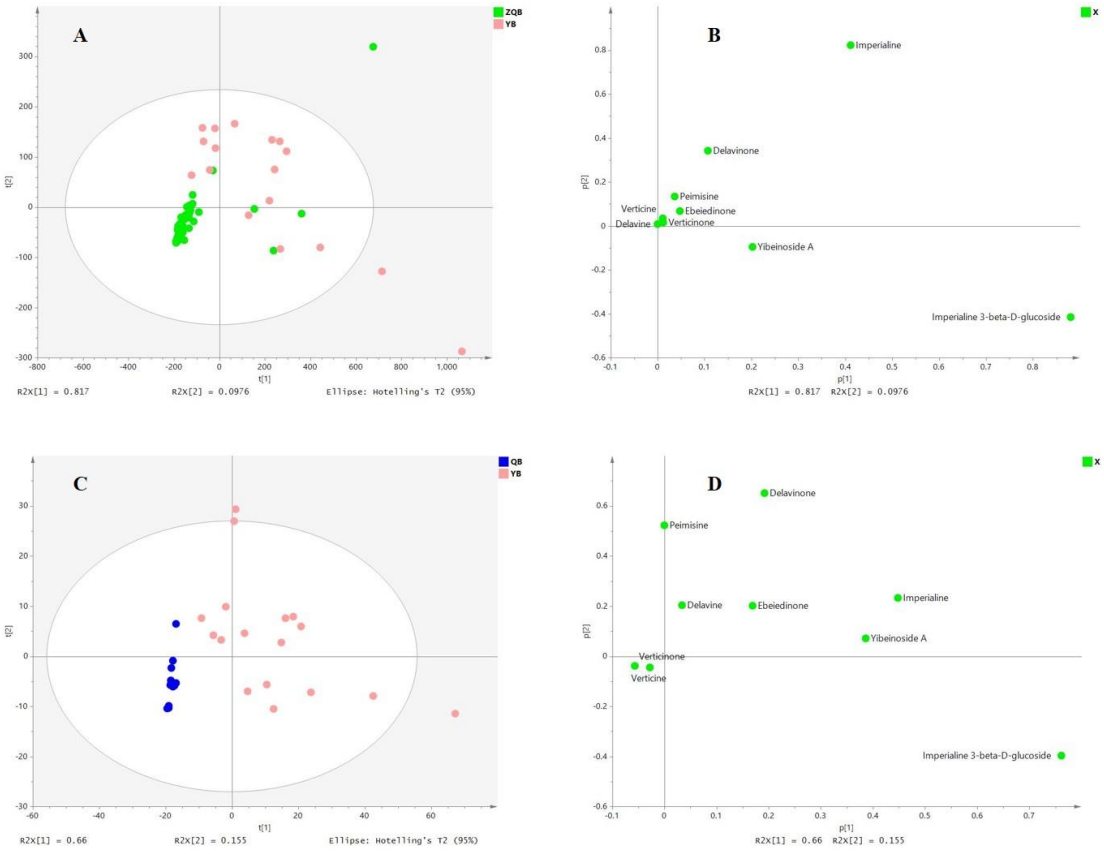

Figure 4. The PCA of bulbus of FCB and FPB based on the content of nine alkaloids: score plot (**A**,**C**) and loading plot (**B**,**D**).

3.3.3. PLS-DA and OPLS-DA

PLS-DA and OPLS-DA are supervised recognition patterns, which can reduce and weaken the differences in data within groups and highlight the differences between groups [28–30]. In these models, R2 represents the variation described by all components and Q2 is a predictive value of the given model that is calculated by cross-validation (permutation test) [23]. In general, the larger R2 and Q2, the better the interpretation and

prediction capacity of the model will be, as well as the stability and reliability. According to the value of Q2, the model could predict the distribution of unknown related samples.

As shown in Figure 5A,C, we observed obvious separation between FCB (both ZQB and QB) and FPB in the PLS-DA model. The PLS-DA models were both autofitted with two predictive X-Y components, which resulted in R2X (cum) = 0.584, R2Y (cum) = 0.788, Q2 (cum) = 0.724 (Figure 5B) and R2X (cum) = 0.574, R2Y (cum) = 0.842, Q2 (cum) = 0.778 (Figure 5D), respectively. The permutation test was performed to validate the classification model and the procedure was set to be repeated 200 times. The results indicated that the model was not over-fitted. The permutation plot is shown in the Supplementary Materials (Figure S4).

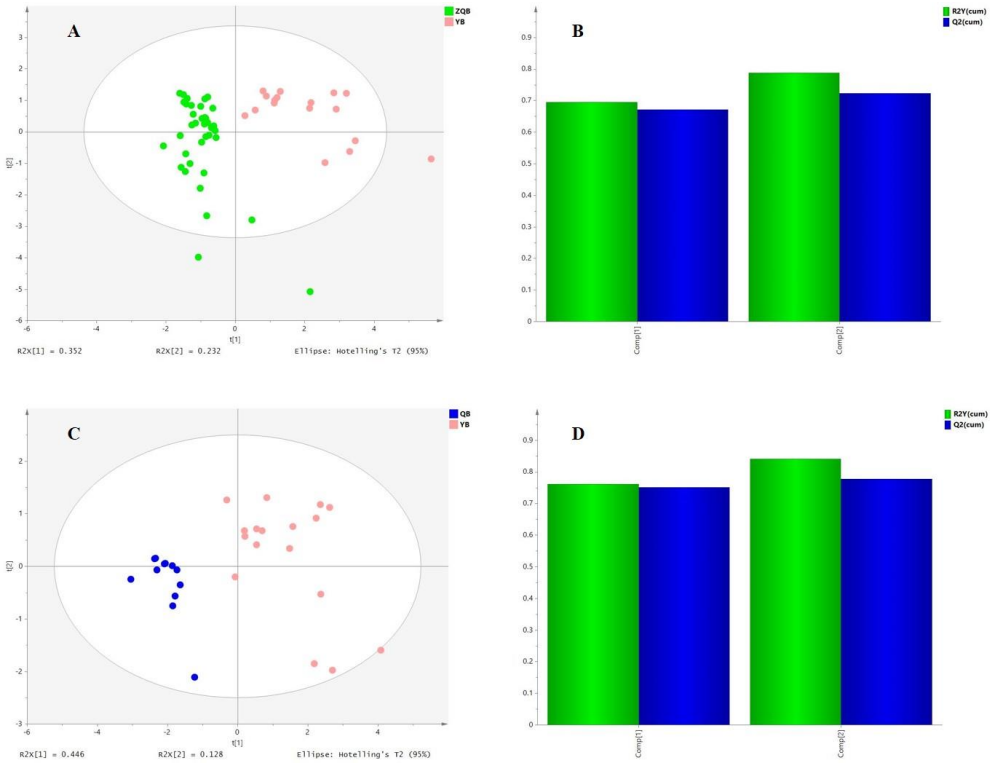

Figure 5. The PLS-DA of bulbus of FCB and FPB based on the content of nine alkaloids: score plot (**A**,**C**) and cumulated percentage variation explained by principal components (**B**,**D**).

Likewise, all batches of FCB (both ZQB and QB) were completely separated from FPB in the OPLS-DA classification model (Figures 6A and 7A). As shown in Figures 6B and 7B, the value of R2 was same as that in the PLS-DA model, but the value of Q2 was 0.734 for ZQB and 0.769 for QB, respectively. In the OPLS-DA model, variable importance in projection (VIP) implied the contribution to the discrimination of each chemical component between groups [30]. The *S*-plot and Student's *t*-test were coupled with the VIP values to select chemical features contributing to the discrimination. In general, variables with VIP > 1 and $p < 0.05$ were considered to be potential chemical markers [23,30]. As shown in Figure 6C,D, combined with the *p* value, yibeinoside A (VIP = 1.35), delavinone (VIP = 1.34), imperialine (VIP = 1.27) and ebeiedinone (VIP = 1.14) were the most important steroidal alkaloids for differentiation between FCB (ZQB) and FPB. The total content of the above four alkaloids was clearly different between ZQB (except for several ZQB samples that were derived from the cultivated bulbus *of F. unibracteata* Hsiao et K.C. Hsia var. wabuensis (S.Y. Tang et S.C. Yue) Z.D. Liu, S. Wang et S.C. Chen, such as ZQB-33 and ZQB-36) and FPB, which amounted to 22.12~315.43 μg·g-1 and 316.49~692.99 $\mu g \cdot g^{-1}$, respectively. Meanwhile, as shown in Figure 7C,D, imperialine (VIP = 1.53), ebeiedinone (VIP = 1.32), delavinone (VIP = 1.24) and yibeinoside A (VIP = 1.07) were also the most

valuable steroidal alkaloids for discrimination between FCB (QB) and FPB. The total content of the above four alkaloids was 8.72~105.55 μg·g-1 in QB, which was also quite different from FPB.

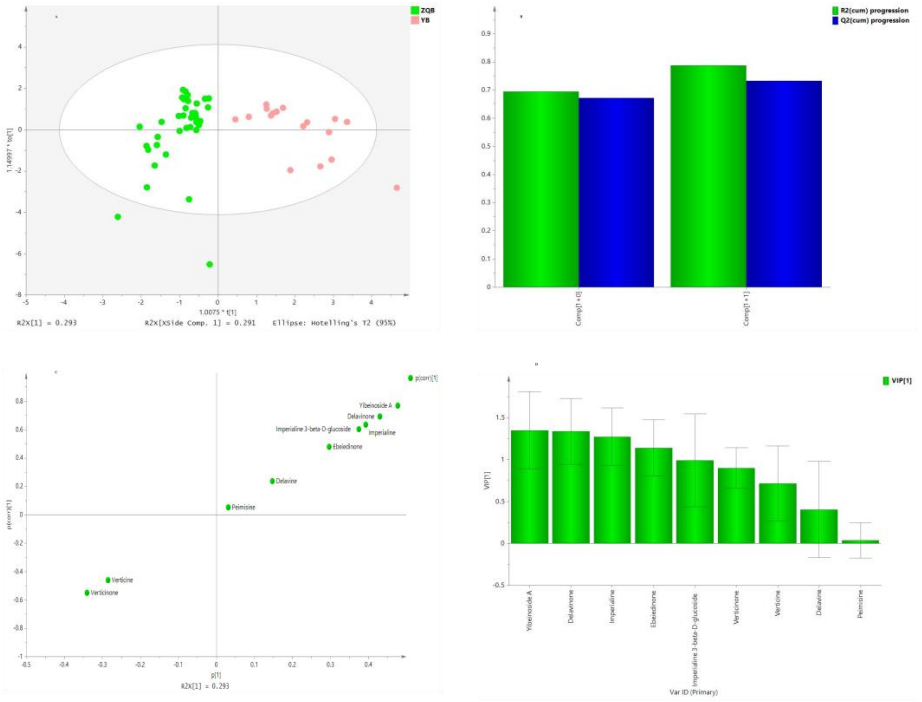

Figure 6. The OPLS-DA of bulbus of FCB (ZQB) and FPB based on the content of nine alkaloids: score plot (**A**), cumulated percentage variation explained by principal components (**B**), *S*-plots (**C**), VIP (**D**).

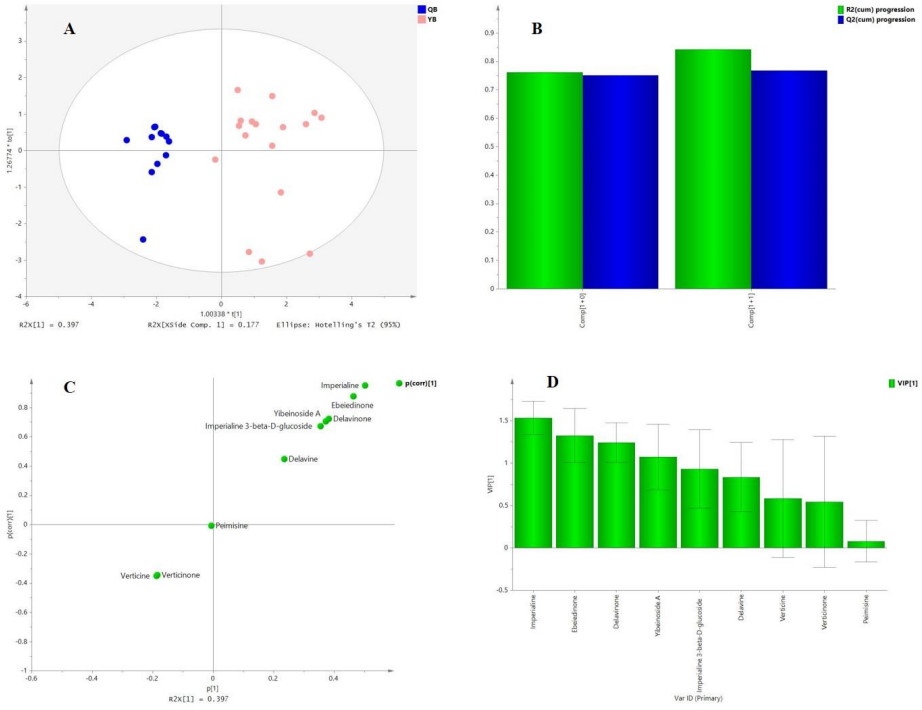

Figure 7. The OPLS-DA of bulbus of FCB (QB) and FPB based on the content of nine alkaloids: score plot (**A**), cumulated percentage variation explained by principal components (**B**), *S*-plots (**C**), VIP (**D**).

These above results demonstrated that the PLS-DA and OPLS-DA models could enlarge the differences between FCB and FPB and successfully separate these two types of bulbus from each other based on the content of the nine steroidal alkaloids, which firstly provided a new approach for the authentication of FCB and FPB, and is also suitable for the quality control of FCB. Additionally, four steroidal alkaloids (yibeinoside A, ebeiedinone, delavinone and imperialine) were ultimately determined as potential markers to distinguish FCB from FPB.

4. Conclusions

In the present study, the content of nine steroidal alkaloids in FCB and FPB was firstly simultaneously measured by a UPLC–ESI–MS method within a short time. Under the condition of sufficient samples, the composition and content features of nine steroidal alkaloids were analyzed and compared between FCB and FPB. Importantly, based on the content of the nine steroidal alkaloids, FCB and FPB were well classified through PLS-DA and OPLS-DA models, and four steroidal alkaloids were determined as valuable markers for the discrimination of FCB and FPB. This study provided a good approach for the authentication of FCB using its active components, and we successfully distinguished FCB and FPB using our established method. To summarize, this study not only offers a further scientific basis for the quality control of FCB, but also provides a new idea for the authentication of similar herbal medicines with complicated origins. However, further studies are still necessary to confirm our findings with more relevant samples.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10020075/s1. Figure S1: Mass spectrums of nine standard alkaloids; Figure S2: Total ions chromatogram of nine alkaloids in mixed standard solution using the multiple-reaction monitoring (MRM) mode; Figure S3: Representative total ions chromatograms of FCB and FPB samples (A: FCB-ZQB.; B: FCB-QB, C: FPB-YB); Figure S4: The permutation plot of the PLS-DA models (A,B: validate model for Figure 5B–D: validate model for Figure 5D); Table S1: Linear regression data, linear range, LOD and LOQ, intra- and inter-day precision, repeatability, stability and recovery of nine alkaloids; Table S2: The content of nine steroidal alkaloids in FCB and FPB samples.

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