Research Article

Constituent Analysis and Quality Evaluation of Epimedii Folium from Different Species

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Epimedii Folium (EF) is a commonly used traditional Chinese drug that includes many species. In Chinese Pharmacopoeia (2020 edition), EF includes *Epimedium brevicornu* Maxim, *E. Sagittatum* (Sieb. et Zucc) Maxim, *E. pubescens* Maxim, *E. koreanum* Nakai, and *Epimedium Wushanense* T.S. Ying. It has been reported that the active constituents in EF are different, resulting in the uneven quality of commercial medicinal materials. To explore the specific differences and make a comprehensive quality evaluation of EF, we established an analytical method to simultaneously detect 45 constituents including 23 flavonoids, 4 phenolic acids, 12 amino acids, 5 nucleosides, and 1 alkaloid in 5 species of EF, based on ultrafast performance liquid chromatography coupled with triple quadrupole-linear ion trap mass spectrometry (UFLC-QTRAP-MS/MS). In addition, orthogonal partial least squares discriminant analysis (OPLS-DA), analysis of variance (ANOVA), and grey correlation analysis (GRA) were used to discriminate and evaluate different species of samples. The results showed that EF could be divided into three categories and EBM was superior to the other four species. This study provides data basis for the comprehensive evaluation and a new perspective on the quality control of EF from different species.

1. Introduction

Epimedii Folium (EF), also known as Xianlingpi and Gangqian, is first recorded in Commentary of Shennong's Herbal Classic of Materia Medica [1]. The medicinal part of EF is leaf. EF belongs to the family of *Epimedium* of *Berberidaceae*, which has the traditional functions of invigorating the kidney and strengthening the yang, strengthening bones and muscles, dispelling wind, and removing dampness [2]. In Pharmacopoeia (2020 edition), there are 5 species of EF, including *Epimedium brevicornu* Maxim (EBM), *E. Sagittatum* (Sieb. et Zucc) Maxim (ESM), *E. pubescens* Maxim (EPM), *E. koreanum* Nakia (EKN), and *E. Wushanense* T.S. Ying(EWY) [3]. Among them, EWY, as a plant of the same genus, is listed separately. About 68 species of Epimedium were found in the world, and there were 58 species in China [4]. EF is widely distributed in the regions of central, southwest, northwest, and northeast China [5], and 5 species are included in Pharmacopoeia (2020 version).. In recent years, new species have also been reported, such as *E. pseudowushanense* B. L. Guo, *E. qingchengshanense* G. Y. Zhong and Guo et al. [6], and *E. myrianthum* Stearn [7].

Over the past few decades, the majority of the literature on EF has primarily focused on pharmacological actions identifying [8], chemical constituents [9], and quality control [10]. The research showed that EF contains a variety of bioactive constituents, but not limited to flavonoids [9, 11], phenolic acids [12], volatile oil [13], polysaccharides [14, 15], nucleic acid, amino acids, alkaloids, and lignans [16, 17]. These bioactive constituents play a significant role in the exceptional clinical function, such as antiosteoporosis [18], bone repair [19, 20], hormone-like effects [21], antitumor [22], anti-inflammatory [23], antiaging [24], immunomodulation [15, 25], and treatment of cardiovascular and cerebrovascular diseases [26, 27]. Flavonoid glycoside derivatives with 8-isopentenyl flavonol as the basic mother nucleus, represented by icaritin and icariin, have

been proved to be the main substances that exert the above pharmacological effects. Therefore, it is essential to ensure the quality of EF for its remarkable clinical effect.

Nowadays, there are many reports on the analysis and quality assessment of chemical components in EF, such as the use of reversed-phase high-performance liquid chromatography (RP-HPLC) [28], gas chromatography-mass spectrometry (GC-MS) [13], ultraperformance liquid chromatography (UPLC) [9], and rapid resolution liquid chromatography (RRLC) [29]. However, due to the various species and wide origin of this medicinal material, the quality of EF is uneven and the chemical components content is differential [30]. Previous studies have primarily concentrated on the quantitative and qualitative analysis of flavonoids with significant pharmacological effects in a single species. However, no study has used LC-MS technology to perform a comprehensive quantitative analysis of phenolic acids, amino acids, nucleic acids, and alkaloids in EF for quality assessment. As we all know, the components system of traditional Chinese medicine (TCM) is complex, and the efficacy is the result of the integration and regulation of multiple components [31]. Assessing the quality of herbal medicines based on the determination of a single or a few bioactive constituents is biased and may not fully reflect its intrinsic quality. According to the characteristics of multiple active constituents in EF, establishing a method of simultaneous determination of multiple bioactive constituents was necessary, which was scientific and practical for exploring the comprehensive evaluation system of multiple index constituents.

Hence, the aim of this study was to identify and evaluate the quality of EF from different species, including EBM, ESM, EPM, EKN, and EWY. Simultaneous determination of 45 components in 5 species of EF was conducted using ultrafast liquid chromatography-triple quadrupole-linear ion trap tandem mass spectrometry (UFLC-QTRAP-MS/MS). In addition, the extraction conditions are optimized by single-factor analysis and the Box–Behnken design (BBD) along with response surface methodology (RSM). Orthogonal partial least squares discriminant analysis (OPLS-DA) and analysis of variance (ANOVA) were applied to classify, distinguish, and reveal the differential constituents between different species of EF. Then, the quality of EF was assessed using grey association analysis (GRA). This study not only established a reliable and accurate method based on UFLC-QTRAP-MS/MS for the simultaneous determination of multiple bioactive constituents in EF but also provide a basis for the comprehensive evaluation and intrinsic quality control of EF from different species in Pharmacopoeia. This study not only developed a dependable and precise UFLC-QTRAP-MS/MS approach for the concurrent determination of multiple bioactive constituents in EF but also established a foundation for the comprehensive evaluation and intrinsic quality control of various species of EF in Pharmacopoeia.

2. Materials and Methods

2.1. Plant Materials.
Plant material was collected from nine provinces of China and North Korea, the domestic region including Gansu, Shanxi, Shaanxi, Sichuan,

TABLE 1: Information of EF.

No.	Species	Habitats
S1		Longnan, Gansu
S2		Ankang, Shanxi
S3		Longnan, Gansu
S4		Tianshui, Gansu
S5		Lanzhou, Gansu
S6		Listen, Gansu
S7		Longnan, Gansu
S8	EBM	Longnan, Gansu
S9		Longnan, Gansu
S10		Longnan, Gansu
S11		Longnan, Gansu
S12		Longnan, Gansu
S13		Jincheng, Shanxi
S14		Dingxi, Gansu
S15		Ankang, Shanxi
S16		Longnan, Gansu
S17		Hanzhong, Shanxi
S18		Ankang, Shanxi
S19	ESM	Yongchuan, Chongqing
S20		Leishan, Guizhou
S21		Leishan, Guizhou
S22		Dazhou, Sichuan
S23	ESM	Dazhou, Sichuan
S24		Sansui, Guizhou
S25		Leshan, Sichuan
S26		Hanzhong, Shanxi
S27	EPM	Hanzhong, Shanxi
S28		Hanzhong, Shanxi
S29		Bazhong, Sichuan
S30		Dandong, Liaoning
S31		North Korea
S32	EKN	Baishan, Jilin
S33		Benxi, Liaoning
S34		Benxi, Liaoning
S35		Baishan, Jilin
S36		Dazhou, Sichuan
S37		Ankang, Shanxi
S38	EWY	Dazhou, Sichuan
S39		Bazhong, Sichuan
S40		Enshi, Hubei

Guizhou, Chongqing, Hubei, Liaoning, and Jilin. Table 1 details the geographical habitats of each sample. Figure 1 shows different species of EF. All samples were authenticated by Professor Xunhong Liu (Nanjing University of Chinese Medicine, Nanjing, PR China). The Laboratory of Chinese medicine identification, Nanjing University of Chinese Medicine, deposited the plant material.

2.2. Chemicals and Reagents.
The standards of serine, glutamic acid, threonine, proline, adenosine, valine, leucine, phenylalanine, caffeic acid, hyperoside, epimedin C, and icariin were purchased from the Institute of Food and Drug Administration of China (Beijing, China). Histidine, glycine, alanine, uracil, 2′-deoxyadenosine, inosine, tyrosine, 2′-deoxyinosine, quercitrin, quercetin, and luteolin were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Isoquercitrin was purchased
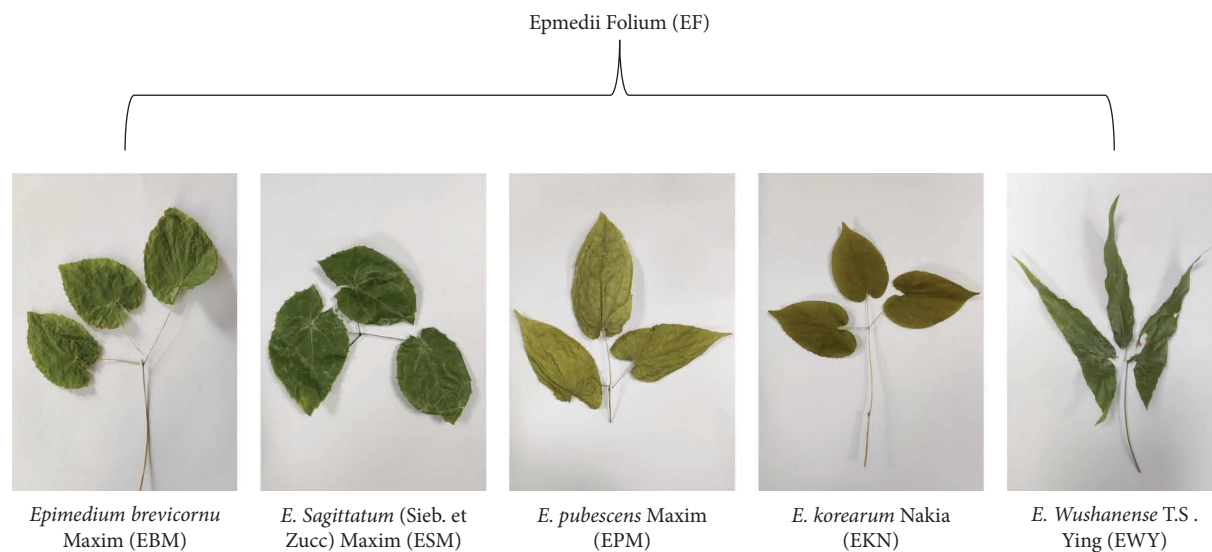
Epmedii Folium (EF)

| *Epimedium brevicornu* Maxim (EBM) | *E. Sagittatum* (Sieb. et Zucc) Maxim (ESM) | *E. pubescens* Maxim (EPM) | *E. korearum* Nakia (EKN) | *E. Wushanense* T.S . Ying (EWY) |

FIGURE 1: Different species of EF.

from Jiangsu Yongjian Pharmaceutical Technology Co., Ltd. (Jiangsu, China). Astragalin was purchased from Chengdu Desite Biotechnology Co., Ltd. (Chengdu, China). Neochlorogenic acid and cryptochlorogenic acid were purchased from Chengdu Purifa Technology Development Co., Ltd. (Chengdu, China). Magnoflorine, trifolin, afzelin, epimedoside A, epimedin A1, epimedin A, epimedin B, sagittatoside A, epimedin-I, ikarisoside A, icariside-I, icaritin, sagittatoside B, $2'$-O-rhamnosyl icariside-II, baohuoside-I, and anhydroicaritin were purchased from Chengdu Alfa Biotechnology Co., Ltd. (Chengdu, China). Lysine and chlorogenic acid were purchased from Baoji Chenguang Biotechnology Co., Ltd. (Baoji, China).

2.3. Preparation of Standard Solutions.
A stock solution of reference standards consisting of 45 compounds was prepared using methanol, and their concentrations were as follows: 1.008, 1.00, 1.180, 1.160, 5.295, 1.100, 1.160, 4.010, 2.478, 0.800, 2.928, 1.700, 2.583, 2.185, 2.615, 1.102, 3.346, 1.055, 1.290, 1.830, 1.052, 1.620, 1.094, 0.936, 1.004, 3.470, 1.032, 1.840, 1.035, 1.545, 1.128, 1.360, 2.420, 2.460, 2.715, 1.190, 1.565, 3.130, 1.770, 2.125, 1.505, 0.735, 1.080, 0.765, and 0.153 mg/ml; the stock solution was then diluted with ethanol to generate calibration curves at various concentrations. All solutions were kept at 4°C until LC-MS analysis.

2.4. Preparation of Sample Solutions.
Approximately 0.5 g of sample powder from various species of EF were weighed accurately and sonicated with 50% ethanol (5 mL) for 60 minutes, followed by cooling to room temperature. And we supplemented the weight loss with 50% ethanol, shaken well, and filtered. Then, the filtrate was centrifuged at 12000 r/min for 10 min, and the supernatant was filtered through a 0.22 μm microporous membrane and stored at 4°C in a refrigerator.

2.5. Chromatographic and Mass Spectrometric Conditions.
The SIL-20A XR system (Shimadzu, Kyoto, Japan) was employed for chromatographic analysis of EF. The chromatographic column used was an XBridge® C18 column (4.6 mm × 100 mm, 3.5 μm) maintained at 30°C, with an injection volume of 2 μL. The mobile phase consisted of a 0.4% formic acid water solution (A) and acetonitrile (B) at a flow rate of 0.8 mL/min, with the following gradient elution: 0~1 min, 5~5% B; 1~14 min, 5~31% B; 14~18 min, 31~41% B; 18~22 min, 41~56% B; 22~26 min, 56~75% B; 26~29 min, 75~83% B; and 29~32 min, 83~5% B.

Detection was carried out using an API5500 triple quadrupole-linear ion trap tandem mass spectrometer (AB SCIEX, Framingham, MA, USA) equipped with an electrospray ionization (ESI) source. The following operating parameters were used: ion source temperature, 550°C; nebulizer gas (GS1) flow, 55 psi; auxiliary gas (GS2) flow, 55 psi; curtain gas (CUR) flow, 40 psi; and spray voltage (IS), 4500 V in positive mode and −4500 V in negative mode. Analyte detection was performed using multiple-reaction mode (MRM).

2.6. Validation of the Method.
The validity of the approach was confirmed through assessments of its linearity, intraday and interday precision, repeatability, stability, recovery, and matrix effect [32]. The standard curve was established by serial dilution of mixed standards, and the equation for linear regression, coefficient of correlation, and range for linearity was computed. The limit of detection (LOD) and limit of quantification (LOQ) for 45 components were determined at signal-to-noise ratios of 3 and 10. Intraday precision was evaluated by injecting the mixed standard solution six times per day, while interday precision was assessed by injecting triplicates of the solution for three consecutive days. To verify the repeatability, six EF samples were precisely weighed, prepared individually according to the aforementioned optimal conditions, and subsequently

analyzed. The stability of the sample was assessed by extracting and analyzing the same solution at 0, 2, 4, 8, 12, and 24-hour intervals, using the previously mentioned chromatographic conditions. The recovery experiment was used to evaluate the accuracy of the method; three standards with different concentration levels were added to the samples with known content, including low concentration (80%), medium concentration (100%), and high concentration (120%). The analysis of spiked samples using UFLC-QTRAP-MS/MS to determine the recovery was conducted in triplicate for each experiment. Matrix effect refers to the interference in the matrix or the enhancement or inhibition of chromatographic signals by blended constituents. The slope comparison method was used to evaluate it. Thus, the matrix effect was computed as the ratio of the slope in the matrix-matched calibration curve to the slope in the solvent standard curve. A slope close to 1.0 indicates a weaker matrix effect.

2.7. Multivariate Statistical Analysis.

Following data preprocessing, OPLS-DA was utilized to observe the overall clustering trend of each group, and SIMCA-P 13.0 software (Umetrics AB, Umea, Sweden) was employed to visualize their distribution. The quantitative detection outcomes for the 45 active components were subjected to variance analysis using SPSS Statistics 22.0 software. Additionally, OriginPro 2021b (OriginLab, Northampton, MA, USA) was used to provide a more intuitive visualization of the classification and content changes of the samples. Excel for Mac 2019 (Microsoft Corporation, Seattle, WA, USA) was used to evaluate the quality of various EF species based on their content of 45 active components, using GRA. OriginPro 2021b (OriginLab, Northampton, MA, USA) was employed to generate histograms for all data.

3. Results and Discussion

3.1. Optimization of Extraction Conditions

3.1.1. Single-Factor Experiment.

The selection of extraction solvent, extraction time, and solid-liquid ratio (g/ml) has a considerable impact on the constituents extracted from EF. According to the Pharmacopoeia (2020 edition), the content of icariin in leaves should not be less than 0.5%. Therefore, the extraction rate of icariin was selected as the index of optimal extraction conditions in this study. In order to obtain suitable extraction efficiency of icariin, the single-factor tests were performed for ethanol volume fraction (20% methanol/ethanol, 30% methanol/ethanol, 40% methanol/ethanol, 50% methanol/ethanol, 60% methanol/ethanol, 70% methanol/ethanol, and 80% methanol/ethanol, respectively), extraction time (30, 40, 50, 60, 70 min, respectively), and liquid-solid ratio (6:1, 8:1, 10:1, 12:1, 14:1, 20:1, 30:1, 1:40:1, and 50:1, respectively). Figure S1 (in the supplementary material) illustrates the impact of ethanol volume fraction, liquid-solid ratio, and extraction time on the extraction yields of icariin.

As shown in Figure S1, the extraction rate of icariin was affected by the concentration of ethanol volume fraction.

When the volume fraction of ethanol was 50%, the maximum extraction rate was 4895.64 μg/g. With the increase of ultrasonic extraction time, the extraction of icariin first decreased and then increased. The extraction rate reached 5052.77 μg/g when the extraction time reached 60 min. The liquid-solid ratio had an irregular effect on the extraction rate of icariin, while at the ratio of 1:10, the extraction rate reached a peak of 4812.28 μg/g.

3.1.2. Box–Behnken Design along with Response Surface Methodology.

After analyzing the outcomes of the aforementioned single-factor experiment, the Box–Behnken design (BBD) was employed in conjunction with response surface methodology (RSM) to design a three-factor, three-level experiment. The extraction rate of icariin was utilized as the response value, with the aim of optimizing the icariin extraction process. The experimental design factors and levels of the response surface method are shown in Table S1, the experimental design and results are shown in Table S2, and the analysis of variance and significance test are shown in Table S3. The results showed that the model can be used to determine the optimal extraction conditions of icariin. Then, the effects of ethanol volume fraction, extraction time, and liquid-solid ratio on the peak area of icariin were analyzed using Design-Expert 8.0.6 software, and results were shown in Figure 2.

Ultimately, using Design-Expert 8.0.6 to solve the regression equation for the data in Table S2, the optimal extraction conditions were obtained when the extraction time was 60.1 min and the liquid-solid ratio was 10:1 for 49.41% ethanol, with the predicted value was 3271.00 μg/g. In order to simplify the experimental operation, the above conditions were adjusted as 50% ethanol volume fraction, 10:1 liquid-solid ratio, and 60 min extraction time. Under this condition, the average extraction yield was 3841.22 μg/g, which was slightly higher than the theoretical value of 3271.00 μg/g. The experimental results supported the theoretical value, indicating that the optimized conditions were feasible and could be used as extraction conditions for EF.

3.2. Optimization of UFLC Parameters.

The UFLC chromatographic conditions, including the column, mobile phase, and column temperature, were optimized to achieve the most favorable separation efficiency for the target constituents in EF and to improve the peak shape. Comparing the separation effects of XBridge® C_{18} column (4.6 mm × 100 mm, 3.5 μm) and Agilent ZORBAXSB-C_{18} column (4.6 mm × 250 mm, 5 μm) on 45 index constituents, it was found that the XBridge® C_{18} column (4.6 mm × 100 mm, 3.5 μm) had relatively high resolution and sensitivity. In addition, four mobile phase systems (water-methanol, water-acetonitrile, water-methanol:acetonitrile (1:1), and 0.1%, 0.4%, 0.8% formic acid water solution-acetonitrile, respectively), flow rates (0.3, 0.4, 0.5, 0.7, 0.8, 0.9, and 1.0 ml/min, respectively), and column temperatures (25, 30, 35, and 40°C, respectively) were examined and compared. The final chromatographic conditions were established using gradient elution with 0.4%
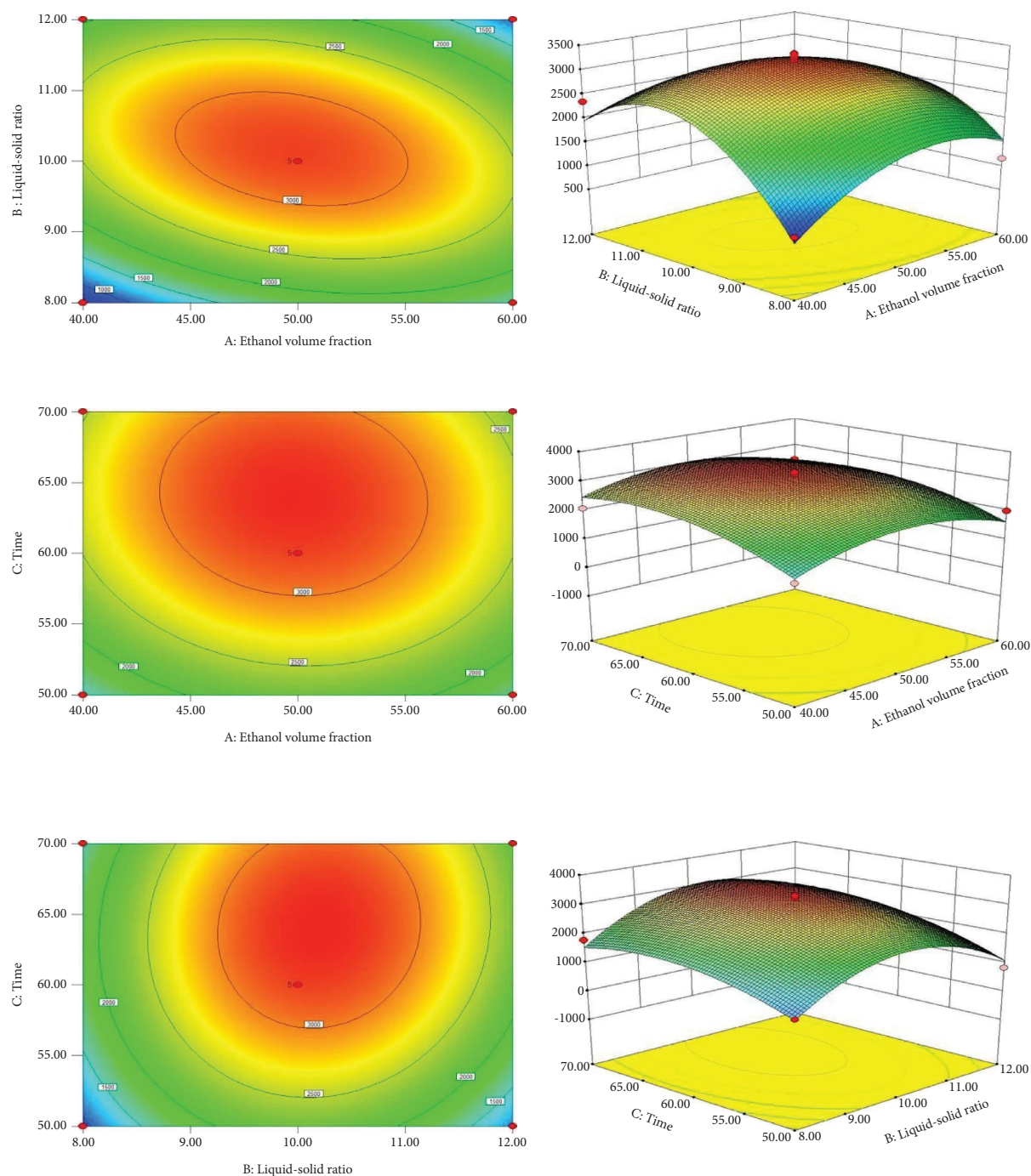
FIGURE 2: The results of response surface.

formic acid as eluent A and acetonitrile as eluent B, at a flow rate of 0.8 ml/min and a column temperature of 30°C.

3.3. Optimization of Mass Spectrometry (MS) Conditions.

Both positive and negative scanning modes are used by the mass spectrometry to detect all standard solutions (about 100 ng/ml) for a sensitive and accurate quantitative method. Following multiple experimental tests, it was determined that amino acids, nucleosides, and magnoflorine exhibit strong sensitivity in positive ion mode, while flavonoids and phenolic acids demonstrate significantly greater reactivity in negative ion mode. Constituents of magnoflorine, epimedoside A, and icariin are correspondingly better in negative ion mode than those in positive ion mode. Thus, this study uses both ESI^+ and ESI^- modes. Although some constituents have similar retention times, they can be accurately quantified based on different precursor and product ions. Figures S2 and S3 show the spectra of the negative and positive ion modes from the mixed standards, respectively. The optimal parameters for the 45 constituents, including retention time (tR), precursor and product ions, declustering

potential (DP), and collision energy (CE), are provided in Table 2. The CAS numbers of 45 target constituents are shown in Table S4. Figure 3 shows the multiple-reaction monitoring (MRM) of the 45 constituents.

3.4. Method Validation. All quantitative method validation was performed using the above method. The results of the verification of each method are shown in Table 3. Each standard calibration curve was constructed by plotting the peak area (Y) versus the corresponding concentration (X). All analytes showed good linearity with an appropriate coefficient of determination ($r > 0.9990$). The ranges of LOD and LOQ range from 0.085~8.792 ng/ml to 0.283~29.306 ng/ml, respectively. The relative standard deviations (RSDs) of intraday and interday precision are 0.32%~5% and 0.62%~4.99%, respectively. In turn, the RSDs for the repeatability and stability tests were less than 4.94% and 4.81%. The total recovery rate varied from 94.51% to 105.66%, with RSD <4.94%. The recovery of different species of EF and the RSD of recovery for each species are shown in Tables S5 and S6. The slope ratio indicated that the effect of the matrix on analyte ionization was not obvious under optimal conditions. The matrix effect of different species of EF is shown in Table S7.

3.5. Quantitative Analysis of Samples. Information regarding the samples is provided in Table 1. Using the UFLC-QTRAP-MS/MS verification analytical method, 45 constituents, which includes 23 flavonoids, 4 phenolic acids, 12 amino acids, 5 nucleosides, and 1 alkaloid, were simultaneously determined in EF. Table S8 shows quantitative results of 45 constituents. EF samples were all rich in flavonoids, with the content of flavonoid ranging from 5520.32 to 21299.16 μg/g, comprising over 80% of the total analytes analyzed in this study. Icariin, as the quality index component of EF in Pharmacopoeia, ranges from 802.48 to 6419.17 μg/g, with a percentage of 0.08%~0.64%. In particular, the flavonol glycoside derived from 8-isoprenyl flavonol as the basic parent nucleus unique to EF accounted for 99.23% of the total flavonoid content. The flavonoid level varied from sample to sample, with the total flavonoid in the EBM being the highest of all samples. The general content of phenolic acid was 11.50~3039.92 μg/g, of which the substance of chlorogenic acid was the highest, accounting for more than 40.19% of the total phenolic acid. There were obvious differences in the phenolic acid content in different species, and the phenolic acid content in EKN was significantly higher than that in others. The total content of amino acids was 202.26~2201.79 μg/g, of which proline, glutamic acid, and phenylalanine were relatively high. Besides, the level of amino acid in EBM and ESM was significantly higher than that of the other three species. The total content of nucleosides was 35.28~283.65 μg/g, accounting for 0.21%~1.56% of the total content of all constituents. The level of nucleic acid in ESM and EBM was higher than that of other species. The only alkaloid detected was magnoflorine, with a total content of 470.45~2165.07 μg/g; its

content in ESM was significantly higher than that in other species. The content of flavonoids, phenolic acids, amino acids, nucleosides, and magnoflorine in EF is shown in Figure 4.

3.6. OPLS-DA of Samples. To begin with, the quality differentiation among samples of varied EF species was achieved through the application of principal component analysis (PCA). However, the PCA results showed that samples of different species could not be completely distinguished, and it was deemed unsuitable for establishing a foundation to differentiate and assess the quality of EF. Therefore, orthogonal partial least squares discriminant analysis (OPLS-DA) was used. This analytical method is a supervised discriminant analysis statistical method, which can maximize the variation of between-groups and minimize the separation of within-groups. Using OPLS-DA, a model of the relationship between the content of the target constituents and the sample class was successfully established to predict all samples. Figure 5 shows the OPLS-DA scoring plot. The samples of different species of EF were obviously divided into three categories. EBM was the category I, three species, EPM, EKN, and EWY, were grouped into category II, and ESM was the category III. This outcome indicated the presence of disparities in the bioactive constituents of EBM, ESM, and other three species, which was also consistent with the results of HCA. Furthermore, the statistical parameters of OPLS-DA R2X (cum), R2Y (cum), and Q2 (cum) were 0.824, 0.934, and 0.794, respectively, indicating that the model had good reproducibility and predictability. When a variable's VIP is greater than 1, it signifies that the variable plays a significant role in the classification of the samples. As depicted in Figure 6, twenty constituents were identified to have a significant impact on the cluster based on their VIP values, including anhydroicaritin, epimedin-I, crypto-chlorogenic acid, epimedin C, 2′-O-rhamnosyl icariside-II, isoquercitrin, alanine, leucine, epimedin A, epimedin B, luteolin, quercetin, icaritin, sagittatoside A, epimedoside A, tyrosine, icariside-I, ikarisoside A, lysine, and caffeic acid.

3.7. ANOVA of Samples. Analysis of variance (ANOVA) is a technique for analyzing the way in which the mean of a variable is affected by different types and combinations of factors, which can be used to compare any number of groups or treatments [33]. In this study, the content of bioactive constituents was analyzed using ANOVA followed by the least significant difference (LSD) test (assuming equal variance) or Tamhane's test (not assuming equal variance) to evaluate the changes of 45 constituents in 5 EF species. The values with p values less than 0.05 were deemed as having significant differences. As shown in Table 4, among the 45 constituents, there were 5 constituents, including proline, astragalin, trifolin, epimedin A1, and sagittatoside B, showed no significant differences among different varieties ($p > 0.05$), which indicates that the content of the above five constituents was consistent among different species without significant differences. The other 40 constituents showed significant differences among different varieties ($p < 0.05$).

Table 2: Optimized mass spectrometric parameters for MRM of 45 constituents.

No.	Constituents	Formula	t_R (min)	Precursor ion (m/z)	Product ion (m/z)	DP (V)	CE (eV)	CXP (eV)	Ion mode
1	Lysine	$C_6H_{14}N_2O_2$	1.12	147.11	83.91	100	14	14	ESI$^+$
2	Histidine	$C_6H_9N_3O_2$	1.16	156.08	110.03	100	16	14	ESI$^+$
3	Serine	$C_3H_7NO_3$	1.21	106.05	59.99	100	8	14	ESI$^+$
4	Glycine	$C_2H_5NO_2$	1.21	76.04	30.00	73	6	14	ESI$^+$
5	Glutamic acid	$C5H_9NO_4$	1.22	148.10	83.90	12	14	14	ESI$^+$
6	Alanine	$C_3H_7NO_2$	1.22	90.06	44.02	100	10	14	ESI$^+$
7	Threonine	$C_4H_9NO_3$	1.22	120.17	74.00	100	20	14	ESI$^+$
8	Proline	$C_5H_9NO_2$	1.31	116.07	70.02	68	10	14	ESI$^+$
9	Adenosine	$C_{10}H_{13}N_5O_4$	1.35	268.10	136.10	31	23	14	ESI$^+$
10	Uracil	$C_4H_4N_2O_2$	1.4	113.00	70.00	111	21	14	ESI$^+$
11	Valine	$C_5H_{11}NO_2$	1.43	118.09	72.06	100	10	14	ESI$^+$
12	2$'$-deoxyadenosine	$C_{10}H_{13}N_5O_3$	1.72	251.81	136.08	80	9	6	ESI$^+$
13	Inosine	$C_{10}H_{12}N_4O_5$	2.04	269.00	137.07	46	15	14	ESI$^+$
14	Tyrosine	$C_9H_{11}NO_3$	2.17	182.10	136.00	16	16	14	ESI$^+$
15	2$'$-deoxyinosine	$C_{10}H_{12}N_4O_4$	2.35	253.02	136.90	11	11	16	ESI$^+$
16	Leucine	$C_6H_{13}NO_2$	2.9	132.10	86.05	100	16	14	ESI$^+$
17	Phenylalanine	$C_9H_{11}NO_2$	4.99	166.10	120.05	100	14	14	ESI$^+$
18	Neochlorogenic acid	$C_{16}H_{18}O_9$	5.76	353.02	190.96	−90	−24	−21	ESI$^-$
19	Chlorogenic acid	$C_{16}H_{18}O_9$	7.83	353.05	191.03	−120	−22	−13	ESI$^-$
20	Cryptochlorogenic acid	$C_{16}H_{18}O_9$	8.42	353.07	191.01	−105	−20	−21	ESI$^-$
21	Caffeic acid	$C_9H_8O_4$	8.86	179.03	134.60	−125	−20	−15	ESI$^-$
22	Magnoflorine	$C_{20}H_{24}NO_4$	9.83	343.10	298	125	24	14	ESI$^+$
23	Isoquercitrin	$C_{21}H_{20}O_{12}$	13.15	463.00	300.00	−180	−36	−15	ESI$^-$
24	Hyperoside	$C_{21}H_{20}O_{12}$	13.42	463.00	300.00	−160	−36	−15	ESI$^-$
25	Astragalin	$C_{21}H_{20}O_{11}$	14.4	447.10	283.90	−100	−36	−15	ESI$^-$
26	Trifolin	$C_{21}H_{20}O_{11}$	14.98	447.08	284.04	−195	−34	−19	ESI$^-$
27	Quercitrin	$C_{21}H_{20}O_{11}$	14.99	447.00	301.00	−165	−30	−15	ESI$^-$
28	Afzelin	$C_{21}H_{20}O_{10}$	16.75	431.10	285.00	−130	−40	−13	ESI$^-$
29	Epimedoside A	$C_{32}H_{38}O_{15}$	16.98	663.234	355.219	121	35	26	ESI$^+$
30	Quercetin	$C_{15}H_{10}O_7$	18.64	301.10	151.00	−62	−28	−15	ESI$^-$
31	Luteolin	$C_{15}H_{10}O_6$	18.7	285.09	132.98	−170	−40	−15	ESI$^-$
32	Epimedin A1	$C_{39}H_{50}O_{20}$	19.41	837.248	367.13	−185	−52	−45	ESI$^-$
33	Epimedin A	$C_{39}H_{50}O_{20}$	19.8	837.238	675.249	−175	−20	−29	ESI$^-$
34	Epimedin B	$C_{38}H_{48}O_{19}$	20.01	807.241	645.212	−170	−26	−31	ESI$^-$
35	Epimedin C	$C_{39}H_{50}O_{19}$	20.33	821.261	659.271	−225	−14	−29	ESI$^-$
36	Sagittatoside A	$C_{33}H_{40}O_{15}$	20.56	675.172	366.145	−180	−46	−27	ESI$^-$
37	Epimedin-I	$C_{41}H_{52}O_{21}$	21.43	879.225	367.128	−195	−50	−31	ESI$^-$
38	Ikarisoside A	$C_{26}H_{28}O_{10}$	23.61	499.089	353.141	−185	−36	−19	ESI$^-$
39	Icariside-I	$C_{27}H_{30}O_{11}$	25.06	529.195	367.167	−230	−22	−17	ESI$^-$
40	Icaritin	$C_{21}H_{20}O_6$	25.07	367.148	309.046	−240	−34	−37	ESI$^-$
41	Icariin	$C_{33}H_{40}O_{15}$	25.17	677.21	369.219	181	37	28	ESI$^+$
42	Sagittatoside B	$C_{32}H_{38}O_{14}$	25.51	645.254	366.162	−250	−44	−26	ESI$^-$
43	2$'$-O-Rhamnosyl icariside-II	$C_{33}H_{40}O_{14}$	25.69	659.186	366.116	−300	−42	−33	ESI$^-$
44	Baohuoside-I	$C_{27}H_{30}O_{10}$	26.06	513.114	366.108	−300	−36	−23	ESI$^-$
45	Anhydroicaritin	$C_{21}H_{20}O_6$	29.31	367.149	352.107	−60	−28	−15	ESI$^-$

Figure 7 shows the box plot of 45 constituents' content in 5 species of EF. It was worth noting that there were 19 constituents, including 10 flavonoids, 8 amino acids, and uracil, which were significantly higher in EBM than other species. The highest content of 14 constituents was found in ESM, including 7 flavonoids, 4 amino acids, 2 nucleosides, and magnoflorine. The content of five constituents in EPM was significantly higher than that in other species, including four flavonoids and proline. The content of 4 phenolic acids, epimedin-I, and 2-deoxyinosine was the highest in EKN, while the content of 2$'$-O-rhamnosyl icariside-II in EWY was the highest.

3.8. GRA of Samples. Grey Correlation Degree Analysis (GRA) is an influence measure in grey system theory that analyzes the uncertain relationship between one major factor in a given system and all other factors. Therefore, according to the content of 45 bioactive constituents, a comprehensive GRA evaluation of different species of EF was performed. Table 4 presents the grey composite evaluation values (ri) and quality rankings obtained from the results. The relative correlation degree reflects the association between the component content and the samples, with those having higher relative correlation considered to be of superior quality.
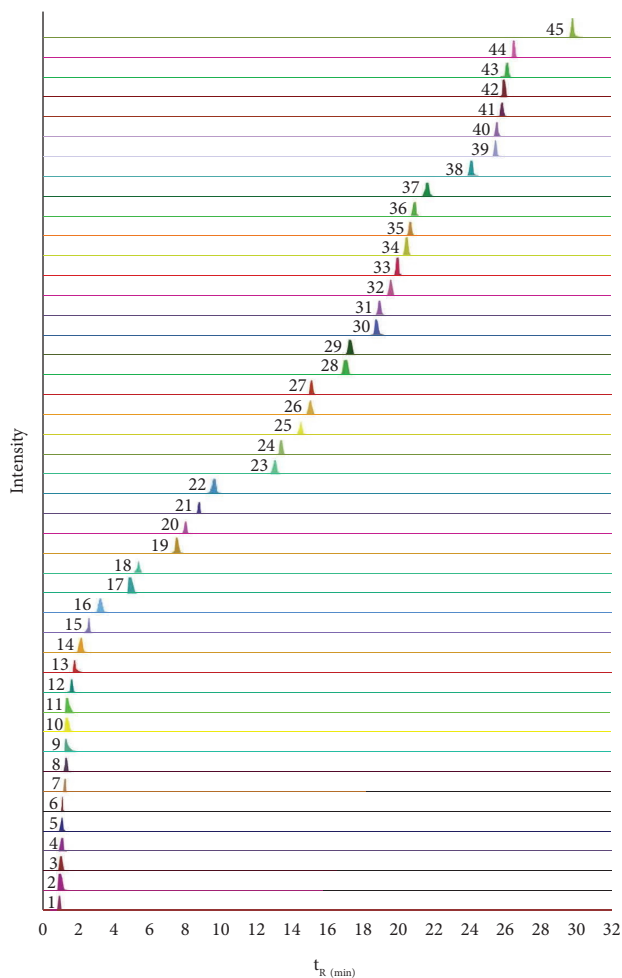
FIGURE 3: The representative extract ion chromatograms (XICs) of multiple-reaction monitoring (MRM) chromatograms of the 45 investigated constituents (the peak numbers denoted are the same as those in Table 2).

Table 5 shows the grey composite evaluation values (r_i) and quality rankings. Overall, the quality of EBM ranked first among the 5 species, which was followed by ESM. While the interspecific quality difference of EPM, EKN, and EWY was not significant, and the overall quality of the three species was inferior to that of EBM and ESM. In addition, there were also differences in the quality of samples of EF of the same species, which may be related to differences in the growing environment, climate, and harvest time. For example, in EBM, samples from Longnan, Gansu Province, were better than those from other sources. It can also be seen that the r_i value of each sample varies greatly, with a maximum value of 22.41%, which effectively differentiates the quality among the 5 species of EF. In summary, GRA can successfully assess the quality of different species of EF based on the content of multiple constituents.

3.9. Discussion. As mentioned above, EF is widely used in clinical practice with significant pharmacological effects [18, 19, 21, 23]. Nonetheless, there are a limited number of comprehensive studies on the quality of EF both domestically and internationally, particularly articles that provide a comprehensive analysis of the bioactive constituents of various species. The majority of studies have concentrated on the quantitative and qualitative analysis of flavonoids with significant pharmacological effects and high content in a single species. However, it is widely recognized that the efficacy of TCMs is the outcome of the integrated adjustment of multiple components [31, 32], emphasizing the need for ensuring the quality of TCMs to achieve their remarkable clinical effects. Yin et al. [34] established a method based on UFLC-QTRAP-MS/MS technology for simultaneous determination of multiple active components in Abelmoschi Corolla, which provided an objective method reference for comprehensive evaluation and comprehensive control of Abelmoschi Corolla internal quality. Wei et al. [35] explored the distribution patterns of multiple active constituents in the pericarp, stalk, and seed of Forsythiae Fructus, which offers a scientific foundation for further exploration of the quality formation mechanism of Forsythiae Fructus. Overall quality control of TCMs is more in line with the characteristics of complex system of TCMs. Hence, given the characteristics of multiple active constituents in EF, the method for simultaneous determination of multiple active constituents was established, which was of practical and scientific significance for exploring the comprehensive evaluation system of multiple marker components.

This study established an effective and dependable UFLC-QTRAP-MS/MS method, which can simultaneously determine 23 flavonoids, 12 amino acids, 4 phenolic acids, 5 nucleosides, and 1 alkaloid in 40 different samples of EF. Single-factor analysis and the Box–Behnken design (BBD) along with response surface methodology (RSM) were used to optimize the extraction conditions (Figure 2). OPLS-DA results showed that EBM, ESM, EPM, EWY, and EKN can be clearly divided into 3 categories (Figure 5). The VIP values indicated that there were significant differences in 20 bioactive constituents, which can be thought of as chemical markers that distinguish different species of EF (Figure 6). The results of ANOVA showed that the content of 40 of the 45 target constituents was significantly different among different species ($p < 0.05$). In addition, based on the r_i value of the samples in the GRA analysis, the overall quality of the EBM was optimal, followed by ESM. While the interspecific quality difference of EPM, EKN, and EWY was not significant, and the overall quality of the three species was inferior to that of EBM and ESM. This suggests that the species is indeed an important factor influencing the accumulation of bioactive constituents. However, the GRA analysis found that there were also significant differences between samples of the same EF species, such as the content of total phenolic acids in different samples of EWY was 11.50–3068.36 μg/g, which may be related to external factors such as the growing environment and harvest time. Some articles have also reported on this [12, 36], but there are still shortcomings of researching single species, which cannot form a comprehensive evaluation system.

Table 3: Regression equations, limits of detection (LOD), limits of quantification (LOQ), precision, repeatability, stability, recovery, and matrix effect of 45 constituents.

No.	Constituents	Regression equation	r	Liner range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Precision (RSD, %) Intraday (n=6)	Precision (RSD, %) Interday (n=9)	Repeatability (RSD, %) (n=6)	Stability (RSD, %) (n=6)	Recovery (%) Mean	Recovery (%) RSD	Matrix effect
1	Lysine	$Y=1470X-315000$	0.9996	304–10100	2.563	8.542	1.19	2.37	4.78	1.24	97.78	0.99	0.94
2	Histidine	$Y=3850X-37500$	0.9994	32.4–5020	1.364	4.545	3.17	2.33	1.67	1.85	94.51	4.94	0.99
3	Serine	$Y=414X-3220$	0.9998	49.7–5950	3.371	11.238	3.89	3.77	2.97	2.38	97.09	2.96	0.96
4	Glycine	$Y=73.8X-925$	0.9993	96.8–11600	0.179	0.598	3.40	3.22	2.17	1.75	94.77	3.41	0.99
5	Glutamic acid	$Y=875X-70300$	0.9994	156–10600	2.371	7.903	1.80	4.52	3.09	2.61	102.17	0.66	0.94
6	Alanine	$Y=549X-782$	0.9999	281–10900	2.374	7.914	3.92	3.63	3.11	0.96	101.29	1.29	1.01
7	Threonine	$Y=481X-14800$	0.9998	104–11700	2.148	7.160	4.67	1.33	4.73	4.81	102.24	1.49	1
8	Proline	$Y=2320X-18600$	0.9995	323–12100	1.710	5.701	1.46	1.26	2.80	1.24	100.22	2.17	1.01
9	Adenosine	$Y=2610X-26700$	0.9993	442–12300	1.602	5.341	1.91	2.09	2.22	3.39	101.38	4.28	0.97
10	Uracil	$Y=376X-5390$	0.9999	31–8040	1.905	6.349	3.67	2.77	5.46	4.13	104.31	2.02	0.99
11	Valine	$Y=2010X-260000$	0.9993	435–14600	1.729	5.764	0.32	4.99	0.79	3.57	103.95	3.67	1.01
12	2'-deoxyadenosine	$Y=1060X-790$	0.9997	37.1–4280	0.365	1.216	0.39	2.59	2.71	1.10	97.37	3.69	1
13	Inosine	$Y=2360X-728000$	0.9993	514–13000	1.872	6.239	5	2.53	4.38	4.45	96.91	2.00	0.96
14	Tyrosine	$Y=3530X-115000$	0.9998	320–11000	0.725	2.417	4.12	2.33	3.35	3.48	97.29	2.52	1.02
15	2'-deoxyinosine	$Y=2560X-251000$	0.9998	361–13000	0.929	3.097	1.43	3.15	0.54	0.71	96.89	1.71	1.05
16	Leucine	$Y=2800X-45900$	0.9995	169–5530	0.738	2.460	2.79	2.86	1.43	1.71	97.54	1.92	0.98
17	Phenylalanine	$Y=3320X+135000$	0.9999	29–8410	1.033	3.442	1.59	1.99	5.44	0.82	99.14	0.62	0.96
18	Neochlorogenic acid	$Y=333X-1850$	0.9999	29.9–5300	8.792	29.306	2.33	1.56	3.34	4.76	102.21	4.13	0.99
19	Chlorogenic acid	$Y=3260X-54.4$	0.9995	35.5–6440	0.364	1.212	1.98	2.48	1.59	4.53	102.51	2.67	0.98
20	Cryptochlorogenic acid	$Y=1020X-27200$	0.9994	62.4–9140	1.517	5.055	1.52	4.80	3.50	3.20	95.20	2.81	0.98
21	Caffeic acid	$Y=4260X-15100$	0.9993	30.0–5260	3.757	12.524	1.09	3.36	1.72	3.22	97.87	2.40	0.95
22	Magnoflorine	$Y=895X+2620000$	0.9996	840–32400	0.387	1.291	1.77	3.73	1.95	3.87	101.27	1.38	1
23	Isoquercitrin	$Y=6750X-44.2$	0.9997	23.8–5400	0.293	0.977	1.26	3.30	2.84	1.35	95.33	3.59	1.02
24	Hyperoside	$Y=9890X-15100$	0.9999	23.6–2340	0.117	0.390	1.43	3.42	2.72	1.49	98.86	3.40	0.94
25	Astragalin	$Y=909X-30600$	0.9997	78.4–5040	0.534	1.780	2.95	3.29	3.51	2.31	105.66	2.14	0.97
26	Trifolin	$Y=3060X-57400$	0.9999	45.1–3450	0.105	0.351	0.97	1.41	1.97	2.75	99.45	0.64	0.97
27	Quercitrin	$Y=4610X-17400$	0.9996	32.1–5170	0.111	0.369	1.10	4.27	2.07	4.08	103.14	1.91	0.99
28	Afzelin	$Y=2050X-138000$	0.9995	173–13800	0.460	1.533	1.07	1.46	3.11	0.92	103.42	1.97	0.95
29	Epimedoside A	$Y=1880X-132$	0.9993	6.08–10400	0.274	0.912	3.56	1.96	3.06	4.03	97.24	2.05	0.96
30	Quercetin	$Y=4650X-44700$	0.9999	44.3–7760	0.351	1.170	1.12	3.28	4.94	4.06	104.31	3.52	0.99
31	Luteolin	$Y=8600X-207000$	0.9997	74.9–5680	0.085	0.283	1.76	2.91	4.84	2.43	96.65	2.06	0.97
32	Epimedin A1	$Y=1190X-110000$	0.9999	208–27300	0.404	1.347	0.87	3.84	2.04	3.99	99.07	4.72	1.01
33	Epimedin A	$Y=637X-13500$	0.9994	164–36200	0.524	1.745	0.47	1.89	1.77	2.42	97.89	4.87	1.02
34	Epimedin B	$Y=669X-59000$	0.9996	217–18500	0.363	1.210	0.55	2.25	1.71	2.21	103.85	3.32	1.04
35	Epimedin C	$Y=794X-22900$	0.9999	111–20600	0.464	1.546	1.37	0.62	1.08	1.49	104.77	3.92	0.99
36	Sagittatoside A	$Y=326X-1250$	0.9995	241–23800	0.194	0.647	1.92	0.71	2.31	1.14	98.80	3.11	0.92
37	Epimedin-I	$Y=666X-1630$	0.9994	7.14–31400	0.817	2.722	1.41	1.79	1.97	3.88	99.35	1.49	0.94
38	Ikarisoside A	$Y=3330X-1590$	0.9999	187–14700	0.161	0.535	1.08	1.68	1.55	2.86	97.39	4.43	1
39	Icariside-I	$Y=3820X-3940$	0.9998	17.6–8900	0.275	0.917	1.41	3.58	3.56	2.63	96.12	4.92	0.99
40	Icaritin	$Y=2500X-51700$	0.9997	207–10600	0.763	2.545	1.53	1.68	2.97	4.29	99.15	1.00	0.99
41	Icariin	$Y=1070X-13200$	0.9998	133–30100	0.144	0.479	3.96	4.58	2.17	3.44	104.42	1.42	1.04

TABLE 3: Continued.

| No.	Constituents	Regression equation	r	Liner range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Precision (RSD, %) Intraday ($n=6$)	Interday ($n=9$)	Repeatability (RSD, %) ($n=6$)	Stability (RSD, %) ($n=6$)	Recovery (%) Mean	RSD	Matrix effect
42	Sagittatoside B	$Y = 2820X - 2660$	0.9993	46.8–11100	0.158	0.526	1.37	2.22	2.57	2.84	101.63	2.10	1.02
43	2′-O-Rhamnosyl icariside-II	$Y = 1050X - 24000$	0.9999	134–16300	0.180	0.601	2.85	4.86	4.85	0.43	101.00	3.81	1.04
44	Baohuoside-I	$Y = 3080X - 316000$	0.9998	141–7680	0.356	1.186	1.78	1.75	1.78	1.67	99.69	3.63	1.02
45	Anhydroicaritin	$Y = 6590X - 267000$	0.9996	97.6–10400	0.152	0.506	2.51	1.31	2.51	2.69	97.71	4.82	0.97
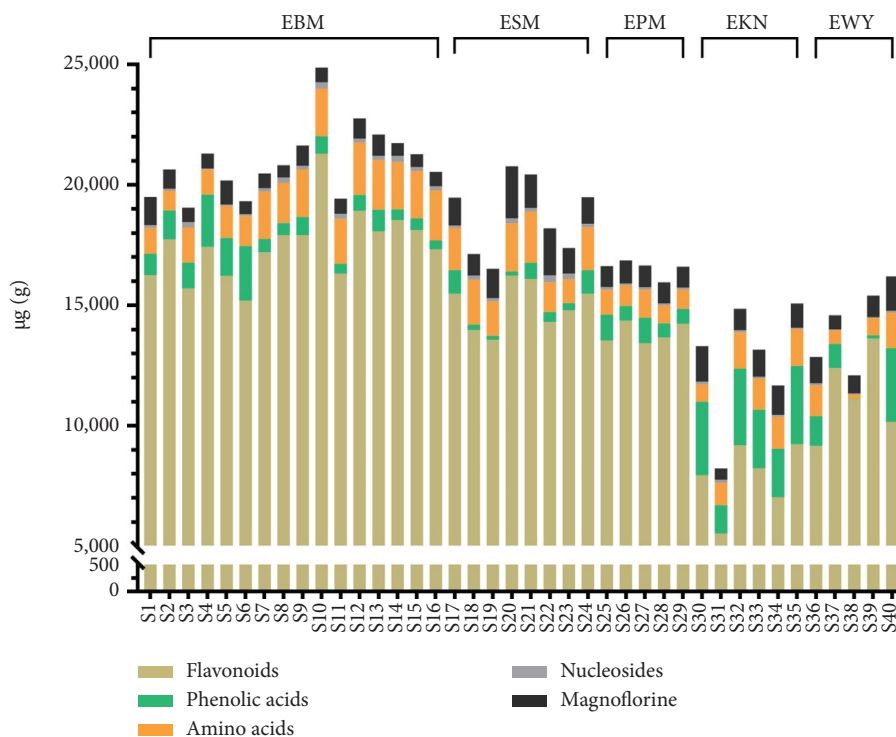
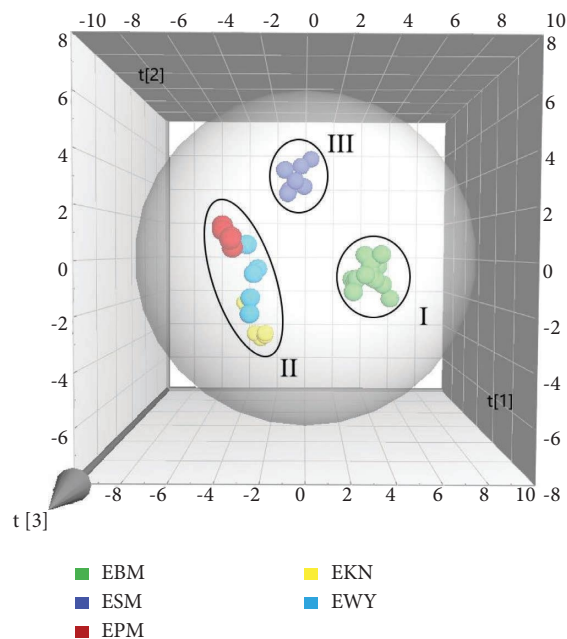Figure 4: The content of five kinds of chemical constituents in EF ($n = 3$).

Figure 5: The OPLS-DA model for the classification of 5 species of EF based on the content of 45 constituents.

In Pharmacopoeia (2020 edition), the content of icariin is used as the index component for quality identification of EF, and it is required that icariin should not be less than 0.50%. However, in our research, the content determination results showed that only 5% of the samples met the Pharmacopoeia standard, and the content of icariin was significantly different among different species. The same problem

was found in the previous articles [37, 38]. Therefore, in order to further establish a more comprehensive quality evaluation system for EF, multiple investigations should be carried out. Besides the detection of icariin, the constituent's determination may be added as new indexes for the quality control of EF. In our research, in addition to icariin, epimedin A, epimedin B, epimedin C, sagittatoside A, and
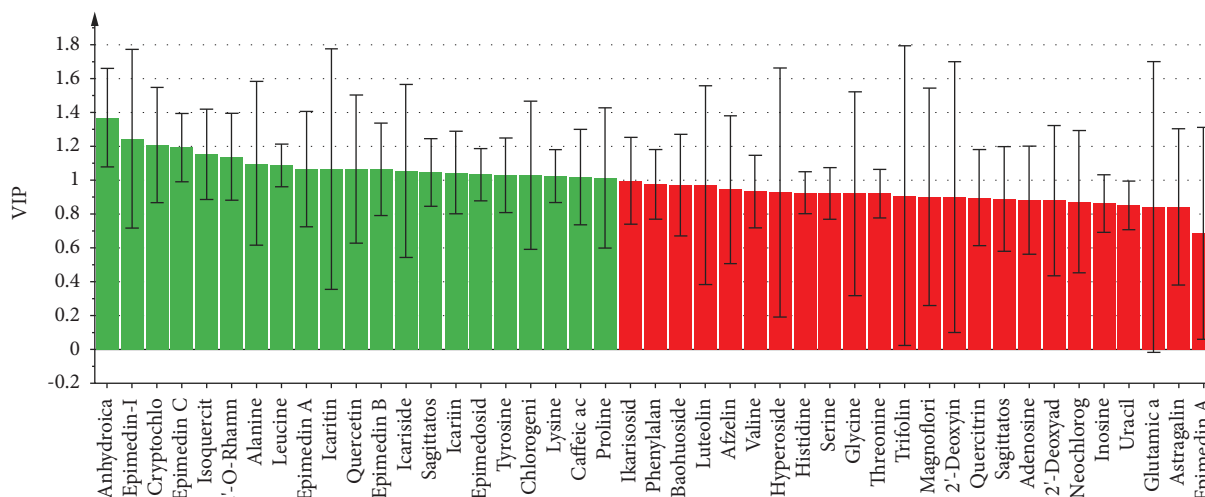
Figure 6: The VIP value for classification of EF. The X axis numbers denoted are the same as those in Table 2.

Table 4: ANOVA results of different species of EF.

Constituents	Mean and standard deviation					F	p
	EBM ($n = 16$)	EKN ($n = 6$)	EPM ($n = 5$)	ESM ($n = 8$)	EWY ($n = 5$)		
Lysine	198.24 ± 91.89	29.10 ± 6.27	21.02 ± 2.09	152.09 ± 66.04	27.93 ± 15.75	13.653	<0.001
Histidine	17.79 ± 6.84	9.38 ± 4.30	8.08 ± 1.26	15.28 ± 7.88	6.88 ± 4.19	5.29	0.002
Serine	75.72 ± 34.54	41.10 ± 17.08	15.49 ± 1.56	50.45 ± 28.90	21.32 ± 22.41	6.776	<0.001
Glycine	15.79 ± 4.95	9.61 ± 3.97	6.73 ± 2.01	21.99 ± 12.58	10.46 ± 7.68	4.849	0.003
Glutamic acid	137.58 ± 35.69	147.36 ± 53.47	169.08 ± 29.17	297.65 ± 189.22	171.46 ± 56.67	4.279	0.006
Alanine	118.50 ± 29.45	126.60 ± 30.36	87.64 ± 36.55	238.12 ± 84.59	119.02 ± 107.76	7.411	<0.001
Threonine	95.93 ± 39.97	59.90 ± 19.34	31.41 ± 16.26	85.47 ± 43.27	38.32 ± 27.91	5.146	0.002
Proline	304.12 ± 70.04	310.99 ± 162.30	377.14 ± 42.67	282.05 ± 95.87	203.27 ± 129.76	2.029	0.112
Adenosine	55.31 ± 45.23	16.72 ± 5.23	11.56 ± 7.17	74.53 ± 39.85	15.51 ± 15.56	4.717	0.004
Uracil	79.48 ± 47.51	51.13 ± 23.85	58.35 ± 11.03	79.21 ± 30.50	26.11 ± 12.41	2.74	0.044
Valine	173.75 ± 86.98	137.64 ± 43.24	65.58 ± 23.86	179.91 ± 71.88	86.25 ± 73.08	3.454	0.018
2'-deoxyadenosine	4.13 ± 3.80	1.54 ± 1.72	0.84 ± 0.26	7.94 ± 4.26	0.74 ± 0.50	6.306	0.001
Inosine	15.53 ± 1.32	13.91 ± 0.77	14.85 ± 0.33	14.00 ± 0.67	14.26 ± 1.18	4.499	0.005
Tyrosine	121.80 ± 51.56	43.78 ± 37.75	11.13 ± 1.17	18.93 ± 8.39	21.93 ± 19.51	16.949	<0.001
2'-deoxyinosine	3.96 ± 0.02	4.09 ± 0.14	4.00 ± 0.01	3.96 ± 0.01	4.06 ± 0.11	6.13	0.001
Leucine	167.86 ± 54.89	153.70 ± 66.47	32.65 ± 19.62	151.58 ± 50.45	55.51 ± 48.05	9.588	<0.001
Phenylalanine	242.16 ± 58.19	146.99 ± 45.44	95.18 ± 6.37	159.69 ± 83.34	75.99 ± 69.41	10.793	<0.001
Neochlorogenic acid	364.42 ± 470.81	1036.99 ± 545.10	159.37 ± 28.01	159.76 ± 76.23	660.73 ± 842.11	3.976	0.009
Chlorogenic acid	455.10 ± 111.93	664.78 ± 94.39	557.55 ± 178.10	249.06 ± 304.20	266.07 ± 285.79	5.364	0.002
Cryptochlorogenic acid	94.14 ± 75.51	794.70 ± 312.83	63.13 ± 44.63	72.60 ± 45.43	158.85 ± 165.23	31.375	<0.001
Caffeic acid	17.55 ± 10.38	18.73 ± 7.44	5.69 ± 0.41	4.31 ± 1.92	2.98 ± 2.69	8.278	<0.001
Magnoflorine	706.85 ± 191.35	1034.29 ± 340.86	901.91 ± 35.07	1369.82 ± 452.24	951.86 ± 325.19	7.049	<0.001
Isoquercitrin	97.65 ± 63.25	171.46 ± 63.62	315.17 ± 45.65	288.36 ± 52.58	118.45 ± 133.47	15.271	<0.001
Hyperoside	13.02 ± 11.66	41.74 ± 15.52	63.12 ± 28.89	71.62 ± 62.26	44.68 ± 54.18	4.316	0.006
Astragalin	98.85 ± 86.20	147.72 ± 100.58	121.48 ± 33.61	201.18 ± 153.95	99.97 ± 103.21	1.465	0.234
Trifolin	21.41 ± 13.59	27.89 ± 8.81	11.06 ± 5.64	33.86 ± 34.83	8.59 ± 6.61	2.076	0.105
Quercitrin	93.94 ± 94.28	32.88 ± 14.47	162.34 ± 53.70	180.32 ± 81.70	97.56 ± 105.87	3.456	0.018
Afzelin	147.71 ± 89.52	4.57 ± 2.54	15.75 ± 7.95	98.40 ± 63.97	53.43 ± 39.28	7.423	<0.001
Epimedoside A	604.53 ± 141.49	138.82 ± 63.48	61.60 ± 5.85	379.66 ± 222.82	153.80 ± 86.09	23.951	<0.001
Quercetin	14.82 ± 19.63	22.55 ± 17.41	61.95 ± 16.95	36.69 ± 23.52	19.24 ± 39.39	4.558	0.005
Luteolin	5.54 ± 3.39	10.24 ± 12.48	18.89 ± 2.80	30.99 ± 17.15	7.55 ± 11.67	9.387	<0.001
Epimedin A1	636.93 ± 577.67	48.93 ± 23.28	133.78 ± 48.09	738.22 ± 1018.00	75.40 ± 38.87	2.411	0.068
Epimedin A	2138.93 ± 253.61	1408.67 ± 393.67	1694.20 ± 79.36	1254.84 ± 548.43	1301.71 ± 500.77	10.715	<0.001
Epimedin B	2370.28 ± 330.44	1453.11 ± 345.07	1734.47 ± 43.65	1643.32 ± 634.64	1111.82 ± 480.92	12.293	<0.001
Epimedin C	2184.46 ± 390.62	726.32 ± 164.88	3941.52 ± 60.72	3453.26 ± 250.88	2326.50 ± 750.61	65.116	<0.001
Sagittatoside A	3219.18 ± 593.34	1338.45 ± 400.74	1222.17 ± 124.57	2086.52 ± 423.88	1345.09 ± 388.82	32.323	<0.001
Epimedin-I	23.96 ± 10.58	723.21 ± 382.72	11.59 ± 1.04	13.52 ± 7.11	6.36 ± 1.31	30.316	<0.001
Ikarisoside A	200.98 ± 96.18	35.32 ± 20.38	19.68 ± 2.59	97.92 ± 66.21	125.93 ± 125.26	7.538	<0.001
Icariside-I	27.54 ± 6.75	10.79 ± 4.77	12.99 ± 2.50	36.24 ± 17.06	4.76 ± 3.73	14.272	<0.001
Icaritin	29.37 ± 6.90	22.44 ± 8.84	56.16 ± 6.39	73.76 ± 37.52	17.45 ± 4.35	12.966	<0.001
Icariin	4252.89 ± 904.90	1155.98 ± 335.53	2417.97 ± 243.80	2707.15 ± 644.07	2528.38 ± 1331.47	18.564	<0.001
Sagittatoside B	367.88 ± 192.28	178.17 ± 61.54	389.67 ± 31.82	213.12 ± 187.19	315.48 ± 191.67	2.379	0.07
2'-O-rhamnosyl icariside-II	739.84 ± 346.90	86.63 ± 30.74	1211.52 ± 43.89	1124.24 ± 319.00	1316.00 ± 509.61	14.789	<0.001
Baohuoside-I	215.37 ± 79.69	64.84 ± 35.87	117.36 ± 21.79	215.04 ± 98.49	202.05 ± 136.37	4.676	0.004
Anhydroicaritin	7.52 ± 8.08	8.44 ± 10.41	48.10 ± 2.61	9.48 ± 4.85	3.43 ± 2.05	37.024	<0.001
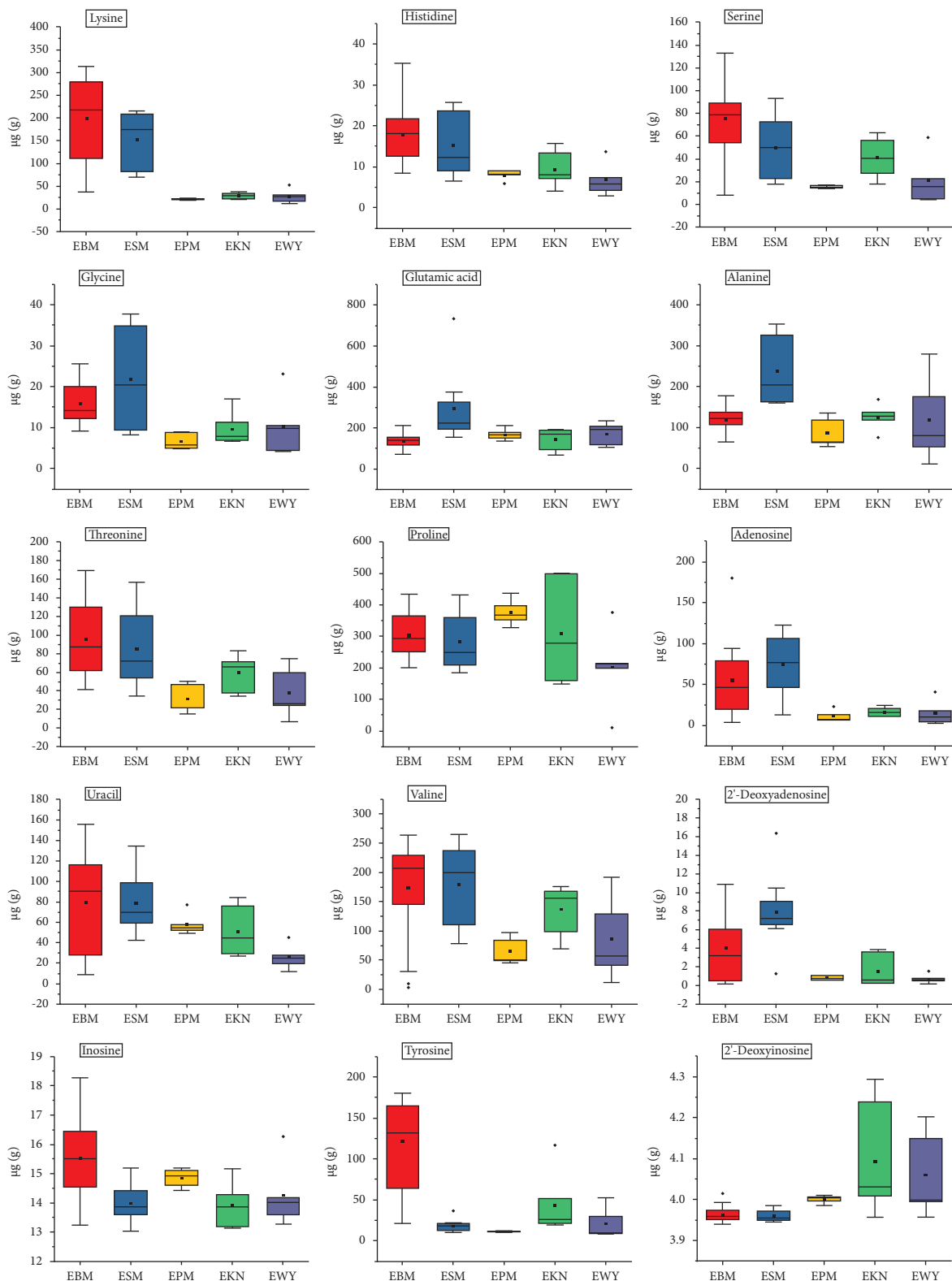
(a)

Figure 7: Continued.
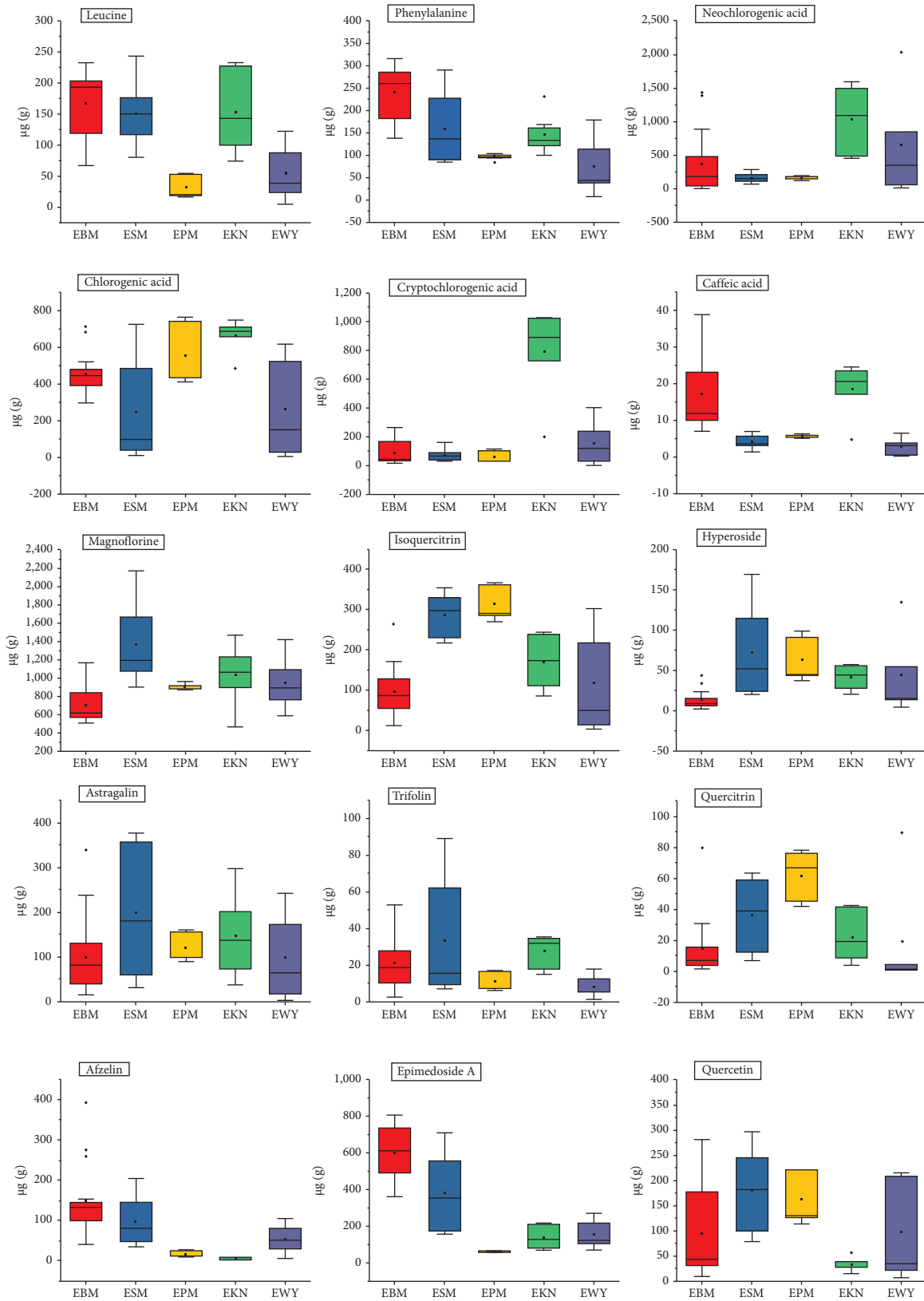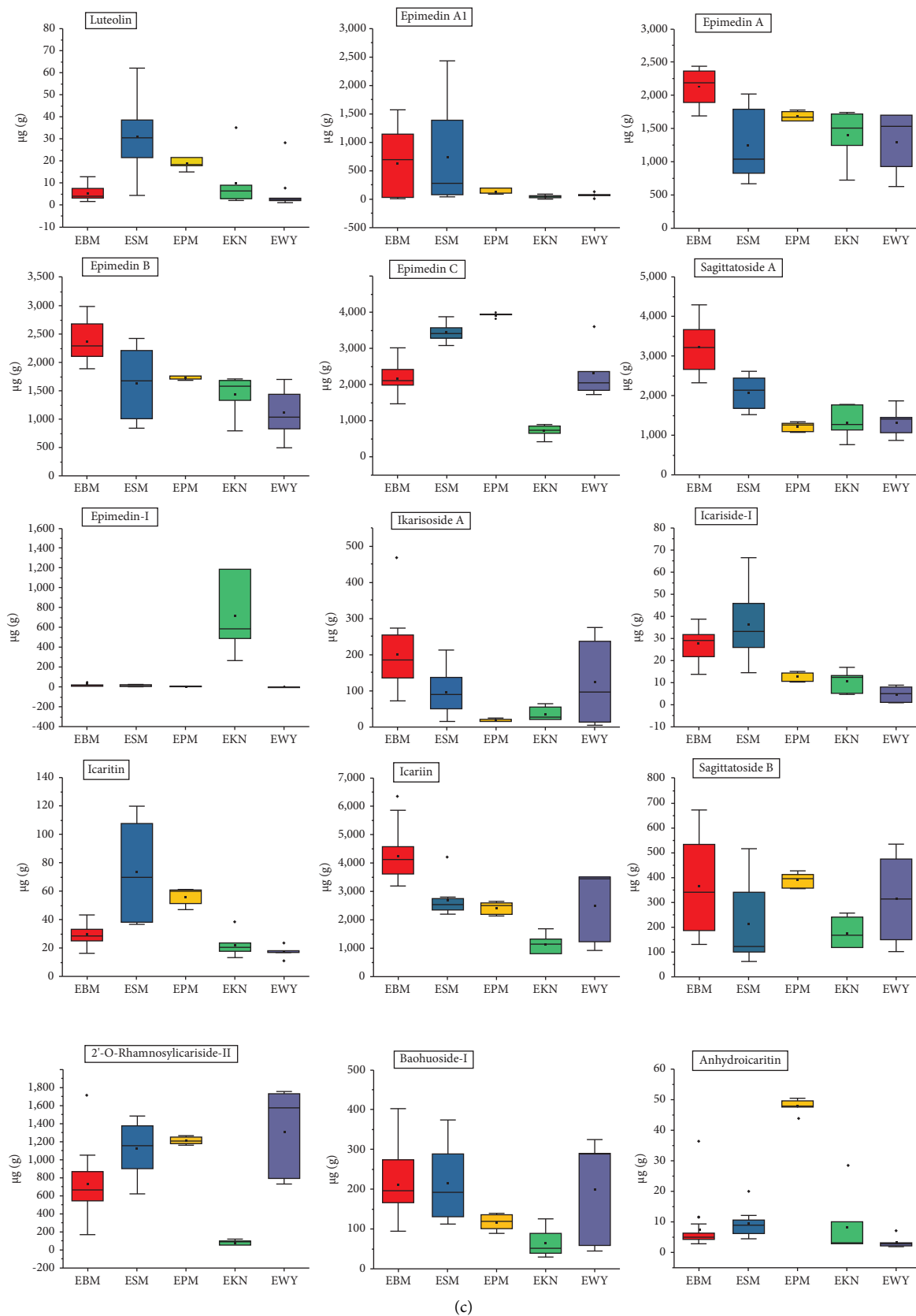
(b)

Figure 7: Continued.

Figure 7: The box plot of 45 constituents' content in 5 species of EF (diamonds represent extremes and squares represent mean value).

TABLE 5: Quality sequencing of the 40 tested samples.

No.	r_i	Ranking	Difference of r_i (%)
S1	0.3840	38	17.73
S2	0.3778	39	19.06
S3	0.3974	35	14.85
S4	0.417	16	10.51
S5	0.4120	24	11.74
S6	0.4012	30	14.05
S7	0.4317	9	7.53
S8	0.4232	12	9.34
S9	0.4497	5	3.66
S10	0.4427	6	5.16
S11	0.4030	29	13.67
S12	0.4539	4	2.76
S13	0.4359	7	6.62
S14	0.4342	8	6.99
S15	0.4310	10	7.67
S16	0.4131	20	11.50
S17	0.4584	3	1.81
S18	0.4256	11	8.83
S19	0.4081	27	12.58
S20	0.4181	15	10.43
S21	0.4622	2	0.99
S22	0.4126	22	11.62
S23	0.4060	28	13.03
S24	0.4668	1	0.00
S25	0.4133	19	11.46
S26	0.4003	31	14.25
S27	0.4150	17	11.11
S28	0.3975	34	14.85
S29	0.3986	32	14.60
S30	0.3959	36	15.19
S31	0.4133	18	11.45
S32	0.4123	23	11.68
S33	0.4127	21	11.58
S34	0.4096	26	12.26
S35	0.4107	25	12.01
S36	0.3942	37	15.56
S37	0.4186	14	10.33
S38	0.3622	40	22.42
S39	0.3981	33	14.71
S40	0.4188	13	10.28

magnoflorine also had high content, with the maximum content of 0.24%, 3%, 0.4%, 0.43%, and 0.2%, respectively. In some samples, the content of epimedin C and sagittatoside A was higher than that of icariin. Also, the above constituents have been proved to have obvious pharmacological effects [39–41]. Therefore, in the future research and application, the composition determination may be considered as new quality control indexes of EF.

Compared with previous studies, our research not only analyzed the flavonoids with obvious pharmacological effects but also carried out quantitative analysis of magnoflorine, phenolic acids, amino acids, and nucleosides, which filled the blank of quantitative analysis of constituents with nonobvious pharmacological effects in EF [42]. In addition, the UFLC-QTRAP-MS/MS method was faster than the previous HPLC method, and mass spectrometer detector adopted the positive and negative ion mode for simultaneous determination, which was more sensitive than the conventional detector and more accurate in determining the molecular weight of the target components [43].

It has been reported that China is the center of modern differentiation and diversity of Epimedium. A total of 68 species have been published and 58 from China, among which 57 are unique to China. In addition to the 5 species included in Pharmacopoeia as EF, more than 10 species are commonly used in the folk [44]. Therefore, in the future research, for the problem of uneven quality of commercial medicinal materials on the market, the established method can be applied to the quality evaluation of more species of EF, which can establish a more perfect quality control and evaluation system of EF. In short, the combination of the established UFLC-QTRAP-MS/MS method and multivariate statistical analysis furnished fundamental insights into the quality assessment of EF from diverse species.

4. Conclusion

We established an efficient and reliable UFLC-QTRAP-MS/MS method to simultaneously determinate 23 flavonoids, 12 amino acids, 4 phenolic acids, 5 nucleosides, and 1 alkaloid in 40 different samples of EF. Single-factor analysis and the Box–Behnken design (BBD) along with response surface methodology (RSM) were used to optimize the extraction conditions. Multivariate statistical analysis made use of OPLS-DA, ANOVA, and GRA to classify and distinguish the differential constituents between different species of EF. The experimental results showed that the overall quality of the EBM was optimal, followed by ESM while the interspecific quality difference of EPM, EKN, and EWY was not significant and the overall quality of the three species was inferior to that of EBM and ESM. This study not only established a reliable and accurate method for simultaneous determination of multiple bioactive components in EF by UFLC-QTRAP-MS/MS but also provided a basis for the comprehensive evaluation and intrinsic quality control of EF from different species.

Data Availability

The data that support the findings of this study are included in the supplementary material of this article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

J.X., C.C., and X.L. conceived and designed the experiments; J.X., Y.Z., H.C., J.Y., S.Y., and Y.S. performed the experiments; J.X., J.Y., W.Y., N.W., and Y.S. analyzed the data; J.X. and H.C. wrote the manuscript; Z.C., C.C., X.L., and J.C. revised the manuscript; X.L. and L.Z. provided the samples of Epimedii Folium. All authors read and approved the final manuscript.

Acknowledgments

Supplementary Materials

Supplementary information is available online. Figure S1: effects of ethanol concentration, solid-liquid ratio, and extraction time on extraction yields of icariin (extraction yield (%) = weight of analyte (mg)/weight of dried sample (g) × 100). Figure S2: the spectra of the negative ion modes from the mixed standards. The numbers denoted are the same as those in Table 2. Figure S3: the spectra of the positive ion modes from the mixed standards. The numbers denoted are the same as those in Table 2. Table S1: experimental design factors and levels of response surface experiment. Table S2: experimental design and results of Box–Behnken. Table S3: variance analysis and significance test of response surface experiment. Table S4: the CAS numbers of 45 target constituents. Table S5: the recovery of different species. Table S6: the RSDs of recovery for each specie. Table S7: the matrix effect of different species of EF. Table S8: the content of 45 constituents in samples (μg/g, $n = 3$). (*Supplementary Materials*)

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