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Study of the Relationship between Genetics and Geography in Determining the Quality of Astragali Radix

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Astragali Radix (AR), prepared from the roots of *Astragalus membranaceus* (FISCH. *ex* LINK) BUNGE or its variey, *A. membranaceus* (FISCH. *ex* LINK) BUNGE var. *mongholicus* (BUNGE) HSIAO., is one of the most used and valuable traditional Chinese medicines (TCMs). Historically, Hunyuan, Shanxi Province in China is the geo-authentic producing area of AR and crude AR from here called "geo-authentic." According to tradition, geo-authentic TCMs define both authenticity and quality. However, no scientific investigation has ever determined whether the superior quality of Hunyuan AR is due to the genetic characteristics or to the local environment. In our study, seeds of 30 AR samples representing the two varieties from different regions were cultivated in Hunyuan under the same conditions. A method, using ultra-performance liquid chromatography coupled with photodiode array detector and evaporative light scattering detectors, was developed to evaluate the quality through a simultaneous determination of four major isoflavonoids and four major saponins. The two AR varieties were successfully distinguished by principal component analysis while samples of the same species with different seeds origins could not be distinguished. A genetic study demonstrated that the internal transcribed spacer sequences of the nuclear ribosomal DNA in *A. membranaceus* var. *mongholicus* samples from different geographical regions were highly conservative. These results indicate that the content of active components in AR depends on the interaction of genotype and environment. At the varietal level, genetic properties appear to be more important for pharmaceutical quality than environmental factors, while on the intraspecific level environmental factors might be more important than genetic properties.

Key words Astragali Radix; geo-authentic; principal component analysis; ultra performance liquid chromatography; internal transcribed spacer sequence

Astragali Radix (AR), known as Huangqi, is one of the oldest and still most commonly used traditional Chinese medicines (TCMs). In the China Pharmacopoeia, AR is listed as being prepared from the dried roots of two types of Astragalus: the species, *Astragalus membranaceus* (FISCH. *ex* LINK) BUNGE, and its variey, *A. membranaceus* (FISCH. *ex* LINK) BUNGE var. *mongholicus* (BUNGE) HSIAO. In terms of TCM, AR is used to treat deficiency of "Qi" (the vital energy). In Western medical terms, it is widely used as an immunostimulating, cadiotonic, hepatoprotective, antidiabetic, and antiviral drug.[1—3)] Pharmacological studies have demonstrated it to have cardioprotective, hepatoprotective, hypotensive, immunostimulant, anti-ageing, anti-oxidant, anti-diabetic, and anti-inflammatory activities.[4—7)] Studies in recent years have determined that the constituents most often associated with the activities of AR are flavonoids, saponins, polysaccharides, amino acids and various trace elements.[8,9)]

AR is mostly obtained from cultivated plants, as wild ones are increasingly scarce.[10)] Nowadays, most of the AR sold commercially in the herbal markets is from cultivated *A. membranaceus* var. *mongholicus*.[11)] *A. membranaceus* is cultivated in the northeastern province of Heilongjiang and the southwestern province of Sichuan of China while *A. membranaceus* var. *mongholicus* is cultivated mainly in the northern provinces (Shanxi, Neimenggu, Hebei and Gansu) in China. Historically, Hunyuan, Shanxi Province in China is the geo-authentic producing area of AR. AR cultivated in Hunyuan is recognized to be geo-authentic AR. For each traditional Chinese medicine (TCM), there is a specific region where the best quality of that TCM is reputed to originate. The English translation of the Chinese term for this region is the herb's "geo-authentic production area." According to tradition, geo-authentic TCMs define both authenticity and quality for that herb. The geo-authentic herbs are believed to present the highest quality in all samples from different regions; this means that they are the most effective in pharmaceutical use, and should mean that they contain the highest contents of active constituents. In the case of AR, scientific data has confirmed that AR from Hunyuan, Shanxi Province does have the highest total concentration of active isoflavonoids and saponins when compared with drugs from other regions.[12,13)]

Phytochemical studies have shown that the main active components in medicinal plants are secondary metabolites which are produced during the growth and development of plants as they adapt to the environment.[14)] These components are usually used as markers for identification and quality evaluation of TCMs. The content of the active components also belongs to one of the phenotypes of geo-authentic TCMs, depending on the interaction of genotype and environment. We would like to know whether the potency of geo-authentic TCMs is due to the genetic characteristics of the seeds or to geography (*i.e.*, the specific growing conditions of a particular location). Therefore, in this study, we examined the relationships between pharmaceutical quality and genetics in the varieties of AR using molecular genetics and qualitative analysis of samples.

We collected 30 samples of AR seeds, representing both varieties, from different AR main cultivation regions and planted them in the same area in Hunyuan, Shanxi Province, which is, by tradition, the geo-authentic production area for this herb. An ultra-performance liquid chromatography cou-

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pled with photodiode array detector (PDA) and evaporative light scattering detectors (UPLC-UV-ELSD) method was developed for the quality assessment of AR through simultaneous determination of four major active isoflavonoids and four major saponins in a single run. Previous studies used principal component analysis (PCA), a method of multivariate data analysis, to successfully distinguish AR samples from different cultivated regions based on the contents of constituents or elemental composition data.[15,16] We used this method in order to evaluate the effect of environment on genetic expression. The major active isoflavonoids and saponins of the 30 samples were determined by UPLC-UV-ELSD and analysed by PCA.

We also studied the genetic properties of the samples by sequencing the intraspecific variations on the internal transcribed spacer (ITS) sequences of AR from different geographical regions. The ribosomal DNA (rDNA) ITS sequences including ITS-1, 5.8S rDNA and ITS-2 of *A. membranaceus* var. *mongholicus* from 23 geographical regions were analyzed and a phylogenetic tree based on ITS and 5.8S rDNA sequences data showed intraspecific variation at the molecular level. The molecular results give the genetic characteristics of *A. membranaceus* var. *mongholicus*.

These results provide useful information on the relative importance of environmental factors and the genetic nature to quality in the cultivation of AR. The method can be widely applied in assessing not only TCMs, but other herbs of importance to the pharmaceutical industry.

MATERIALS AND METHODS

Samples, Chemicals and Reagents Eighteen samples of seeds numbered A1—A18 of *A. membranaceus* var. *mongholicus* and 12 samples numbered B1—B12 of *A. membranaceus* were collected or purchased from different farms or main herbal markets in Shanxi, Gansu, Neimenggu, Ningxia, Jilin, Heilongjiang, Shandong, Shaanxi and Hebei provinces in China. All seeds were planted and cultivated in the same area in Hunyuan, Shanxi Province in June, 2006. For the repeatability of our experiments, seeds of each sample had been planted into three groups, and every group had two or three individual plants. Their roots were collected and dried at the same time in October, 2008. The dried roots were identified by Dr. Hu-Biao Chen, School of Chinese Medicine, Hong Kong Baptist University. The voucher specimens are deposited at the Herbarium, School of Chinese Medicine, Hong Kong Baptist University. The details are summarized in Table 1.

Fresh leaves of *A. membranaceus* var. *mongholicus* were collected form different regions in China including Shanxi, Neimenggu, Gansu and Qinghai. All fresh materials were put in silica gel immediately after collection. The plant materials were identified by Dr. Hu-Biao Chen, School of Chinese Medicine, Hong Kong Baptist University. The voucher specimens are deposited at the Herbarium, School of Chinese Medicine, Hong Kong Baptist University. The details are summarized in Table 2.

The authentic reference compounds of formononetin (>98.0%), astragaloside IV (>98%) were purchased from Jiangxi Herbfine Pharmaceutical Co., Ltd. (Jiangsu, China). Calycosin (>98%) was purchased from Phytomarker Ltd. (Tianjin, China). Calycosin-7-*O*-β-D-glucopyranoside (>98%) and ononin (>98%) were purchased from Hong Kong Jockey Club Institute of Chinese Medicine Ltd. (Hong Kong, China). Other reference compounds, namely, astragaloside I (>98%), astragaloside II (>98%) and astragaloside III (>98%), were purchased from Shanghai Tauto Biotech

Table 1. Details of Astragali Radix Analyzed

Sample No.	*Astragalus* spp.	Sources of seeds
A1	*A. membranaceus* var. *mongholicus*	Tianzhen, Shanxi
A2		Datong, Shanxi
A3		Hunyuan, Shanxi
A4		Hunyuan, Shanxi
A5		Hunyuan, Shanxi
A6		Yingxian, Shanxi
A7		Yingxian, Shanxi
A8		Xunyi, Shaanxi
A9		Longxi, Gansu
A10		Weiyuan, Gansu
A11		Weiyuan, Gansu
A12		Minxian, Gansu
A13		Longxi, Gansu
A14		Guyang, Neimenggu
A15		Guyang, Neimenggu
A16		Elunchunqi, Neimenggu
A17		Wuchuan, Neimenggu
A18		Yinchuan, Ningxia
B1	*A. membranaceus*	Linjiang, Jilin
B2		Changchun, Jilin
B3		Jiagedaqi, Heilongjiang
B4		Juancheng, Shandong
B5		Weifang, Shandong
B6		Wendeng, Shandong
B7		Wendeng, Shandong
B8		Elunchunqi, Neimenggu
B9		Xunyi, Shaanxi
B10		Xunyi, Shaanxi
B11		Xunyi, Shaanxi
B12		Anguo, Hebei

Table 2. Origins of the 23 Fresh Samples

Code No.	Locality[a)]	Source	Voucher No.
2	Fansi, Shanxi	Wild	050726-1
3	Fansi, Shanxi	Cultivated	050726-2
4	Yingxian, Shanxi	Cultivated	050726-3
5	Yingxian, Shanxi	Cultivated	050726-4
9	Yingxian, Shanxi	Cultivated	050727-1
10	Yingxian, Shanxi	Cultivated	050727-2
11	Yingxian, Shanxi	Cultivated	050727-3
16	Yunyuan, Shanxi	Cultivated	050728-1
20	Keshenketengqi, Neimenggu	Wild	050807-1
23	Wuchuan, Neimenggu	Cultivated	050810-1
26	Wuchuan, Neimenggu	Cultivated	050810-2
28	Guyang, Neimenggu	Cultivated	050810-3
30	Guyang, Neimenggu	Cultivated	050810-4
32	Hangjinqi, Neimenggu	Wild	050811-1
37	Dingxi, Gansu	Cultivated	050816-1
39	Longxi, Gansu	Cultivated	050817-1
43	Longxi, Gansu	Cultivated	050817-2
45	Longxi, Gansu	Cultivated	050817-5
51	Zhangxian, Gansu	Cultivated	050817-6
56	Dangchang, Gansu	Cultivated	050818-2
60	Minxian, Gansu	Cultivated	050819-2
75	Datong, Qinghai	Cultivated	050822-1
78	Ping'an, Qinghai	Cultivated	050823-1

a) All are cities in provinces in Mainland China.

Co., Ltd. (Shanghai, China).

Methanol and acetonitrile of HPLC grade were obtained from Lab-scan (Bangkok, Thailand). Formic acid was purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q water system (Millipore, MA, U.S.A.).

Apparatus and Conditions Chromatographic separation was performed using a Waters ACQUITY^{TM} Ultra Performance Liquid Chromatograph system (Waters Corp., Milford, MA, U.S.A.), equipped with a binary solvent delivery system, an auto-sampler, a PDA with an ELS Detector. An Acquity UPLC BEH C_{18} column (1.7 μm, 2.1×100 mm) together with an Acquity UPLC BEH C_{18} column guard column (1.7 μm, 2.1×5 mm) (Waters Corp., Milford, MA, U.S.A.) were used to separate the components. The UV detection wavelengths were set at 280 nm. The drift tube temperature for ELSD was set at 99 °C and gas (N_2) pressure was 500 psi.

The column temperature was kept constant at 40 °C. The mobile phase consisted of 0.1% formic acid water (A) and 0.1% formic acid acetonitrile (B) using a gradient elution of 7—50% B at 0—30 min, and the re-equilibration time of gradient elution was 2 min. The mobile phase flow rate was 0.3 ml/min.

Sample Preparation The dried roots were powdered by a mill and the powders were screened through 380 μm sieves. Each sample of fine powder (1 g) was accurately weighed and extracted twice with methanol–H_2O (25 ml, 7 : 3) by ultrasonication at room temperature for 30 min. After centrifugation, the combined solution was evaporated *in vacuo* to dryness. The residue was dissolved in 5 ml of methanol–H_2O (7 : 3) using a volumetric flask and filtered through a syringe filter (0.2 μm, Alltech, Beerfield, IL, U.S.A.). An aliquot of 2 μl of the filtrate was injected into UPLC for analysis.

Data Analysis The data were statistically analyzed using SPSS for Window, Version 11.5.0 (SPSS, Inc., Chicago, IL, U.S.A.), and SIMCA-P 11.5 (Umetrics, Umea, U.S.A.). The difference in isoflavonoid and saponin contents between two species was evaluated using one-way analysis of variance (ANOVA). Any p value less than 0.05 was considered statistically significant. Quantitative analysis data were analyzed using principle component analysis (PCA) by SIMCA-P software.

Genomic DNA Extraction and Polymerase Chain Reaction (PCR) Amplification Total DNA was extracted from 20—50 mg dried leaves using a DNeasy Plant Mini Kit (QIAGEN, Germany). DNA quality and quantity were determined by electrophoresis on 1.0% agarose gel stained with ethidium bromide.

For amplification of the ITS1-5.8S-ITS2 rDNA region, ITS-1 forward primer (5′-AGAAGTCGTAACAAGGTTTC-CGTAGG-3′) and ITS-2 reverse primer (5′-TCCTCCGCT-TATTGATATGC-3′) were used as forward and reverse primers, respectively.[17]

PCR was performed in a 30 μl reaction mixture, containing 0.01 mM of each primer (Invitrogen, U.S.A.), 2.5 mM dNTPs, 23 mM $MgCl_2$, 10×PCR buffer, 1 U *Taq* DNA polymerase (Roche Biochemicals, Indianapolis, IN, U.S.A.) and 1.0 μl of template DNA. Amplification was carried out under the following conditions: pre-cycling at 95 °C for 4 min; 30 cycles of denaturation at 95 °C for 45 s, annealing at 54 °C for 45 s and elongation at 70 °C for 60 s; a final elongation at 72 °C for 7 min. The PCR products were purified using Quantum Prep PCR Kleen Spin Columns (BIO-DAD, U.S.A.). The 1/10 volume of resulting PCR products was detected by 1.0% agarose gel electrophoresis.

ITS Sequencing Analysis Sequences of PCR products were detected by an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). After comparison with the GenBank database, the obtained sequence data were edited and aligned by the Clustal X program (Version 1.83, Conway Institute UCD Dublin, Ireland), and adjusted manually when necessary.

RESULTS AND DISCUSSION

Chemical Profile and Quantitative Analysis Extracts of AR Ultra-performance liquid chromatography (UPLC) is a powerful tool for phytochemical analysis since it can quickly, efficiently separate compounds and provide online UV.[18] UPLC using 1.7 μm particles and a properly holistically designed system provide significantly higher resolution while reducing run times and improving sensitivity for the analysis of many compound types.[19] Representative UPLC-UV-ELSD chromatograms of the eight reference compounds and the extracts of one sample from Hunyuan, Shanxi Province under the detection conditions are shown in Fig. 1. Four major isoflavonoids namely, calycosin-7-*O*-β-D-glucopyranoside (**1**), ononin (**2**), calycosin (**3**), formononetin (**4**), and four major saponins namely, astragaloside I (**5**), astragaloside II (**6**), astragaloside III (**7**), astragaloside IV (**8**) were identified by comparing their retention time and UV spectra with those of each reference compound which was eluted in parallel with a series of mobile phases. In addition, spiking samples with the reference compounds further confirmed the identities of the peaks. Chemical structures of these compounds are shown in Fig. 2.

Calibration Curves, Limits of Detection and Quantification, Reliability and Recovery Stock solution of a mixture of the eight reference standards was prepared by dissolving accurately weighed portions of the standards in methanol, transferring to a 5 ml volumetric flask, and then adding methanol to make up to the volume. The concentration of each compound was as follows: 0.602 mg/ml (**1**), 0.604 mg/ml (**2**), 0.600 mg/ml (**3**), 0.306 mg/ml (**4**), 1.086 mg/ml (**5**), 0.580 mg/ml (**6**), 0.206 mg/ml (**7**) and 0.202 mg/ml (**8**). The stock solution was further diluted with methanol to make serial concentrations for the calibration curves. The solutions were brought to room temperature and filtered through a 0.2 μm membrane filter and an aliquot of 2 μl was injected into UPLC for analysis. The regression equations for compounds **1**, **2**, **3** and **4** by UV detection were calculated in the form of $y=bx+a$, while the regression equations for the four saponins (compounds **5**—**8**) were determined by ELSD and described as: $\ln y=b \ln x+a$, where y and x were peak area and amount of compound injected, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were determined at a signal-to-noise (S/N) ratio of 3 and 10, respectively. The results are shown in Table 3.

The intra-day and inter-day variations were evaluated by determining the contents of eight compounds in three different working solutions prepared from the same sample during a single day; the analysis was repeated on three different

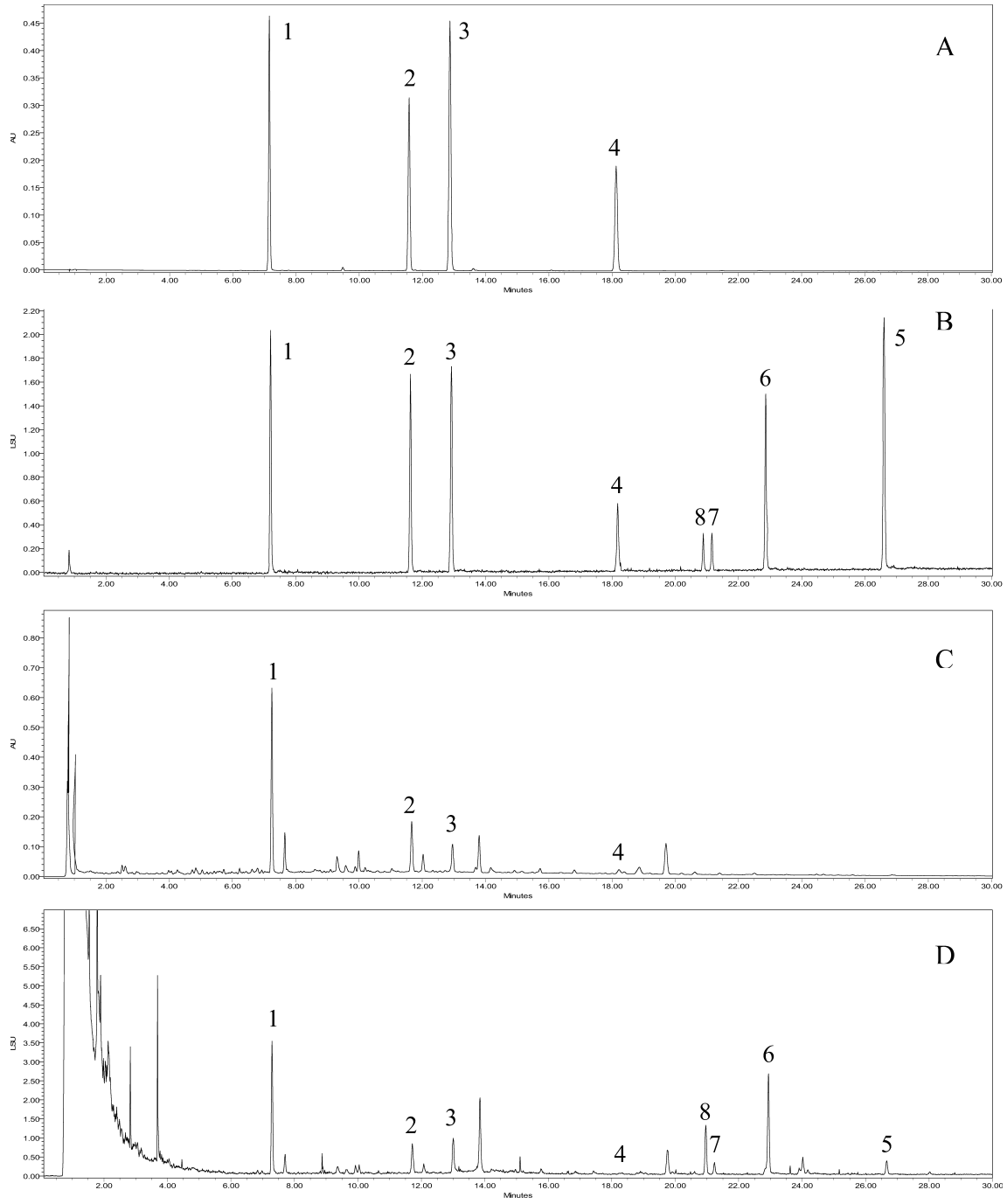


Fig. 1. Typical UPLC-UV-ESLD Chromatograms of the Eight Reference Standards and Extracts of a Sample from Hunyuan, Shanxi Province under the Detection Conditions

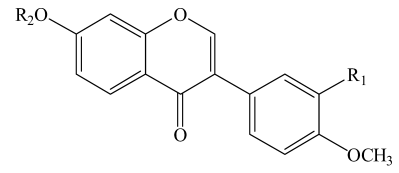
(A) and (B) represent UV chromatograms monitored at 280 nm and ELSD chromatograms of eight reference compounds. (C) and (D) present UV chromatograms monitored at 280 nm and ELSD chromatograms of extracts of the sample. Peaks were identified as: (**1**) calycosin-7-*O*-*β*-D-glucopyranoside, (**2**) ononin, (**3**) calycosin, (**4**) formononetin, (**5**) astragaloside I, (**6**) astragaloside II, (**7**) astragaloside III and (**8**) astragaloside IV.

days. As shown in Table 4, the RSD values for method repeatability were found to be within the range 0.22—4.67% for intra-day assays and 0.42—2.79% for inter-day assays.

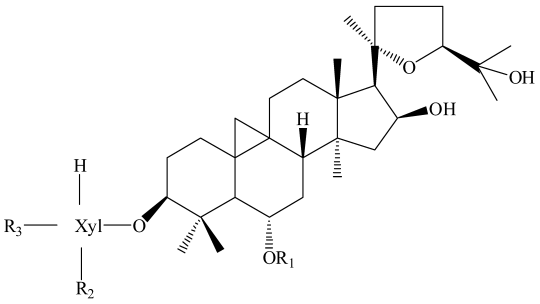
The recovery test was used to evaluate the accuracy of this method. Accurate amounts of four isoflavonoids and four saponins were added to 1 g of one sample from Hunyuan, Shanxi Province in triplicate; then the spiked samples were extracted and analyzed as described in sample preparation section. The results are given in Table 5. The percent of average recoveries ranged from 96.45—103.85%. The RSD values for the recovery test were found to be within the range 0.490—2.411%.

Therefore, we concluded that the UPLC-DAD-ELSD method is accurate, precise and sensitive enough for simultaneous quantitative evaluation of the four major active isoflavonoids and four major saponins in AR.

Content Analysis of Isoflavonoids and Saponins and Quality Assessment The newly established UPLC-UV-ELSD assay method was subsequently applied to a simultaneous determination of main isoflavonoids and saponins and



Peak No.	Compound	R_1	R_2
1	calycosin-7-*O*-β-D-glucopyranoside	OH	Glc
2	ononin	H	Glc
3	calycosin	OH	H
4	formononetin	H	H



Peak No.	Compound	R_1	R_2	R_3
5	astragaloside I	Glc	Ac	Ac
6	astragaloside II	Glc	Ac	H
7	astragaloside III	H	Glc	H
8	astragaloside IV	Glc	H	H

Fig. 2. Chemical Structures of Four Isoflavonoids and Four Saponins from Astragali Radix

Table 3. Calibration Curves, Test Range, LODs, and LOQs for Four Isoflavonoids and Four Saponins in UPLC-DAD-ELSD

Analyte	Calibration curve	r^2	Linear range (μg/ml)	LOD (ng)	LOQ (ng)
1	$y=10861x+66778$	0.9996	0.50—602.00	0.19	0.62
2	$y=10781x+84078$	0.9995	0.50—604.00	0.16	0.59
3	$y=14500x+125873$	0.9994	0.50—600.00	0.28	0.87
4	$y=16547x+17897$	0.9995	1.28—183.60	0.34	0.98
5	$y=1.5022x+1.1255$	0.9994	27.15—1086.00	18.1	59
6	$y=1.7128x+0.1833$	0.9995	29.00—580.00	9.7	28
7	$y=1.2675x+1.9865$	0.9997	10.30—206.00	10.3	31
8	$y=1.4667x+1.4673$	0.9994	10.10—202.00	10.1	29

Table 4. Determination of the Reliability of the Described Method for 8 Maker Compounds

Analyte	Assay on day 1		Assay on day 3		Assay on day 5		Inter-day RSD (%)
	Mean (mg/g)	RSD (%)	Mean (mg/g)	RSD (%)	Mean (mg/g)	RSD (%)	
1	0.251	1.89	0.250	0.81	0.248	0.33	0.59
2	0.101	3.16	0.100	2.69	0.101	1.04	0.65
3	0.038	2.14	0.037	2.66	0.038	1.36	1.14
4	0.021	1.42	0.021	1.31	0.022	0.22	2.79
5	0.650	1.97	0.645	1.83	0.645	0.78	0.42
6	0.278	1.15	0.268	2.26	0.265	3.80	2.49
7	0.170	4.67	0.168	3.88	0.164	3.72	1.81
8	0.426	0.44	0.424	0.70	0.420	1.47	0.65

Table 5. Recoveries of the 8 Marker Compounds

Analyte	Original mean (mg)	Spiked mean (mg)	Found mean (mg)	Recovery (%)	RSD (%) ($n=3$)
1	0.183	0.242	0.416	96.45	0.490
2	0.053	0.104	0.156	98.96	2.041
3	0.030	0.037	0.066	97.07	2.170
4	0.017	0.016	0.032	99.33	1.001
5	0.386	0.522	0.899	98.28	2.411
6	0.203	0.260	0.468	101.61	0.856
7	0.173	0.150	0.329	103.85	2.030
8	0.390	0.134	0.520	96.66	1.629

Table 6. Contents of Isoflavonoids and Saponins in Various Astragali Radix Samples (n=3)

Sources of seeds	Sample No.	Content (mg/g)									
		Four major isoflavonoids					Four main saponins				
		1	**2**	**3**	**4**	TIF[a)]	**5**	**6**	**7**	**8**	TAS[b)]
Shanxi	A1	0.723	0.177	0.066	0.010	0.976	1.152	0.433	0.076	0.076	1.736
	A2	0.873	0.242	0.099	0.021	1.235	0.535	0.221	tr[c)]	0.048	0.804
	A3	0.677	0.189	0.046	0.011	0.924	1.459	0.465	0.072	0.092	2.088
	A4	0.886	0.262	0.087	0.023	1.258	1.066	0.279	0.083	tr	1.428
	A5	1.119	0.314	0.109	0.016	1.559	1.316	0.398	0.097	0.079	1.890
	A6	1.079	0.264	0.120	0.016	1.479	0.974	0.311	tr	0.078	1.363
	A7	0.754	0.170	0.070	0.009	1.004	1.220	0.346	0.061	0.072	1.699
Shaanxi	A8	1.133	0.250	0.135	0.016	1.533	0.521	0.300	tr	0.052	0.872
Gansu	A9	1.032	0.238	0.105	0.022	1.397	1.154	0.261	tr	0.071	1.487
	A10	1.524	0.348	0.153	0.024	2.049	0.932	0.390	0.066	0.068	1.456
	A11	0.660	0.182	0.038	0.012	0.891	0.596	0.313	tr	0.088	0.997
	A12	0.859	0.244	0.091	0.029	1.222	0.828	0.321	tr	0.079	1.227
	A13	1.146	0.286	0.127	0.021	1.581	0.892	0.243	tr	tr	1.135
Neimenggu	A14	0.645	0.160	0.034	0.009	0.847	0.934	0.530	0.057	0.089	1.610
	A15	0.574	0.140	0.048	0.008	0.770	1.034	0.419	tr	0.076	1.530
	A16	0.683	0.153	0.036	0.006	0.879	0.839	0.343	tr	0.063	1.245
	A17	0.680	0.174	0.047	0.018	0.919	0.737	0.216	tr	tr	0.954
Ningxia	A18	0.840	0.180	0.065	0.010	1.095	1.131	0.454	0.083	0.066	1.733
Jilin	B1	0.583	0.119	0.136	0.006	0.844	0.351	0.208	0.096	0.065	0.720
	B2	1.021	0.177	0.089	0.006	1.293	0.533	0.354	0.064	0.092	1.042
Heilongjiang	B3	0.768	0.123	0.118	0.006	1.015	0.589	0.341	0.098	0.080	1.107
Shandong	B4	0.748	0.123	0.120	0.006	0.997	0.824	0.463	0.147	0.147	1.581
	B5	1.006	0.245	0.122	0.016	1.390	0.542	0.482	0.202	0.181	1.408
	B6	0.175	0.067	0.009	0.010	0.261	0.704	0.442	0.215	0.106	1.466
	B7	0.251	0.100	0.037	0.022	0.410	0.650	0.257	0.159	0.426	1.492
Neimenggu	B8	0.623	0.082	0.071	0.009	0.784	0.540	0.516	0.222	0.227	1.504
Shaanxi	B9	0.664	0.097	0.068	0.004	0.833	0.456	0.341	0.053	0.072	0.921
	B10	0.667	0.170	0.067	0.009	0.914	0.557	0.368	0.198	0.111	1.234
	B11	0.535	0.093	0.117	0.003	0.749	0.401	0.232	0.086	0.076	0.794
Hebei	B12	0.498	0.090	0.047	0.006	0.641	0.429	0.256	0.125	0.131	0.942

a) TIF represents content of total isoflavonoids determined in the samples. *b*) TAS represents content of total saponins determined in the samples. *c*) Trace (less than LOQ).

quality evaluation of AR. The quantitative analytical results are summarized in Table 6.

According to the latest edition of the "Pharmacopoeia of the People's Republic of China" (2010), *A. membranaceus* var. *mongholicus* and *A. membranaceus* are the two varieties that can be used as the plant origin of AR. These are the crude drugs of AR sold in the herbal markets. In this study, all seeds of samples were identified and collected from local farms or herbal markets. They were planted in the same area, cultivated under the same conditions and collected at the same time. The data presented in Table 6 shows that, in the samples of *A. membranaceus* var. *mongholicus*, the total isoflavonoid content ranged from 0.770 to 2.049 mg/g with an average of 1.201±0.342 mg/g and the total saponin content ranged from 0.804 to 2.088 mg/g with an average of 1.403±0.361 mg/g. In the samples of *A. membranaceus*, the total isoflavonoid content ranged from 0.261 to 1.390 mg/g with an average of 0.8441±0.322 mg/g and the total saponin content ranged from 0.720 to 1.581 mg/g with an average of 1.184±0.303 mg/g. In all samples, calycosin-7-*O*-β-D-glucopyranoside (**1**) and astragaloside I (**5**) were the most abundant of the isoflavonoids and saponins, respectively. Statistically, the contents of calycosin-7-*O*-β-D-glucopyranoside (**1**), ononin (**2**), formononetin (**4**), astragaloside I (**5**), total isoflavonoids and total saponins found in *A. membranaceus* var. *mongholicus* samples were significantly higher than those found in *A. membranaceus* samples. However, the contents of astragaloside III (**7**) and astragaloside IV (**8**) found in *A. membranaceus* var. *mongholicus* samples were significantly lower than those found in *A. membranaceus* samples, the difference reaching 2—4 folds. Moreover, astragaloside III (**7**) was too low to quantify in ten *A. membranaceus* var. *mongholicus* samples. Because the plants were cultivated in the same environment, the differences observed must be due to the genetics.

The concentration of each quantified compound was considerably different in individual samples. In *A. membranaceus* var. *mongholicus* samples, the highest amount (2.049 mg/g) of total isoflavonoids was found in the sample A10 from Weiyuan, Gansu Province while the highest amount (2.088 mg/g) of total saponins was found in the sample A5 from Hunyuan, Shanxi Province. On the other hand, in *A. membranaceus* samples, the highest amount (1.390 mg/g) of total isoflavonoids was found in the sample B5 from Weifang, Shandong Province while the highest amount (1.581 mg/g) of total saponins was found in the sample B4 from Juancheng, Shangdong Province. It is interesting to find that samples grown form seeds from Hunyuan, Shanxi Province did not have the highest contents of each isoflavonoid and each saponin. Statistical analysis shows that no significant differences were found for the eight qualified components when comparing different samples grown from

A. membranaceus seeds from the same geographic region source ($p<0.05$). In *A. membranaceus* var. *mongholicus* samples, only the contents of calycosin (**3**) showed significant differences between different samples grown from seeds from the same geographic region.

Principal Component Analysis PCA is a method of multivariate data analysis. It provides a way to identify the most important directions of variability in a multivariate data matrix and make a multivariate classification of the analyzed objects. This concept is the basis for chemometric methods such as soft modeling and multivariate calibration methods, among others.[20] PCA has been applied to quality assessment of plant extracts and natural products upon the data of HPLC chromatography.[21—24] PCA was performed. PCA is based on the derivation of linear combinations of the measured descriptors to produce new variables calls principal components (PCs) that are uncorrelated. PCs are obtained sequentially: the first PC (PC1) represents the largest portion of explainable variability in the measured data; the second PC (PC2) represents the next largest portion of explainable data variability, and so forth. Data plots using PCs as variables could reduce the dimensionality to visualize the data trends of the data matrix.[25]

To analyze the differences in main isoflavonoid and saponins contents difference among the AR samples, PCA was performed. Initially, data of eight measured constituents in all samples whose seeds belong to the two varieties were analyzed with PCA. A well-fitting PCA-X model ($R^2X=0.919$, $Q^2=0.845$) was constructed which clearly divided the 30 samples into two groups. The first two PCs account for 65.8% of the total variance (PC1, 48.1%; PC2, 17.7%). The obtained PCA scores plot is shown in Fig. 3, where each AR sample represents a marker. The scores plot clearly indicates that the patterns of *A. membranaceus* var. *mongholicus* and *A. membranaceus* are significantly different based on contents of main isoflavonoids and saponins, even though they were cultivated in the same environment. This result indicates that between the two AR varieties genetic properties are more important in determining the pharmaceutical quality of AR than geography and cultivation factors.

Data of eight measured constituents in *A. membranaceus* var. *mongholicus* and *A. membranaceus* samples were analyzed with PCA. For *A. membranaceus* var. *mongholicus*, 18 samples fall into 5 classes according to seed sources. A PCA-X model ($R^2X=0.997$, $Q^2=0.684$) was constructed. The first two PCs account for 86.2% of the total variance (PC1, 47.7%; PC2, 38.5%). For *A. membranaceus*, 12 samples fall into 6 classes according to seed sources. A PCA-X model ($R^2X=0.943$, $Q^2=0.212$) was constructed. The first two PCs account for 76.7% of the total variance (PC1, 45.2%; PC2, 31.5%). It is hard to distinguish these samples by PCA. The results show little difference in class between samples from different geographical origins. It has been reported that dividing the measured isoflavonoids and saponins into different groups based on their chemical structures would improved specificity of differentiation during PCA analysis.[15] Thus, we categorized our determined constituents into three groups: Group TF1, contained calycosin-7-*O*-β-D-glucopyranoside (**1**) and calycosin (**3**); Group TF2, contained ononin (**2**) and formononetin (**4**); and Group TAS including four saponins. PCA analysis was performed again using the contents of the categorized constituents in the *A. membranaceus* var. *mongholicus* and *A. membranaceus* samples. However, the results could not distinguish them. This did not improve the specificity of differentiation for our samples. These results further indicate that, for both *A. membranaceus* var. *mongholicus* and *A. membranaceus*, plants grown in the same place from seeds collected form different places were not significantly different in terms of bioactive components. But all samples of the two different varieties grown under the same conditions could be well differentiated from each other. These results indicate that the genetic properties of AR might be more important in determining the quality of AR than environmental factors such as weather, geographic location,

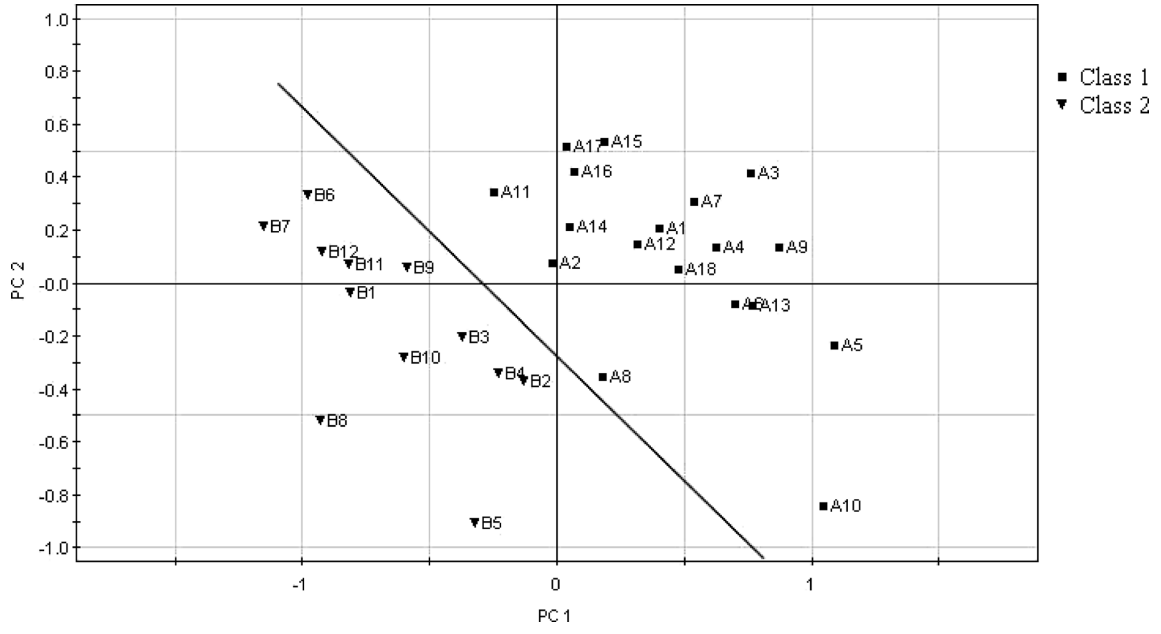


Fig. 3. PCA Scores Plot of *A. membranaceus* var. *mongholicus* and *A. membranaceus* Samples

Class 1: *A. membranaceus* var. *mongholicus*; class 2: *A. membranaceus*.

Table 7. Comparison of Variable Sites in ITS Sequences of *A. membranaceus* var. *mongholicus* from Different Regions

Code No.	91	92	95	453	474	489	552	572	GenBank No.
2	G	G	T	G	C	G	G	G	JF736665
3	·	·	·	·	·	·	·	·	JF736665
4	·	·	·	·	·	·	·	·	JF736665
5	·	·	·	·	·	·	·	·	JF736665
9	·	·	·	·	·	·	·	·	JF736665
10	·	·	·	·	·	·	·	·	JF736665
11	·	·	·	·	·	·	·	·	JF736665
16	·	·	·	·	·	·	·	·	JF736665
20	·	·	A	·	T	·	·	·	JF736666
23	·	·	·	·	·	·	·	·	JF736665
26	·	·	·	·	T	·	·	·	JF736667
28	·	·	·	·	·	·	·	·	JF736665
30	·	·	·	·	·	·	·	·	JF736665
32	·	·	·	·	·	·	·	·	JF736665
37	C	T	·	·	·	·	·	·	JF736668
39	·	·	·	·	·	·	·	·	JF736665
43	·	·	·	·	·	·	·	·	JF736665
45	·	·	·	·	·	·	C	A	JF736669
51	·	·	·	·	·	·	·	·	JF736665
56	·	·	·	·	·	·	·	·	JF736665
60	·	·	·	·	·	·	·	·	JF736665
75	·	·	·	·	·	·	·	·	JF736665
78	·	·	·	·	·	·	·	·	JF736665

"·" indicates the same base as the first row. Numbers indicate variable sites.

soil conditions, methods of cultivation and processing. According to some other studies, the AR cultivated in different regions could be successfully distinguished by PCA.[15] However, those studies examined only *A. membranaceus* var. *mongholicus*, and did not indicate whether the differences were due to environmental factors or genetic properties.

Comparison of ITS Sequence Molecular genetic methods for identifying *Astragalus* species related to AR have been developed; these include random amplified polymorphic DNA (RAPD),[26,27] 3′ untranslated region sequence-based amplified polymorphism (UAP),[28] 5S gene spacer, ITS and 18S rRNA analysis.[29,30] However, variation in the ITS sequences of *A. membranaceus* var. *mongholicus* among the different regions has rarely been reported.[29] At the same time, Chinese medicinal materials cultivated in different localities are believed to differ in the therapeutic potency. Theoretically, they could differ in biochemical composition due to environmental factors of cultivation, genetics and/or methods of collection and processing.[31] Scientific assessment is needed to confirm traditional belief.

The internal transcribed spacer (ITS) region of 18S—26S rDNA comprises three components: the 5.8S subunit, an evolutionarily highly conserved sequence, and two spacers designated as ITS-1 and ITS-2.[32]

In this study, complete sequences of the ITS region containing ITS-1, 5.8S, ITS-2 were determined. Twenty-three sequences obtained were loaded into Clustal X with sequences AF359749 and AF359750 from GenBank database for sequence alignment. Five-terminal of forward primer is the nucleotide position of No. 1 in ITS sequence. All amplified ITS sequences showed the same length of 603 bp, while 228 bp of ITS-1, 164 bp of 5.8S and 211 bp of ITS-2 had 7 variation sites. Nineteen samples, namely code Nos. 2, 3, 4, 5, 9, 10, 11, 16, 23, 28, 30, 32, 39, 43, 51, 56, 60, 75, and 78, appeared to have the same sequence (GenBank No. JF736665).

The detail of variable sites is shown in Table 7. The difference between the ITS sequences of 23 samples ranged from 0 to 0.0033%. There were 3 variable sites in the ITS-1 sequence and 4 variable sites in the ITS-2. The 5.8S rDNA sequences (229—392 bp) were of the same bases. The 5.8S rDNA as the spacer domain is highly conserved.

The genetic distance between each pair of samples ranged from 0 to 0.007 according to ITS sequences. The spacer domains among all samples were highly conserved. Thus these results show that the ITS sequences of samples of *A. membranaceus* var. *mongholicus* from different regions are highly conservative at an intraspecific level. It suggests that the highly conservative ITS sequence is not such an important characteristic of authenticity of AR. It may indicate that the differences in the quality of AR samples from different cultivated regions may be a result of environmental factors rather than a product of their DNA sequences.

CONCLUSION

In conclusion, our studies provide scientific information on the relationship between genetics and geography in determining the pharmaceutical quality of AR. The UPLC-UV-ELSD method we used was successful when applied through a simultaneous determination of four isoflavonoids and four saponins in a single run. Furthermore, we have established a reliable multivariate statistical approach for the quality evaluation of AR by using this method. The chemical contents of AR samples from different genetic and geographical origins were analyzed to determine the chemical profile of the authenticity of AR, as affected by both environmental factors and genetics. The method could efficiently differentiate AR samples from different varieties but could not differentiate AR samples of a single variety whose seeds came from different cultivated regions. Our study on molecular identification showed that the ITS sequences of samples of *A. membranaceus* var. *mongholicus* from different regions were highly conservative at an intraspecific level. These results indicate that on the species level, the genetic properties of AR might be more important factors for the quality of AR than environmental factors while on the intraspecific level, environmental factors appear to be more important than the genetics. This means that the local environment, including weather, geographic location, soil conditions, and methods of cultivation and processing, plays a significant role in determining the quality of this TCM; the same could be true for other TCMs and indeed other herbs. These results have relevance and implications for the growing of botanicals for the pharmaceutical industry.

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