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Discriminating Astragali Radix from Its Adulterants Using HPLC Coupled with Chemometric Clustering Techniques

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ABSTRACT

A simple high-performance liquid chromatography (HPLC) method coupled with principal component analysis (PCA) and hierarchical cluster analysis (HCA) was developed for distinguishing Astragali Radix from its adulterants. Five species, including *A. membranaceus* var. *mongolicus*, *A. membranaceus*, *H. polybotrys*, *A. chrysopterus* and *A. ernestii*, were analyzed by this method. At the same time, four major bioactive isoflavonoids, namely calycosin-7-*O*-D-glucopyranoside, ononin, calycosin and formononetin, were simultaneously determined in Astragali Radix. This method was proven to have good precision, repeatability, stability and recovery. It was successfully applied to distinguish Astragali Radix from its adulterants species and can be used for the quality control of Astragali Radix.

Key words: Astragali Radix, isoflavonoids, HPLC, principle component analysis, hierarchical cluster analysis

INTRODUCTION

Astragali Radix (huang-qi in Chinese) is a Chinese medicinal herb commonly used in traditional Chinese medicine for the treatment of many diseases. Pharmacological studies and clinical practice have demonstrated that Astragali Radix possesses many biological activities, including hepatoprotective, anti-oxidative, anti-viral, anti-hypertensive and immunostimulant activities[1-5]. In addition to its medicinal use, Astragali Radix has been used in food products, such as herbal tea, soft drinks and soups[6]. Demand for Astragali Radix is increasing throughout the world, especially in China, Japan and Southeast Asia. Although *Astragalus membranaceus* (Fisch.) Bge. and *A. membranaceus* var. *mongholicus* (Bge.) Hsiao are the original species of Astragali Radix in the Chinese Pharmacopeia, there are many other species used as Astragali Radix by Chinese medicine practitioners. These adulterants are also sold as Astragali Radix in the market, including *Hedysarum polybotrys* Hand.-Mazz., *A. chrysopterus* Bge. and *A. ernestii* Comb. The morphological appearances of Astragali Radix and its adulterants show a great resemblance, but they do not have the same pharmacological and clinical indications[7,8]. The misuse of these species may weaken the medicinal efficacy or lead to adverse health effects. Therefore, a global and reliable quality control method of Astragali Radix is required.

Previously, HPLC methods have been developed for the quality evaluation of Astragali Radix through the determination of flavonoids, astragalosides or saponin[9-11]. In the Chinese Pharmacopeia, astragaloside IV and isoflavonoids (calycosin-7-O-D-glucopyranoside) are used as reference markers. However, these published methods only use one or two of constituents for the differentiation of different species. Herbal medicines have complex chemical compositions and characteristic chromatographic fingerprints were developed[12-15]. As the fingerprint chromatograms are comprehensive multivariate data sets due to the complexity of herbal medicines, minor differences between very similar chromatograms might be overlooked. Thus, chemometrics clustering techniques, such as principal component analysis (PCA) and hierarchical cluster analysis (HCA), should be taken into consideration for reasonable definition of the class of herbal medicine. PCA and HCA are unsupervised methods, which constitute a first step in data analysis. Without assuming any previous knowledge of sample class, these methods enable the visualization of the data in a reduced dimensional space built on the dissimilarities between samples with respect to their chemical compositions.

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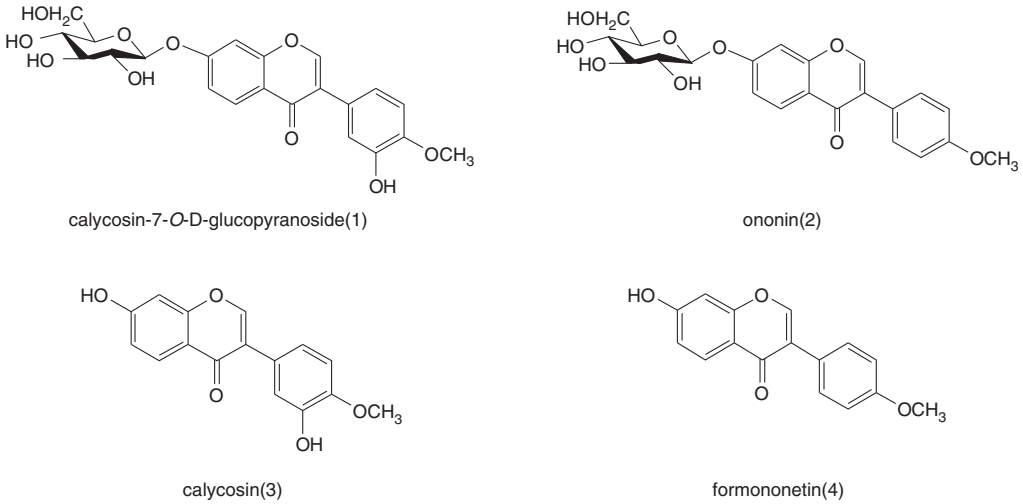

Figure 1. Chemical structures of reference compounds.

In this study, to emphasize the comprehensive properties of Astragali Radix, a HPLC method coupled with chemometric clustering techniques was developed for discriminating Astragali Radix from its adulterants. The contents of four active isoflavonoids in different species were also compared.

MATERIALS AND METHODS

I. *Chromatography*

Quantitative and fingerprint analysis were performed on an Agilent 1100 chromatography system with DAD detector. A Kromasil RP-C_{18} analytical column (4.6 × 250 mm, 5 μm, Eka, Sweden) and a RP-C_{18} guard column were used. The flow rate was set at 1.0 mL/min and the column temperature was set at 25°C. The mobile phase consisted of 0.3% aqueous formic acid (v/v, A) and acetonitrile (B). The HPLC eluting conditions were optimized as follows: linear gradient from 10% to 25% B (0 - 15 min), isocratic 25% B (15 - 20 min), linear gradient from 25% to 34% B (20 - 35 min), 34% to 40% B (35 - 40 min), isocratic 40% B (40 - 50 min), 40% to 70% B (50 - 60 min) and then hold for 5 min. The UV detection wavelength was set from 190 to 400 nm.

II. *Reagents*

The authentic reference compounds of calycosin-7-*O*-D-glucopyranoside (1), ononin (2), calycosin (3) and formononetin (4) were supplied by the Hong Kong Jockey Club Institute of Chinese Medicine. The structures of these compounds were shown in Figure 1. Acetonitrile (LC grade) used for the liquid chromatographic mobile phases was purchased from Merck (Darmstadt, Germany). Milli-Q plus water (Millipore, Bedford, MA, USA) was used throughout this study. Analytical grade methanol from Huada Chemical Reagent Co., Inc. (Guangdong, China) was used for sample preparation.

III. *Materials*

Seventeen samples of Astragali Radix and its adulterants were collected from traditional Chinese medicinal markets in China. All of them were identified by Prof. Qin Songyun from Chongqing Academy of Chinese Materia Medica. Voucher samples were deposited in the State Key Laboratory of Chinese Medicine and Molecular Pharmacology (Shenzhen, China).

IV. *Sample Preparation*

The roots were powdered to a homogeneous size by mill. An accurately weighed sample of powder (1.5 g) was introduced into a flask and refluxed with 60 mL of methanol for 2 h. The extracts were filtered while hot and evaporated under vacuum, and then diluted to volume with methanol in a 5-mL volumetric flask. The solution was filtered through a 0.45-μm syringe filter before analysis. A 10-μL aliquot of solution was injected for HPLC analysis.

The four reference compounds were weighed, dissolved and diluted with methanol in a volumetric flask to obtain standard stock solutions and the concentration of each compound was 1.00, 0.80, 0.40 and 0.40 mg/mL respectively. The stock solution was further diluted to different concentration ranges. The calibration curve was performed with at least six appropriate concentrations in triplicate.

V. *Data Analysis*

Different data pre-processing methods were utilized to minimize interference effects, baseline shift and enhance the data quality for model establishment. These include mean centering, auto-scaling, standard normal variate (SNV),

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Table 1. Linear regression data, LOD and LOQ of the four isoflavonoids in HPLC-DAD

Analyte[a]	Calibration curve[b]	r^2	Linear range (μg/mL)	LOD[c] (μg/mL)	LOQ[d] (μg/mL)
1	Y = 18.513 x + 15.426	0.9999	12.5 - 400	0.15	0.50
2	Y = 17.960 x + 32.084	0.9999	6.25 - 200	0.31	1.04
3	Y = 29.674 x + 23.588	0.9998	3.12 - 100	0.21	0.90
4	Y = 26.414 x + 23.047	0.9997	6.25 - 100	0.21	0.90

[a] Analyte names: 1: calycosin-7-*O*-D-glucopyranoside; 2: ononin; 3: calycosin; 4: formononetin.
[b] Y is the peak area in UV chromatograms monitored at 280 nm, x is the concentration of the standard.
[c] LOD refers to the limit of detection.
[d] LOQ refers to the limit of quantification.

derivative, smoothing, multiplicative scatter correction (MSC) and de-trending. Chemometric clustering techniques, PCA and HCA, were used to analyze the spectral data obtained by HPLC. All computer programs used in this study were coded in MATLAB 7.0.

RESULTS AND DISCUSSION

I. *Development of Extraction Method*

In order to achieve repeatable and quantitative extraction, factors including the type of solvent, extraction method and time were considered during the extraction procedure. As the standard compounds had varied polarities in different solvents, different concentrations of methanol and ethanol solutions were investigated as extraction solvents. The results showed that 100% methanol was the preferred choice for most isoflavonoids, which were co-extracted effectively. For the extraction method, extraction by reflux was the best with the highest yield of the four isoflavonoid components, compared with steeping at room temperature and ultrasonic treatment. The influence of extraction time was also investigated. Samples were extracted with methanol for 0.5 h, 1 h, 2 h and 3 h, respectively. The results suggested that similar amounts of isoflavonoids were obtained with the extraction time of 2 h and 3 h. Therefore, 2 h was chosen as the optimum extraction time.

II. *Optimization of Chromatographic Conditions*

To simultaneously detect the four isoflavonoids in the sample, the wavelength for their detection was selected by using a DAD detector, which was set at 280 nm for obtaining chromatograms. UV spectral data were acquired from 190 to 400 nm. The mobile phase was optimized by comparing different solvents, solvent ratio and gradient profiles. When compared with other solvents, acetonitrile achieved the best separation and lowest column pressure. An acidified mobile phase could minimize peak tailing, improve resolution and facilitate ionization. Thus, acetonitrile and 0.3% aqueous formic acid were chosen as mobile phases with a flow rate of 1.0 mL/min in the gradient elution described in Materials and Methods section.

III. *Method Validation*

The limits of detection and quantification of the method were determined as the concentration with peak area ratio of signal-to-noise (S/N ratio) at no less than 3 and 10, respectively (Table 1). The intra- and inter-day precisions were determined by analyzing the same sample (No. 1) on a single day and on 5 different days, respectively. To confirm the repeatability, six different working solutions prepared from the same sample (No. 1) were analyzed. The RSD value was taken as a measure of precision and repeatability, and the results were shown in Table 2. Quintuplicate samples of Astragali Radix (No. 1) were accurately spiked with known amounts of four reference compounds, and then extracted and analyzed. The results were shown in Table 3.

The linear calibration curve with r^2, linear range, LOD and LOQ values of each compound were shown in Table 1. All calibration curves showed good linearity ($r^2 > 0.999$) the tested ranges. The ranges of LOD and LOQ for the four isoflavonoids were from 0.15 to 0.31 μg/mL and 0.50 to 1.04 μg/mL, respectively. The results for precision and repeatability of the 4 analytes were shown in Table 2. The RSD of the overall intra- and inter-day variations were less than 3%. Besides, validation studies of this method showed good reproducibility with RSD values of less than 5% for the 4 analytes. Table 3 shows that the developed method has good accuracy with an overall recovery of 96.3% to 103.4% for the analytes concerned. Therefore, the HPLC-DAD method is sufficiently accurate and sensitive for the simultaneous quantitative evaluation of the four major active isoflavonoids.

IV. *Quantification and Comparison of Four Compounds in Different Samples*

The contents of four investigated compounds in Astragali Radix and its adulterants were determined by HPLC-DAD (Figure 2). Peaks 1, 2, 3 and 4 are calycosin-7-*O*-D-glucopyranoside, ononin, calycosin and formononetin, respectively. The results were shown in Table 4. It was

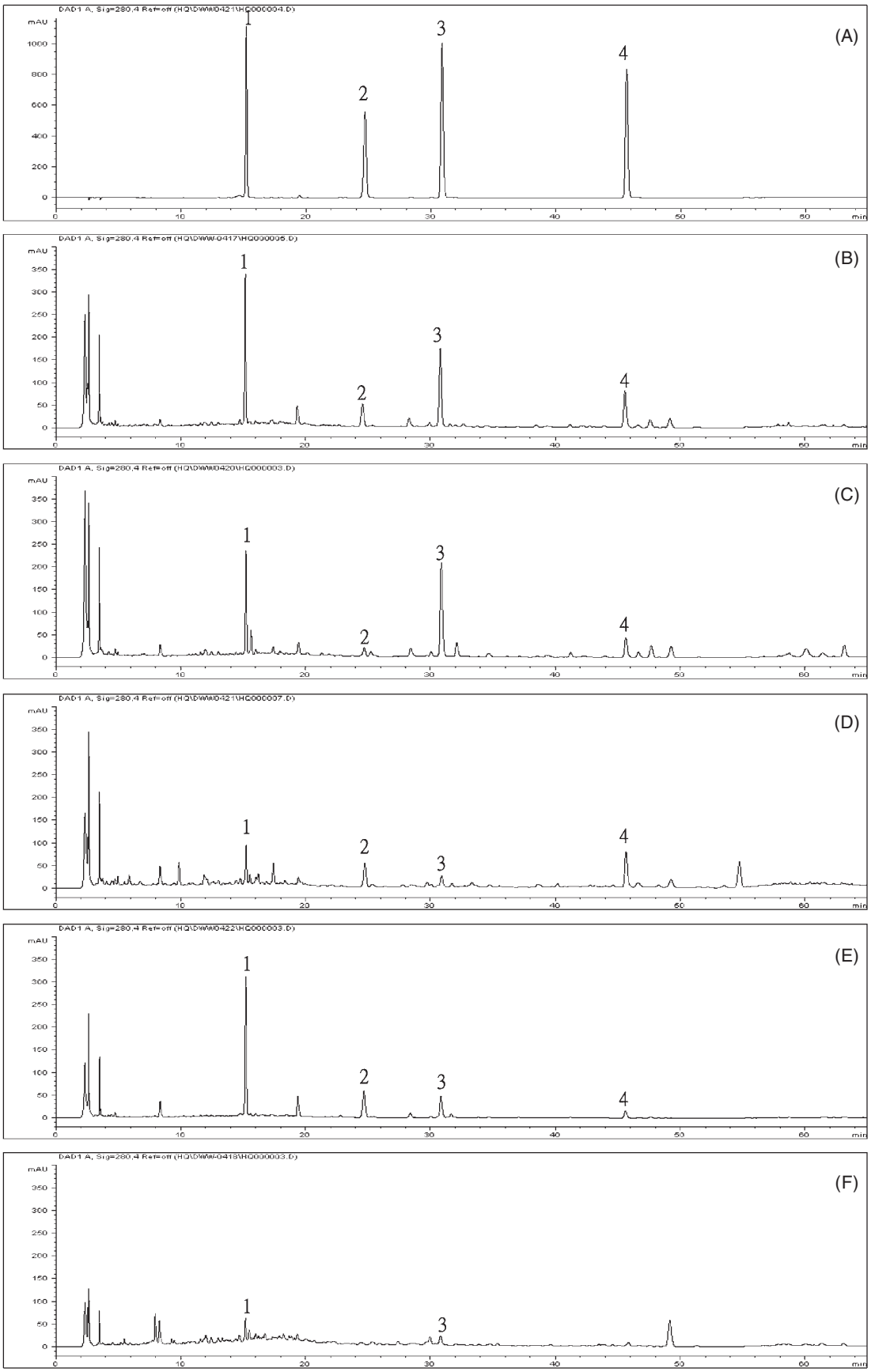

Figure 2. HPLC-DAD chromatograms of samples from different species. (A) reference compounds: 1: calycosin-7-*O*-D-glucopyranoside; 2: ononin; 3: calycosin; 4: formononetin; (B): *A. membranaceus* var. *mongholicus*; (C): *A. membranaceus*; (D) *H. polybotrys*; (E) *A. chrysopterus*; (F) *A. ernestii.*

Table 2. Precision and repeatability of the four isoflavonoids

Analyte[a]	Precision, RSD (%)				Repeatability, RSD (%)	
	Intra-day (n = 6)		Inter-day (n = 5)		(n = 6)	
	RT[b]	PA[c]	RT	PA	RT	PA
1	0.42	0.82	0.41	1.54	0.54	3.92
2	0.53	0.82	0.51	0.79	0.18	3.69
3	0.53	0.90	0.51	1.54	0.16	3.92
4	0.48	0.86	0.47	0.96	0.11	3.71

[a] Analyte names: 1: calycosin-7-*O*-D-glucopyranoside; 2: ononin; 3: calycosin; 4: formononetin.
[b] RT is the retention time.
[c] PA is the peak area.

Table 3. Recovery

Analyte[a]	Original (μg)	Spiked (μg)	Found (μg)	Recovery[b] (%)	RSD (%); n =5
1	473.5	450.0	938.7	103.4	1.44
2	141.9	140.0	285.5	102.6	1.60
3	397.8	345.0	729.9	96.3	1.54
4	211.1	160.0	365.2	96.3	1.67

[a] Analyte names: 1: calycosin-7-*O*-D-glucopyranoside; 2: ononin; 3: calycosin; 4: formononetin.
[b] Recovery (%) = (Amount found – Original amount)/Amount spiked × 100%. The data were presented as the average of five independent experiments.

Table 4. Quantitative results of various Astragali Radix samples

No.	Original plant	Location	Mean concentration of compounds 1 - 4 (μg/g[a]) (n = 3)			
			1[b]	2	3	4
1	*A. membranaceus* var. *mongholicus*	Gansu Province	315.62	91.17	265.2	140.78
2	*A. membranaceus* var. *mongholicus*	Shanxi Province	161.31	28.77	260.18	119.27
3	*A. membranaceus* var. *mongholicus*	Gansu Province	478.80	149.24	229.88	195.24
4	*A. membranaceus* var. *mongholicus*	Inner Mongolia	153.37	45.98	13.08	tr[c]
5	*A. membranaceus* var. *mongholicus*	Gansu Province	445.04	133.61	128.65	93.74
6	*A. membranaceus* var. *mongholicus*	Shanxi Province	310.78	114.80	55.50	25.81
7	*A. membranaceus* var. *mongholicus*	Gansu Province	145.54	34.82	122.11	75.99
8	*A. membranaceus*	Shanxi Province	292.92	47.76	319.80	78.83
9	*H. polybotrys*	Gansu Province	tr	166.39	nd[d]	116.96
10	*H. polybotrys*	Gansu Province	tr	61.92	tr	261.57
11	*H. polybotrys*	Gansu Province	119.47	133.45	30.10	142.96
12	*A. chrysopterus*	Qinghai Province	447.99	211.23	90.73	76.20
13	*A. chrysopterus*	Qinghai Province	773.17	187.70	37.31	21.62
14	*A. chrysopterus*	Qinghai Province	421.60	151.75	72.12	27.26
15	*A. ernestii*	Sichuan Province	nd	nd	49.79	nd
16	*A. ernestii*	Sichuan Province	29.89	nd	9.92	nd
17	*A. ernestii*	Sichuan Province	66.42	nd	27.14	nd

[a] Refers to the dry weight of Astragali Radix.
[b] Analyte names: 1: calycosin-7-*O*-D-glucopyranoside; 2: ononin; 3: calycosin; 4: formononetin.
[c] Trace.
[d] Not detected.

observed that the amounts of the four investigated compounds were obviously different in the Astragali Radix samples. The amounts of calycosin-7-*O*-D-glucopyranoside and calycosin were much lower in the species of *H. polybotrys* and *A. ernestii* than in *A. membranaceus*, *A. membranaceus* var. *mongolicus* and *A. chrysopterus*, while ononin and formononetin were not detected in *A. ernestii*. Quantification of the four compounds could differentiate *H. polybotrys* and *A. ernestii* from other species. However, the amounts of these four compounds in *A. membranaceus*, *A. membranaceus* var. *mongolicus* and *A. chrysopterus* were very similar.

V. *Classification of Astragali Radix Samples Using PCA*

PCA is one of the key tools in multivariate statistical analysis, which is a sophisticated technique widely used for reducing the dimensions of multivariate problems. Applications involving PCA have proliferated to analyze the spectral data obtained by HPLC. The differentiations based on different species are clear in the score plot using first, second and third factors, as shown in Figure 3. For the different species, the scores were divided moderately into four groups. *A. membranaceus* and *A. membranaceus* var. *mongolicus* belonged to area A, while *H. polybotrys, A. chrysopterus* and *A. ernestii* belonged to areas B, C and D, respectively.

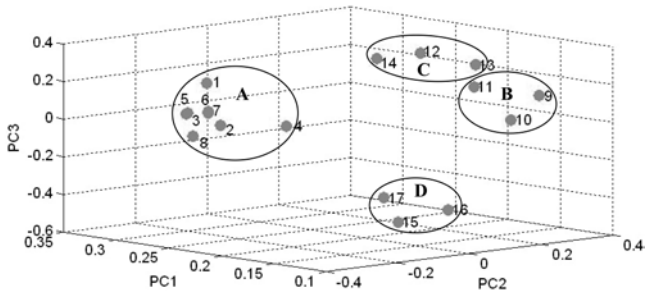

Figure 3. Score plots of the first, second and third principal components for all samples. Samples numbers are the same as in the Table 4.

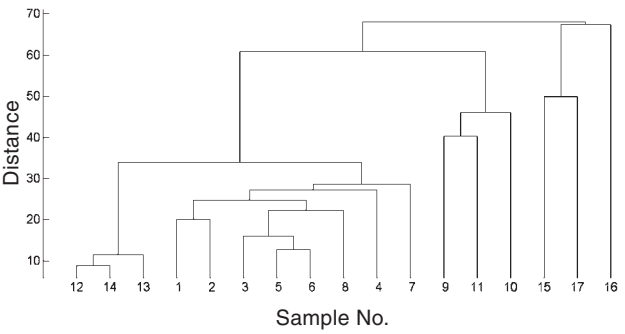

Figure 4. Dendrogram results of the 17 samples tested using hierarchical cluster analysis. Samples numbers are the same as in the Table 4.

VI. *Classification of Astragali Radix Samples Using HCA*

The integrated contents of constituents of 17 samples were classified by HCA. Firstly, the euclidean distances between pairs of the samples were computed with the observations in rows and the variables in columns. Then, single linkage algorithm, namely the widely-used nearest neighbor calculating the smallest distance between objects in the two clusters, was employed to create a hierarchical cluster tree. The final dendrogram plot of the hierarchical was generated by the binary cluster tree to visually represent the correlation among the samples. Figure 4 showed that 17 samples were divided into four main clusters, *A. membranaceus* and *A. membranaceus* var. *mongolicus*, *H. polybotrys*, *A. chrysopterus* and *A. ernestii*, respectively. Based on the data obtained, it was easy to distinguish Astragali Radix from its adulterants.

CONCLUSIONS

Astragali Radix is a well-known and commonly used Chinese herb. However, its adulterants, *A. chrysopterus, H. polybotrys* and *A. ernestii* are also found in the local markets. In this study, we found that the contents of four active isoflavonoids compounds (calycosin-7-*O*-D-glucopyranoside, ononin, calycosin and formononetin) in *A. membranaceus*, *A. membranaceus* var. *mongolicus* and *A. chrysopterus* were very similar, while *H. polybotrys* showed low or undetectable contents of calycosin-7-*O*-D-glucopyranoside and calycosin, and *A. ernestii* showed low or undetectable contents of all four marker compounds. When using chromatographic fingerprints of HPLC-DAD profiles together with HCA and PCA, Astragali Radix and other three adulterants could be successfully differentiated. However, the two official species of Astragali Radix, *A. membranaceus* var. *mongolicus* and *A. membranaceus* could not be differentiated from each other. This is because *A. membranaceus* var. *mongolicus* is a variation of *A. membranaceus* and their close genetic relationship results in similar chemical components, according to chemotaxonomy. The results suggest that HPLC coupled with chemometric treatment is a powerful and practical tool for distinguishing Astragali Radix from its adulterants.

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