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Original Article

Discrimination of Different Part of *Curcuma longa* by HPLC Fingerprints Combined with Multivariate Statistical Analysis

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ABSTRACT

Aim: The purpose of this study is to develop a method to explore the difference between the rhizomes and tuberous roots of *Curcuma longa*, and screen out the difference markers. **Materials and Methods:** The quantitative analysis and fingerprints of rhizomes and tuberous roots were established by HPLC, rhizomes and tuberous roots of *Curcuma longa* were clearly discriminated by Hierarchical Cluster Analysis (HCA), Similarity Analysis (SA) and Principal Component Analysis (PCA). The difference markers were screened out by Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). **Results:** The contents of curcumin, bisdemethoxycurcumin and demethoxycurcumin in all rhizomes were higher than those in tuberous roots. Multivariate statistical analysis shown that the samples of rhizomes were grouped into the same categories and samples of tuberous root were grouped another group in each analysis mode. And the OPLS-DA model had a good productivity and good fit indicated by the value of R^2Y=0.981, Q^2=0.946and R^2X=0.816. The important markers for discrimination samples were the peak 14, peak 10 (demethoxycurcumin) and peak 11 (curcumin). **Conclusion:** The fingerprinting combination of multivariate statistical analysis can be applied to distinguish the rhizomes and tuberous roots of *Curcuma longa*.

Keywords: *Curcuma longa*, Discrimination, HPLC fingerprints, Multivariate statistical analysis, Rhizomes and tuberous root.

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INTRODUCTION

The dried rhizome of *Curcuma longa* is the traditional Chinese medicine of Jianghuang (in Chinese), the tuberous root of *Curcuma longa* is the Chinese medicine of Yujin (in Chinese).[1] The rhizome and tuberous root of *Curcuma longa* have different medicinal value. The rhizome has effects on antitumor, antioxidation, breaking blood stasis, clearing meridians.[2-4] Rhizome are also widely used in industrial dyes, food additives, and health care products.[5,6] The tuberous root of *Curcuma longa* are used in activating blood and stopping energy, relieving depression, dredging the liver and benefiting the gall.[7,8] bisdemethoxycurcumin Curcumin, demethoxycurcumin and are the main active components of them.[9] Jianghuang and Yujin is the same plants of different medicinal part, they are similarities and differences in morphology, character, composition and function, so the application has always been chaotic. Therefore, the pharmacological research and clinical application are affected.[10,11] Further systematic comparative studies of chemical components of these two Chinese herbal medicines are needed to distinguish them, to better guide their rational use in clinic.[11] But it is difficult to identify them because they have the same basic source and have a similar color. This phenomenon causes quality of medicine inferior, affecting their quality, safety, and efficacy.

Most studies on the difference between Jianghuang and Yujin focused on determining one or more chemical component content,[12,13] pharmacological activities,[14] and herbal research.[15] The differences between them were not compared from the whole chemical profiles level. Therefore, to make better use of them, it needs an objective and comprehensive methodology for characterizing their chemical profiles. In this paper, for the first time, HPLC fingerprint combined with multivariate statistical analysis, such as SA, HCA, PCA and OPLS-DA are used to quality assess and distinguish Jianghuang and Yujin, and the difference markers of them are screened out.

MATERIALS AND METHODS

Instruments, reagents and materials

HPLC system (Model LC-2010A HT, Shimadzu Corporation, Japan); Ultrasonic cleaning apparatus (Model KQ3200E, Kunshan Ultrasonic Instrument Co. Ltd.,); Thirteen batches of rhizomes (S1~S13) and tuberous roots (S1-1~S13-1) were collected

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Table 1: Proportion of mobile phase.

Time (min)	Acetonitrile (%)	0.05% phosphoric acid solution (%)	Time (min)	Acetonitrile (%)	0.05% phosphoric acid solution (%)
0.01	20	80	80.00	50	50
30.00	30	70	90.00	60	40
47.00	35	65	95.00	80	20

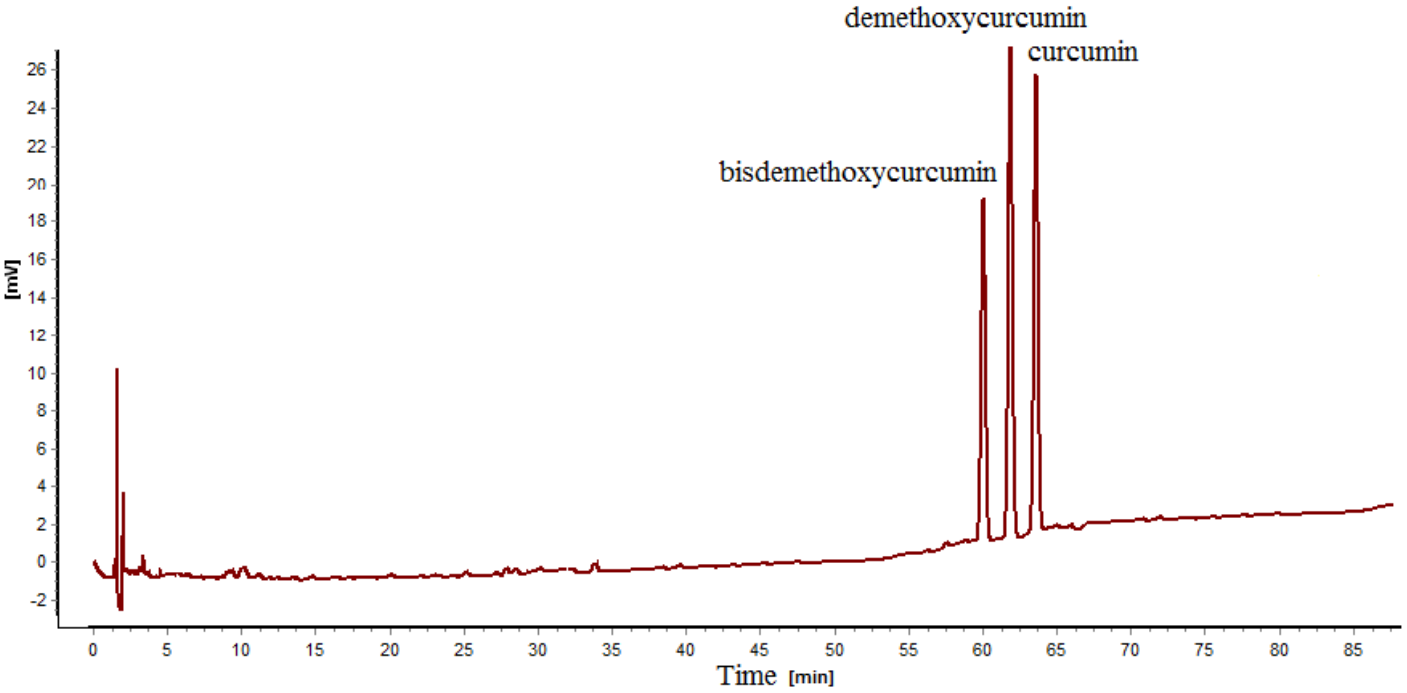

Figure 1: Chromatogram of three curcuminoids.

from different villages in Muchuan County, Sichuan province of China. The rhizome and its corresponding tuberous root were separated and then washed, dried at 40°C and powdered. Standard substances of the three curcuminoids was purchased from the Must Bio-Technology Co. Ltd., (MUST-16031501, MUST-16050404, MUST-16081810).

Reagent preparation

Standard solution of the three curcuminoids were prepared by accurately weighting 0.5000 g bisdemethoxycurcumin, Curcumin and demethoxycurcumin were dissolved and diluted to make a stock solution with 0.5 mg curcuminoids per 1 mL. The standard solution was then diluted to appropriate concentration ranges for the establishment of calibration curves.[14]

Preparation of samples

Collected and cleaned the samples, then separated the rhizomes and tuberous roots. S1 ~ S13 were the rhizomes, and S1-1 ~ S1-13 were the tuberous root corresponding to the rhizomes. Dried at low temperature, and then crushed the samples for preservation.[14,15] Accurately weighed a certain amount of the sample powder, put it into a conical flask with stopper, accurately added 15.00 mL methanol, ultrasonic extracted for 40 min, took it out and placed at room temperature, filled with a 0.45μm microporous membrane, the subsequent filtrate as the test solution.[16-18]

Chromatographic conditions

Based on references,[19] the optimal experimental conditions were follows: Eclipse Plus C_{18} column (5 μm, 150 mm×4.6 mm); the detection wavelength 270 nm; injection volume 10 μL; column temperature 27°C; flow rate phases: B was 0.05% phosphoric acid aqueous solution, A was acetonitrile; flow rate was 1.0 mL/min, gradient elution procedure in Table 1.

RESULTS AND DISCUSSION

Quantitative analysis

Accurately weigh a certain amount of 13 batches of rhizomes and tuberous root samples, prepare the test solution, and determine the content of three curcuminoids. peak 11, 10 and 8 were curcumin, demethoxycurcumin and bisdemethoxycurcumin (Figures 1 and 2).

From Table 2, the average contents of bisdemethoxycurcumin, demethoxycurcumin and curcumin in the rhizomes are 6.72 mg/g, 7.37 mg/g and 36.70 mg/g respectively. The content of curcumin in all rhizomes is much higher than that specified in the pharmacopoeia,[1] indicating that the quality of rhizomes of *Curcuma longa* medicinal materials in Muchuan production area are good. The average contents of bisdemethoxycurcumin, curcumin, and demethoxycurcumin in the corresponding tuberous root are 1.91 mg/g, 14.46 mg/g and 1.69 mg/g. The content of curcuminoids in tuberous roots is very different, and the content of curcumin in some tuberous root samples is lower than the specified in the pharmacopoeia, indicating that the quality of tuberous root samples is unstable.[1]

Fingerprint establishment

The test solution of thirteen batches of rhizomes and corresponding tuberous roots of *Curcuma longa* was prepared, and analyzed by HPLC, and obtained the HPLC fingerprint of all samples (Figure 3). There were obvious differences in the number of peaks, peak heights and peak shapes between the rhizome and tuberous root.[17] Common peaks with better stability were obtained, the curcumin peak as the reference peak (S), the Relative Peak Area (RPA) of each common peak was calculated. According to the relative peak area value, curcumin was the most abundant component in the samples, and the contents of bisdemethoxycurcumin and demethoxycurcumin were also significantly higher than those of other chemical components in the samples. There are different degrees of differences in the relative peak area of each sample, because the chemical components in medicinal materials are closely related to their growth environment, which may be caused by some environmental factors, such as soil pH, N and P content, fertilization conditions.[19] It also shows that the active components of the rhizome and tuberous root of are quite different, and the tuberous root should not be mixed into the rhizome during clinical application, otherwise the quality of the herbal medicine will be affected.

Similarity analysis

The fingerprints of rhizome and tuberous root were imported into the "Chinese Medicine Chromatographic Fingerprint Similarity Evaluation System", and the similarity of each batch of samples was calculated, the results are shown in Table 3.

The similarity of the rhizome samples (S1-S13) were higher than 0.960, and the similarity was relatively stable at 0.960 ~ 0.994,

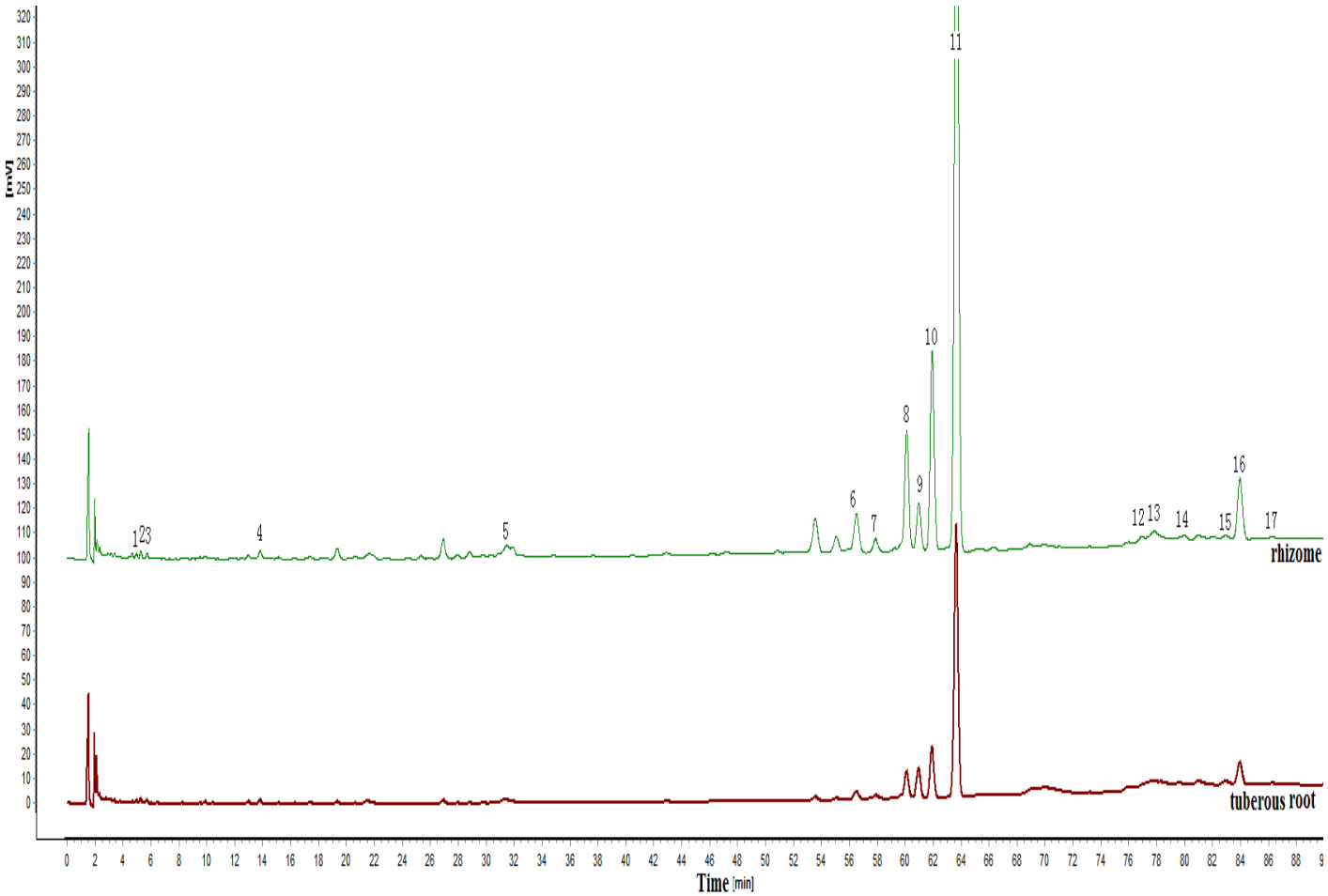

Figure 2: Chromatogram of rhizomeand tuberous root.

Table 2: Content of curcuminoids (mg/g).

Rhizome	Curcumin	Demethoxycurcumin	Bisdemethoxycurcumin
Variation range	24.57~44.14	5.28~8.42	5.22~9.08
Extreme difference	19.57	3.14	3.86
Average	36.70	7.37	6.72
Tuberous root	Curcumin	Demethoxycurcumin	Bisdemethoxycurcumin
Variation range	1.73~21.09	0.22~3.14	0.20~4.12
Extreme difference	19.36	2.92	3.92
Average	14.46	1.69	1.91

Table 3: Similarity of samples.

Sample number	Similarity	Sample number	Similarity	Sample number	Similarity	Sample number	Similarity
S1	0.994	S8	0.991	S1-1	0.981	S8-1	0.951
S2	0.960	S9	0.972	S2-1	0.790	S9-1	0.954
S3	0.990	S10	0.989	S3-1	0.924	S10-1	0.969
S4	0.992	S11	0.992	S4-1	0.323	S11-1	0.978
S5	0.985	S12	0.977	S5-1	0.677	S12-1	0.896
S6	0.981	S13	0.970	S6-1	0.944	S13-1	0.543
S7	0.991			S7-1	0.723		

Table 4: Eigen value and cumulative contribution.

Component	Initial eigen values		
	Characteristic value	Variance contribution (%)	Accumulated contribution (%)
1	11.28	66.36	66.36
2	1.34	7.90	74.26

indicating that the quality of *Curcuma longa* from Muchuan is stable and reliable.[20] The similarity of tuberous root (S1-1-S13-1) samples varied greatly, indicating that the components of the tuberous root samples are very different and the quality is unstable; the similarity between the rhizome and tuberous root are great different, this indicates that there is a big difference between the main components of rhizome and tuberous root.[20]

Hierarchical cluster analysis

HCA was carried out base on the normalization peak area value of 17 common peaks. The samples can be divided into two categories, the rhizome samples (S1 ~ S13) are clustered into one category, and the tuberous root samples (S1-1 ~ S1-13) are clustered another category (Figure 4). The results of cluster analysis showed that tuberous roots could be distinguished from rhizome samples.

Principal component analysis

Using SIMCA14 software, the peak areas of the 17 common peaks of all samples were Z-standardized, and the feature value greater than 1 was used as the extraction standard to obtain the first two principal components, with a cumulative contribution rate of 74.26% (Table 4), which can be used for the quality identification of the rhizome and tuberous root samples. The chromatographic peak 11 (curcumin), peak 10 (demethoxycurcumin) and peak 16 have higher loadings on the main component 1, which indicates that curcuminoids play a major role in the quality identification of rhizome and tuberous root (Table 5).

From eigen vectors (Table 6), the linear equations F1=-0.0050Z1+0.0068Z2+…+0.0045Z17, F2=-0.0853Z1+0.0380Z2-…-0.2314Z17 (the normalized value Zi of the original variable i) are obtained. The comprehensive evaluation function F=0.6636 F1+0.079F2. From Table 7, we can conclude that samples can be divided into two categories, one is the tuberous root samples whose comprehensive principal component values are all less than 0, and another is the rhizome samples whose comprehensive principal component values are all greater than 0 (Table 7). It shows that the content of active components in the rhizome is higher than that in the tuberous root samples, and the

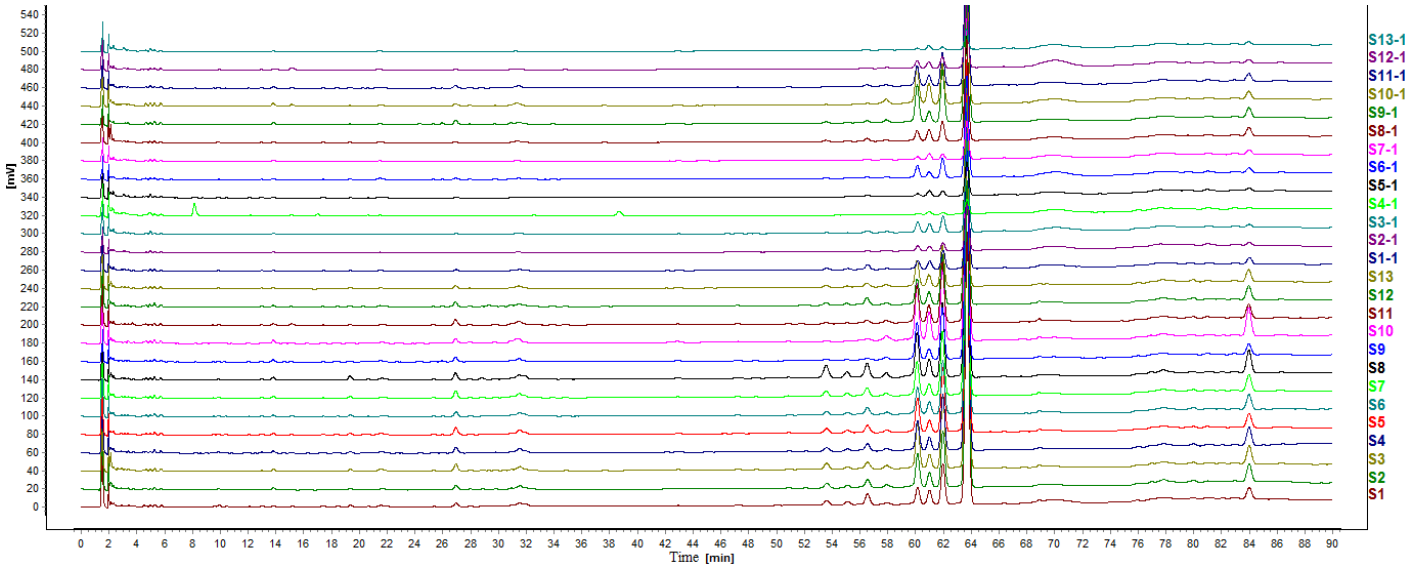

Figure 3: HPLC fingerprint of all samples.

Table 5: Loading matrix.

Peak No	Component 1	Component 2	Peak No	Component 1	Component 2
1	-0.63	-0.15	10	0.98	0.02
2	0.78	0.07	11	0.98	0.01
3	0.85	-0.07	12	-0.02	0.69
4	0.87	-0.07	13	0.61	0.52
5	0.71	-0.43	14	-0.92	-0.11
6	0.70	0.42	15	-0.92	0.00
7	0.95	-0.09	16	0.96	0.05
8	0.95	-0.07	17	0.58	-0.38
9	0.77	-0.15			

Table 6: Feature vector.

Peak No	Component 1	Component 2	Peak No	Component 1	Component 2
1	-0.0050	-0.0853	10	0.0077	0.0084
2	0.0068	0.0380	11	0.0077	0.0054
3	0.0067	-0.0415	12	-0.0002	0.3860
4	0.0069	-0.0396	13	0.0048	0.2879
5	0.0056	-0.2367	14	-0.0072	-0.0588
6	0.0055	0.2344	15	-0.0072	0.0003
7	0.0075	-0.0513	16	0.0076	0.0282
8	0.0075	-0.0404	17	0.0045	-0.2134
9	0.0060	-0.0853			

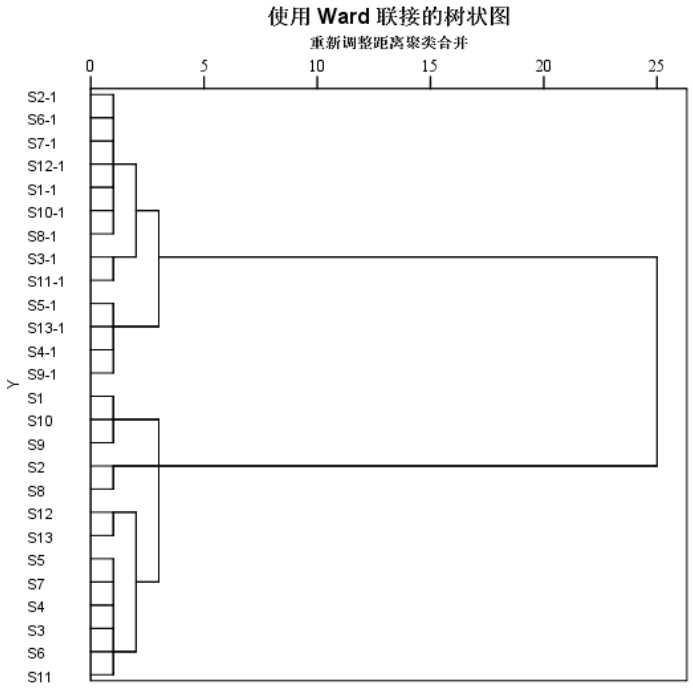

Figure 4: Cluster analysis dendrogram.

comprehensive quality of the rhizome samples is better than the corresponding tuberous root.

The principal component score diagram and loading diagram of the samples are shown in Figure 5A and Figure 5B. Samples can be divided into two groups, rhizome sample as one group, tuberous root samples were classified another group, which was consistent with the cluster analysis results. It can be seen from Figure 5B that the peaks 10, 11 and 16 have the greatest influence on the principal component 1, and the peaks 12 and 13 have the greatest influence on the principal component 2.

OPLS-DA analysis

The OPLS-DA of the turmeric rhizome and tuberous root samples was carried out based on 17 common peaks. The parameters for evaluating the OPLS-DA model are as follows: R^2Y=0.981, Q^2=0.946 and R^2X=0.816. Generally, the closer Q^2 and R^2Y are to 1, the better the stability and predictability of the model.[21] Q^2 = 0.946> 0.5, indicating that the two predicted principal components in the model have an effect strong predictive ability. On the whole, the model fits well.[22] Where R^2X =0.816 indicating that

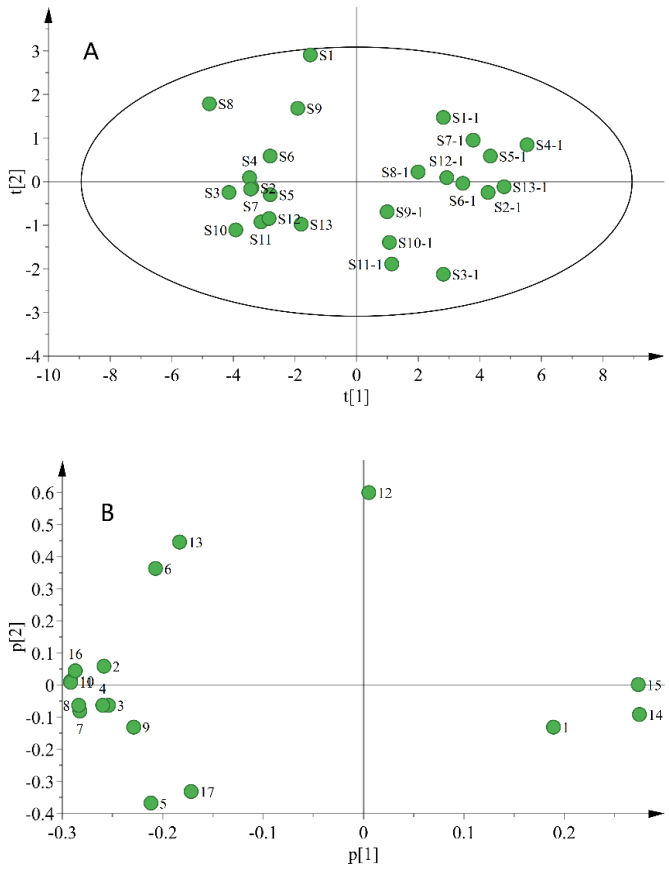

Figure 5: Loading plot (B) and Score plot (A) of PCA.

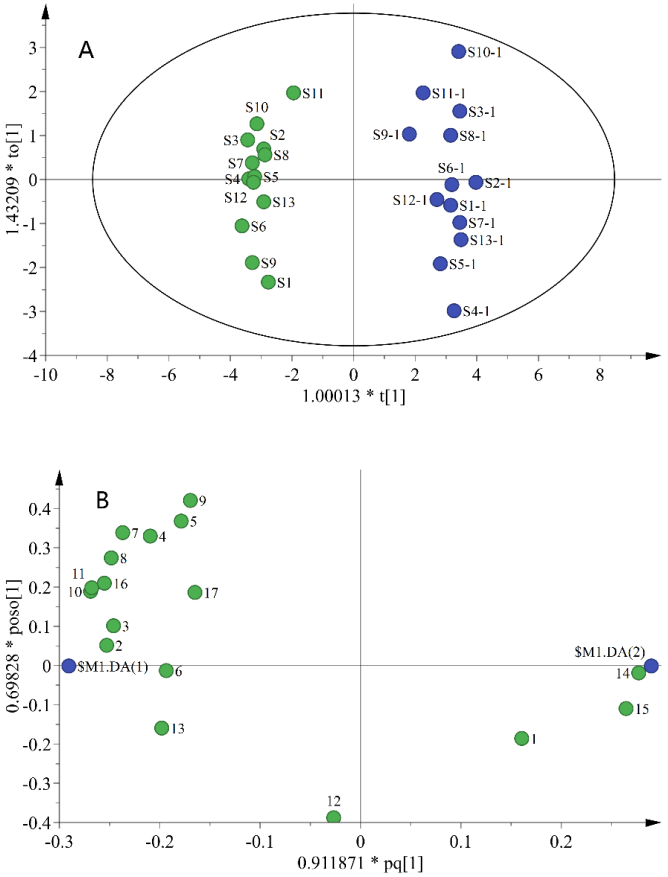

Figure 6: Score plot (A) and lording scatter plot (B) in OPLS-DA.

Table 7: Comprehensive principal value and rank of samples.

Sample No	F1	F2	F	Rank	Sample No	F1	F2	F	Rank
S1	0.0395	1.8784	0.1746	1	S7-1	-0.0999	0.6113	-0.0180	14
S8	0.1262	1.1441	0.1741	2	S1-1	0.0472	-0.6351	-0.0189	15
S9	0.0506	1.0855	0.1193	3	S8-1	-0.0523	0.1391	-0.0237	16
S6	0.0745	0.3759	0.0791	4	S12-1	-0.0772	0.0674	-0.0459	17
S4	0.0917	0.0641	0.0659	5	S5-1	-0.1148	0.3748	-0.0466	18
S3	0.1097	-0.1610	0.0601	6	S9-1	-0.0266	-0.4479	-0.0530	19
S2	0.0901	-0.0943	0.0523	7	S4-1	-0.1466	0.5544	-0.0535	20
S7	0.0906	-0.1166	0.0509	8	S6-1	-0.0907	-0.0311	-0.0626	21
S5	0.0736	-0.1958	0.0334	9	S2-1	-0.1121	-0.1552	-0.0867	22
S13	-0.0741	0.9539	0.0262	10	S13-1	-0.1266	-0.0704	-0.0896	23
S10	0.1037	-0.7168	0.0122	11	S10-1	-0.0285	-0.8987	-0.0899	24
S11	0.0815	-0.5961	0.0070	12	S11-1	-0.0297	-1.2200	-0.1161	25
S12	0.0748	-0.5405	0.0069	13	S3-1	-0.0745	-1.3695	-0.1576	26

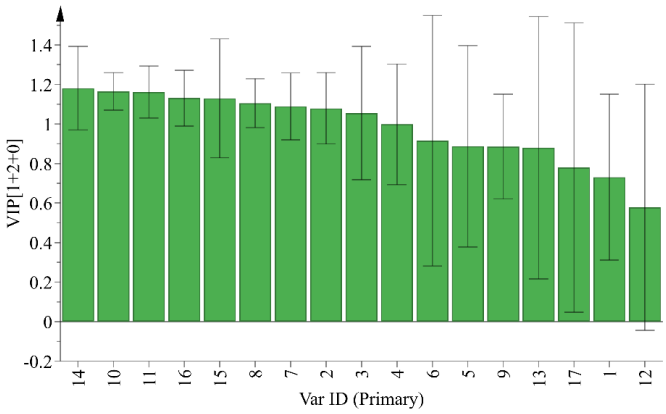

Figure 7: VIP plot of OPLS-DA.

the 17 principal components are paired with X the explanatory ability of the variation is 81.60%;[22] R^2Y = 0.981 indicates that the explanatory ability of the two predicted principal components in the model for the variation of the Y variable is 98.10%.[22] From the first and second principal component score map (Figure 6A), the samples are divided into 2 categories, the rhizome samples and the tuberous root samples are distinguished, and the samples from the same part have obvious aggregation trends, which is consistent with PCA and HCA analysis. It shows that the contents of key compounds in the tuberous root samples are quite different from those in the rhizome samples.[22] It can be seen from the Figure 6B that the peaks 10, 11, 16, and 14 have a greater contribution to the principal component 1. Differential markers were screened out from the VIP value of the OPLS-DA model (Figure 7), the three compounds with the largest VIP values were selected as peak 14, peak 10, and peak 11, indicating that these three peaks have a greater impact on the discrimination of Jianghuang and Yujin, peak 10 and peak 11 are demethoxycurcumin and curcumin, respectively. It indicated that curcuminoids were the main markers for distinguishing rhizome and tuberous root of *Curcuma longa*.

CONCLUSION

Curcuma longa is a common traditional Chinese medicine widely used in medicine and food.[7] In recent years, as its tuberous roots and other parts are mixed into the rhizome medicinal materials, it has caused a threat to the safety of consumers. In this paper, HPLC fingerprints combined with multivariate statistical analysis showed that there was a significant difference in the quality of rhizome and tuberous root. Combined with quantitative analysis, SA, PCA and HCA methods can discriminate rhizomes and tuberous roots, and combined with OPLS-DA method to screen out characteristic compounds that identify them. Compounds peak 14, demethoxycurcumin and curcumin were the best markers for the identification of tuberous root and rhizome of *Curcuma longa*. It is recommended to pay attention to these three active components during the production process, and it is necessary to further track the changes of these active components in various growth stages of *Curcuma longa*. In this experiment, the analytical results of SA, PCA, HCA and OPLS-DA were consistent and mutually supportive. This study further explains why tuberous root cannot be mixed into rhizome medicinal materials, otherwise it will affect the quality of medicinal materials and drug safety.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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