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Using high performance liquid chromatography (HPLC) fingerprint distinguish producing area and grown years of Shanxi Astragalus

Huihui Zhao[1], Yang Liu[1], Chenglong Zheng[1], Shuwen Guo[1], Baosheng Zhao[1], Na Hou[2], Gang Wu[3], Yangqing Liu[3] and Wei Wang[1]*

[1]Beijing University of Chinese Medicine, Beijing, 100029, China.
[2]Beijing Hospital of Traditional Chinese Medicine, Beijing, 100010, China.
[3]Shanxi College of Traditional Chinese Medicine, Taiyuan 030024, China.

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So far, there has been no advanced method to assure the quality control for Astragalus. However, this paper summarizes a fingerprinting approach, which based on developed high performance liquid chromatography to provide reference for the scientific evaluation of the intrinsic quality of Astragalus. To assess the chemical content of Astragalus in different years and different producing areas, we determined 22 batches of samples through cluster analysis and similar calculations. According to the clustering analysis, we group the two-year-old Astragalus into three categories, which established the common model of RP-HPLC Fingerprint of Astragalus, and identified 12 peaks, two of which were Astragaloside (No. 15 peak) and Formononetin (No. 17 peak) respectively. In addition, from the fingerprints, the variation of chemical composition of Astragalus from the same producing area but different years was also summed up. In conclusion, not only is this method simple, fast and reproducible, but also the established fingerprints have a strong exclusion, providing evidences for the quality control and evaluation of Astragalus.

Key words: Astragalus, HPLC, fingerprint.

INTRODUCTION

Astragalus, alias Alone Root, or Two Lift, is the dried root of the Astragalus membranaceus (Fisch.) Bge. Var. monghoficus (Bge.) Hsiao or Astragalus membranaceus (Fisch.) Bge. It is native to Shanxi province, mainly produced in Hunyuan County (Zhang et al., 2008), and collected in spring and autumn without its fibrous roots and root head, and then dried. It has a sweet and warm taste. It belongs to the lung and spleen Merdian. As one of the important traditional Chinese medicines, Astragalus exhibits a variety of physical efficacies, including complementing qi, fixing surface, diuresis, detoxification, draining pus and growing flesh (National Pharmacopoeia Committee, 2005; Zhao et al., 2010; Li et al., 2010). Recent pharmacological studies of Astragalus indicate that the biological active ingredients are as follows: Saponins: Astragalus saponins A, B, C, soybean saponin I, isoastragaloside II, etc.; flavonoids: Formononetin, calycosin etc.; amino acids: 8 - aminobutyric acid, aspartic amide, etc.; polysaccharides: APS I, II, III and so on (Tian et al., 2008). Astragalosides effective components are effective in reducing fasting blood glucose and albuminuria levels, in reversing the glomerular

*Corresponding author. E-mail: wangwei@bucm.edu.cn.

hyperfiltration state, and in ameliorating the pathological changes of early DN in rat models (Nie et al., 2006). Astragalosides also have the effects on reducing $(Ca^{2+})i$ and SR Ca^{2+} load, enhancing free radical removal and decreasing lipid peroxidation in ISO-treated cardiomyocytes, which might account for their protective effect on myocardial injury (Zhang et al., 2009; Meng et al., 2005). Nowadays, it is reported to be widely used in the clinic for its immunomodulatory properties and for the treatment of cancer, systemic lupus erythematosus (SLE), coronary heart disease, chronic gastricism and so on (Chu et al., 1998; Shao et al., 2004; Sun et al., 1983). The traditional guideline of quality control is generally to determine Astragaloside content (Zhao et al., 2006), but this method hardly reflects the internal quality of the medicine. As for method of HPLC, more and more reports of it have been publicly disclosed, such as to determine the most effective solvent extract of mangosteen, anti-acne inducing bacterial activity and the amount of alpha-mangostin, a major active component in each mangosteen fruit rind extract (Pothitirat et al., 2010; Liu et al., 2006). This method using ultrafiltration to pretreat peritoneal fluid and bile samples is developed to measure meropenem and biapenem concentrations in human peritoneal fluid and bile (Kameda et al., 2010; Liu et al., 2002). Interestingly, we found that it even applies to TCM, as a high performance liquid chromatography–diode array detection–chemiluminescence (HPLC–DAD–CL) method for on-line detection to screen antioxidants in complex Shengmai San extracts (Wu et al., 2010). This paper, similarly, boldly based on HPLC, which had good stability, precision and reproducibility (Chaudhary et al., 2007), established the Astragalus fingerprint and investigated the data relations between batches of chemical composition of Astragalus, so that to improve the comparability among samples.

Currently, the study of Astragalus fingerprints exists great deal of limitation, such as collections were of a small number, fingerprints was not strong, few peaks were identified, quality of fingerprint features was too low, and still lacked the data on special fingerprints from authentic origin (Liu et al., 2006). Here, a collection of 22 batches of Astragalus samples primarily studied the HPLC fingerprint and displayed the chemical content characteristics of Shanxi Astragalus compared to Mongolia and Gansu Astragalus; meanwhile, we confirmed the chemical content characteristics of Shanxi Astragalus from different producing areas and years through contrasting one another.

These results indicated that HPLC possesses significant identification effects on Astragalus fingerprints, which implies that it would be a potential candidate for further investigation as a new scientific approach for its quality evaluation. Besides that, this HPLC fingerprint analysis protocol which is simple, convenient and stable, can also contributes to greater scientific and medical understanding of herbal medicines and promotes a new way of studying TCM (Zhao et al., 2011). What is more, it has revealed that eco-climatic variation affects the chemical constituents of herbs. It is therefore quite promising for further pharmacological and biochemical experiments that focus on evaluating the mechanism by which environmental variation affect the chemical constituents in herbs, namely the quality of a medicinal herb (Chen et al., 2011).

EXPERIMENTAL

Apparatus

DIONEX HPLC analyses were carried out on an Agilent 1100 series instrument equipped with a quaternary pump, a vacuum-line degasser, a column oven, a photodiode array detector (PDA), an autosampler. All data acquired were processed by a Chameleon workstation. A KQ-500 ultrasonic bath (work Frequency: 40K Hz; ultrasonic electric power: 500W) and an analytic electronic scale: EL204 (METTLER.TOLEDO Co., Ltd.) were used during the analysis process.

Reagents

Acetonitrile (chromatographic pure Tianjin Kemi'ou Chemical Reagent Co., Ltd. 20080416), the second distilled water (homemade), methanol and other reagents were of analytical grade.

The contrast substance

Formononetin and astragaloside were both purchased from the National Institute for the Pharmaceutical and Biological Products (Beijing, China).

The test sample

Astragalus herbs used in this study were harvested in person, so as to ensure the reliability of the source ingredients. Samples are shown in Table 1.

METHODS AND RESULTS

Chromatographic conditions

Chromatography was performed on a XTerraTM RP8 (5 μm, 3.9 mm × 150 mm Column) column with a flow rate of 0.8 ml/min and temperature kept at 30.00±0.15℃. The mobile phase consisted of 1% acetic acid water (A) and 1% acetic acid acetonitrile (B). The detector was set at 208 nm for acquiring chromatograms. An aliquot of 5 μl solution was injected into HPLC column for analysis. Gradient elution program is shown in Table 2.

Preparation of contrast solution

The solution were prepared by precisely weighing 0.42

Table 1. Information of Astragalus growth years and producing areas.

No.	The period of growth	Origin
1	2	Shanxi Hunyuan
2	2	Shanxi Hunyuan
3	2	Shanxi Anze
4	2	Shanxi Yingxian
5	2	Shanxi Anze
6	2	Gansu Dingxi
7	2	Shanxi Hunyuan
8	2	Shanxi Fanshi
9	2	Inner Mongolia
10	2	Inner Mongolia
11	3	Shanxi Hunyuan
12	4	Shanxi Hunyuan
13	5	Shanxi Hunyuan
14	1	Inner Mongolia Guyang
15	3	Inner Mongolia Guyang
16	3	Shanxi Yingxian
17	4	Shanxi Yingxian
18	5	Shanxi Yingxian
19	3	Gansu Dingxi
20	3	Shanxi Yingxian
21	3	Shanxi Fanshi
22	3	Inner Mongolia

Table 2. Gradient elution program.

Retention/min	Flow ($ml \cdot ml^{-1}$)	A(%)	B(%)
0	0.8	88	12
18	0.8	81.1	18.9
29	0.8	76.1	23.9
34	0.8	72	28
44	0.8	70.5	29.5
49	0.8	68	32
60	0.8	88	12

mg of formononetin, transferred into a 1 ml volumetric flask; 75% (v/v) methanol was added and finally the solution was diluted to 0.42 mg/ml; then precisely 0.91mg of astragaloside was weighed, transferred into a 1 ml volumetric flask, 75% (v/v) methanol was added and finally the solution was diluted to 0.91 mg/ml.

Preparation of test solution

Precisely 2.5 g Astragalus powder was weighed, 30 ml methanol was added, and the mixture was ultrasonic for 30 min, with amount of cotton plugged in the funnel neck for filtration. Its residue with 20 ml methanol ultrasonic filtrate was combined for 20 min, and waved to till it almost got dried. The residue was dissolved in methanol and the capacity was determined to 5 ml. It stood for 4 hours, then the solution was obtained after which it was filtered through a 0.45 mm millipore filter.

Methodology test

Precision test

One Astragalus test sample (No. 1) was taken following preparation of test solution, for 5 successive injections to measure the retention time and peak area of common peaks, calculating the retention time and peak areas of relatively higher content peaks as RSD values. The calculated results show that the retention time for each chromatographic peak is RSD <0.6%, and peak area

Table 3. Precision results.

Peak number	The retention time (T)					Peak area (A)					T	A
	1	2	3	4	5	1	2	3	4	5	RSD%	RSD%
4	9.747	9.727	9.698	9.827	9.838	98.78	98.52	99.67	99.25	99.27	0.57	0.41
9	20.08	20.05	20.13	20.17	20.19	89.51	90.31	90.30	90.25	92.47	0.26	1.1
11	24.96	24.97	25.01	25.03	25.04	41.54	41.31	41.46	41.93	41.85	0.14	0.57
13	29.71	29.80	29.65	29.65	29.66	74.09	74.34	76.38	76.49	76.43	0.20	1.4
16	40.61	40.69	40.68	40.67	40.67	87.93	84.59	87.53	87.48	87.62	0.071	1.4
17	42.54	42.65	42.45	42.45	42.45	30.40	27.51	30.04	30.12	29.96	0.18	3.6
18	45.55	45.66	45.64	45.62	45.61	118.4	118.9	119.0	119.5	118.7	0.083	0.32

Table 4. Reproducible results.

Peak number	The retention time (T)					Peak area (A)					T	A
	1	2	3	4	5	1	2	3	4	5	RSD%	RSD%
4	9.865	9.855	9.870	9.850	9.840	113.6	114.3	112.2	110.1	112.8	0.11	1.3
9	20.19	20.19	20.19	20.18	20.15	101.1	102.4	103.8	100.6	100.4	0.080	1.3
11	25.05	25.06	25.06	25.04	25.01	45.49	45.83	48.42	46.66	47.80	0.067	2.4
13	29.61	29.39	29.38	29.35	29.32	70.65	71.02	68.65	65.14	66.37	0.35	3.4
16	40.66	40.68	40.66	40.64	40.63	105.7	107.4	105.8	98.3	102.9	0.038	3.1
17	42.26	42.16	42.12	42.10	42.10	38.84	37.66	36.10	36.23	36.78	0.15	2.7
18	45.64	45.67	45.62	45.61	45.61	144.8	145.1	154.2	157.5	147.5	0.047	3.4

Table 5. Stability results.

Peak number	The retention time (T) (h)					Peak area (A) (h)					T	A
	0	2	4	6	8	0	2	4	6	8	RSD%	RSD%
4	10.14	9.923	9.747	9.698	9.838	95.90	97.93	98.78	99.25	99.27	1.6	1.3
9	20.41	20.24	20.08	20.13	20.19	90.15	91.23	89.51	90.25	92.47	0.56	1.1
11	25.25	25.09	24.95	25.01	25.04	41.22	41.99	41.54	41.93	41.85	0.40	0.69
13	29.82	29.76	29.70	29.65	29.66	75.89	76.45	74.09	76.49	76.43	0.22	1.2
16	40.85	40.73	40.61	40.68	40.67	87.45	76.98	87.93	87.48	87.62	0.20	4.9
17	42.58	42.58	42.54	42.45	42.45	30.15	28.45	30.40	30.12	29.96	0.13	2.3
18	45.84	45.70	45.55	45.64	45.61	118.9	119.8	118.4	119.5	118.7	0.22	0.45

RSD ≤ 3.6%, which is consistent with the detecting requirement of fingerprints. The results are shown in Table 3.

Reproducibility test

Following the aforementioned preparation of test solution, five samples of the same batch of Astragalus (No. 1) and the respective retention time and peak area of common peaks were measured; the retention time and peak areas of relatively higher content peaks was calculated as RSD values. The calculated results show that the retention time for each chromatographic peak is RSD ≤ 0.35%, and peak area RSD ≤ 3.4%, which is consistent with the requirements of fingerprints. The results are shown in Table 4.

Stability test

One sample of the same batch of Astragalus (Sample 1) was taken following the aforementioned preparation of test solution, and sampled respectively at 0, 2, 4, 6 and 8 h, to measure the retention time and area of common peaks, calculating the retention time and area of relatively higher content peaks as RSD values. The calculated results show that the retention time for each chromatographic peak RSD is ≤ 1.6%, and peak area RSD is ≤ 4.9%, which is consistent with the requirements of fingerprints. The results are shown in Table 5.

Table 6. Calibration table of total peak number.

No. \ T (min)	2.527	5.998	8.055	9.698	12.590	13.368	16.908	19.253	20.132	24.413	25.012	28.484	29.650	31.673	39.666	40.682	42.453	45.635
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	+	+
3	+	+	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	—	+	+	+	+	+	+	+	+	+	+	—	+	+	+	+
13	+	—	—	+	+	—	—	+	+	+	+	+	+	—	+	+	+	+
14	+	+	+	+	+	—	+	+	+	—	+	+	+	—	+	+	+	+
15	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	+	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Total	22	17	17	22	22	18	21	22	22	21	22	22	22	17	22	22	22	22
No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

+, a peak; -, no peak.

Astragalus fingerprint establishment

The choice of reference peak

Systematic analysis and study was made on 22 batches of the Astragalus HPLC chromatogram; since peak 17 separations is good and content is stable, peak 17 was chosen as a reference peak.

Common peaks calibration

According to the retention time for each chromatographic peak, the common peaks were calibrated. The same retention time peaks were grouped into a category. After systematic analysis and study on 22 batches of the Astragalus HPLC chromatogram, the statistics of 12 common peaks was displayed. Specific information is shown in Tables 6 and 7.

Table 7. Total peak retention time table.

Sample number	Retention time of each peak											
	1	4	5	8	9	11	12	13	15	16	17	18
1	2.492	9.838	12.703	19.310	20.185	25.042	28.522	29.660	39.642	40.668	42.447	45.613
2	2.335	9.857	12.708	19.290	20.168	25.025	28.513	29.657	39.628	40.637	42.650	45.537
3	2.350	9.848	12.686	19.188	20.173	25.025	28.431	29.612	39.615	40.638	42.398	45.582
4	2.352	9.910	12.759	19.350	20.222	25.067	28.734	29.717	39.685	40.690	42.631	45.667
5	2.457	9.867	12.732	19.307	20.185	25.040	28.540	29.739	39.666	40.666	42.672	45.627
6	2.398	9.863	12.733	19.292	20.180	25.030	28.522	29.916	39.625	40.755	42.692	45.652
7	2.632	9.852	12.703	19.295	20.165	25.018	28.583	29.789	39.706	40.665	42.708	45.648
8	2.373	9.850	12.694	19.307	20.183	25.040	28.538	29.712	39.705	40.675	42.686	45.662
9	2.320	9.852	12.695	19.292	20.165	25.020	28.656	29.698	39.657	40.638	42.652	45.607
10	2.360	9.855	12.739	19.305	20.182	25.043	28.547	29.733	39.657	40.658	42.635	45.615
11	2.395	9.845	12.742	19.300	20.168	25.045	28.531	30.148	39.749	40.654	42.719	45.617
12	2.257	9.888	12.769	19.358	20.227	25.092	28.669	30.140	39.740	40.727	42.566	45.682
13	2.225	9.853	12.712	19.324	20.167	25.041	28.563	31.652	39.687	40.677	42.683	45.597
14	2.338	9.848	12.708	19.328	20.193	25.067	28.569	31.662	39.775	40.731	42.723	45.662
15	2.349	9.865	12.746	19.328	20.193	25.052	28.595	31.626	39.753	40.738	42.744	45.655
16	2.387	9.868	12.731	19.343	20.205	25.075	28.651	31.652	39.760	40.731	42.759	45.703
17	2.225	9.902	12.761	19.382	20.240	25.115	28.643	31.766	39.813	40.776	42.774	45.755
18	2.078	9.868	12.722	19.348	20.205	25.078	28.679	31.525	39.754	40.738	42.763	45.722
19	2.337	9.865	12.715	19.351	20.202	25.068	28.650	31.642	39.874	40.783	42.753	45.735
20	2.108	9.853	12.687	19.332	20.192	25.062	28.392	31.711	39.731	40.710	42.835	45.715
21	2.120	9.868	12.765	19.333	20.198	25.082	28.435	31.645	39.762	40.732	42.745	45.728
22	2.337	9.832	12.642	19.290	20.152	25.031	28.430	31.581	39.705	40.648	42.142	45.633

Sample chromatograms

The typical Astragalus HPLC chromatogram is shown in Figure 1; Astragalus fingerpring chromatograms of different ages and producing areas are shown in Figure 2 to Figure 6.

Systematic cluster analysis

Cluster analysis, usually used the average linkage method and the Ward method. Ward's method whose clustering result is better than average linkage (Li et al., 2010) is based on the analysis of variance, and can get partial optimization results (Tian et al., 2008).

Therefore, the application of SPSS software was fully utilized in the experiment, using Ward's clustering method for the 10 batches of herb of two years old from different producing areas, and using squared Euclidean distance as the measure of sample similarity (Nie et al., 2006).

According to the clustering results, 10 batches of herbs can be divided into three categories (Sample 7 as type I, Samples I and 3 as type II, No. 2, 4, 5, 6, 8, 9, 10 as type III). Type II was determined to have the best quality combined fingerprints. Clustering pedigree is shown in Figure 7.

Evaluation of fingerprint similarity

Based on the clustering analysis results, we use A version of the software to create a chromatographic fingerprint pattern; and B version of the software to calculate the similarity of 10 batches of fingerprints. Following the State Pharmacopoeia Commission designated Traditional Chinese herbs chromatographic fingerprint evaluation system; type II herbs (No. 2, 4, 5, 6, 8, 9, 10) evaluate similarity in good effect. The results are shown in Table 8.

Investigation of the characteristics of Astragalus fingerprint

Investigation of the characteristics of Astragalus fingerprint of the same year but different producing areas

The characteristics of two-year-old Astragalus fingerprint from different producing areas:

1. The characteristics of the fingerprint of Shanxi and other provinces Astragalus: From the fingerprint of Shanxi (Figure 8) and other provinces (Figure 9) Astragalus of two years old, it can be seen that quantity

Table 8. Evaluation results.

No.	1	2	3	4	5	6	7	8	9	10
Similarity	0.920	0.983	0.941	0.959	0.949	0.962	0.869	0.959	0.966	0.966

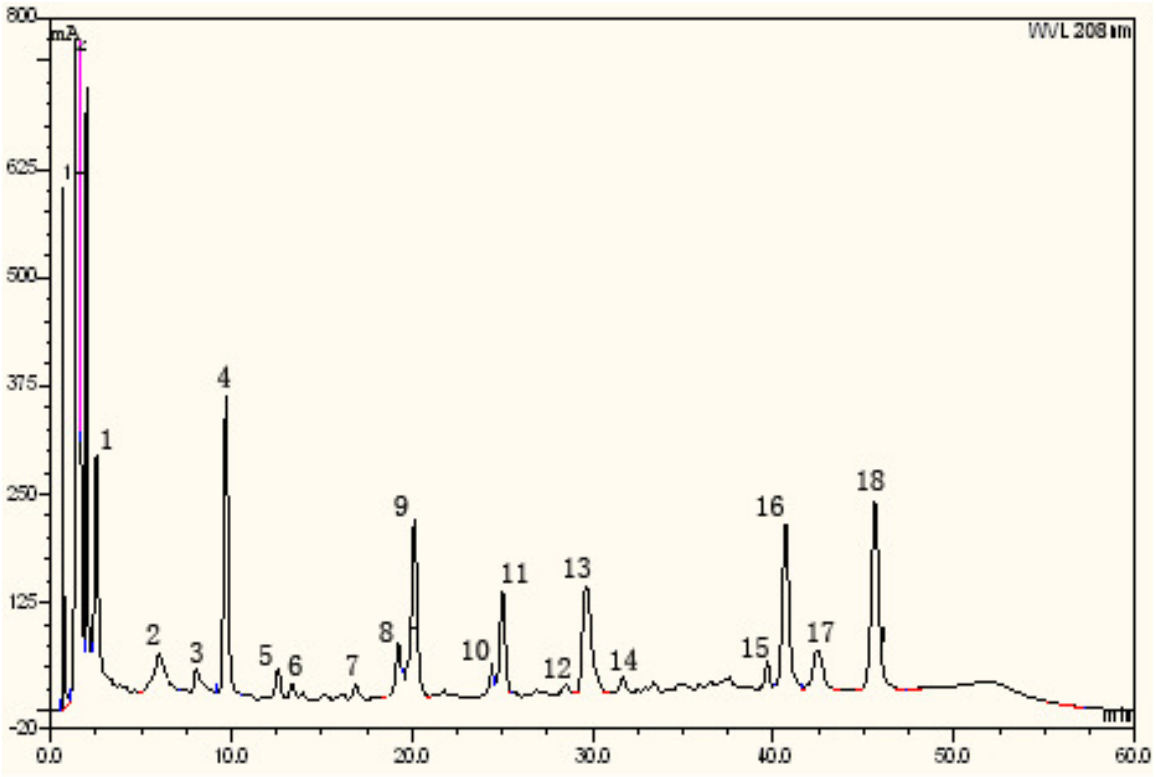

Figure 1. Typical Astragalus HPLC fingerprint chromatogram.

and content of components of Shanxi Astragalus is obviously better than Inner Mongolia and Gansu Astragalus. Besides that, we can also find, from the fingerprint of other provinces Astragalus (Figure 9), that quantity and content of components of Mongolia astragalus is superior to Gansu astragalus.

2. The characteristics of Shanxi Astragalus fingerprints: From the fingerprint of Shanxi (Figure 8) and other provinces (Figure 9), Astragalus of two years old, it can be seen that Hunyuan and Anze Astragalus yielded the best quality among the two-year old Shanxi Astragalus, followed by Fanzhi and Ying Country. As to Hunyuan samples, the content of all components was higher. And No. 1, 4, 5, 8, 9, 11 and 12 peaks of Anze samples were basically of the same intensity as the corresponding peaks of Hunyuan. However, No. 13, 15, 16, 17 and 18 peaks of Anze samples, whose intensity were on the low side compared to the corresponding peaks of Hunyuan samples,. Thus among the Shanxi Astragalus samples, the quality of Hunyuan Astragalus is the best.

The characteristics of three-year-old Astragalus fingerprint from different producing areas:

1. The characteristics of Shanxi and other provinces Astragalus fingerprints: From three-year-old Astragalus fingerprints of the same age but different producing areas (Figure 3), it can be seen, except Hunyuan of Shanxi produced Astragalus, that quantity and content of components of Shanxi Astragalus completely corresponded to that of Gansu Astragalus. But the quantity and content of components of Mongolia was close to that of Shanxi Hunyuan Astragalus, which is higher than that of Shanxi and Gansu produced Astragalus. In detail, different from the other areas produced Astragalus, Mongolia Astragalus is characterized by the basic separation of peaks 8 and 9 and the consistent peak height; whereas, peak 8 and 9 of Shanxi and Gansu Astragalus were not completely separated and a big gap exists between the two peaks height. Gansu Astragalus characterized by the peak height of each common

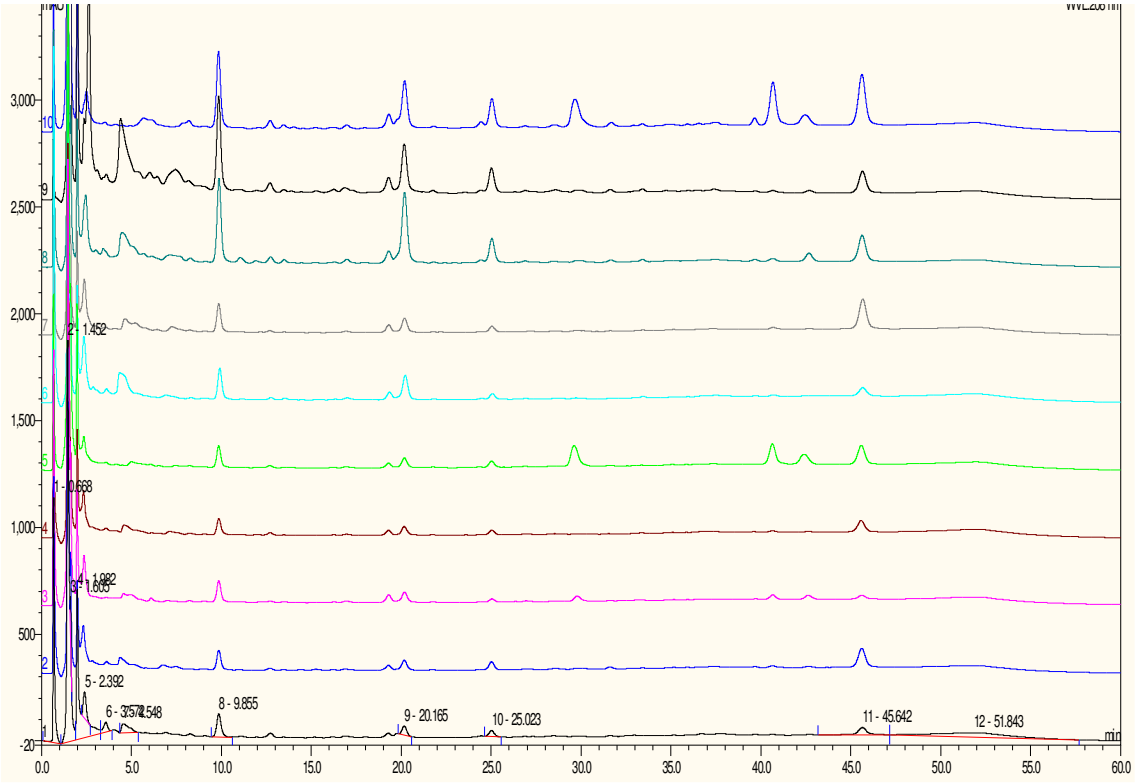

Figure 2. Two-year-old Astragalus of different producing areas.

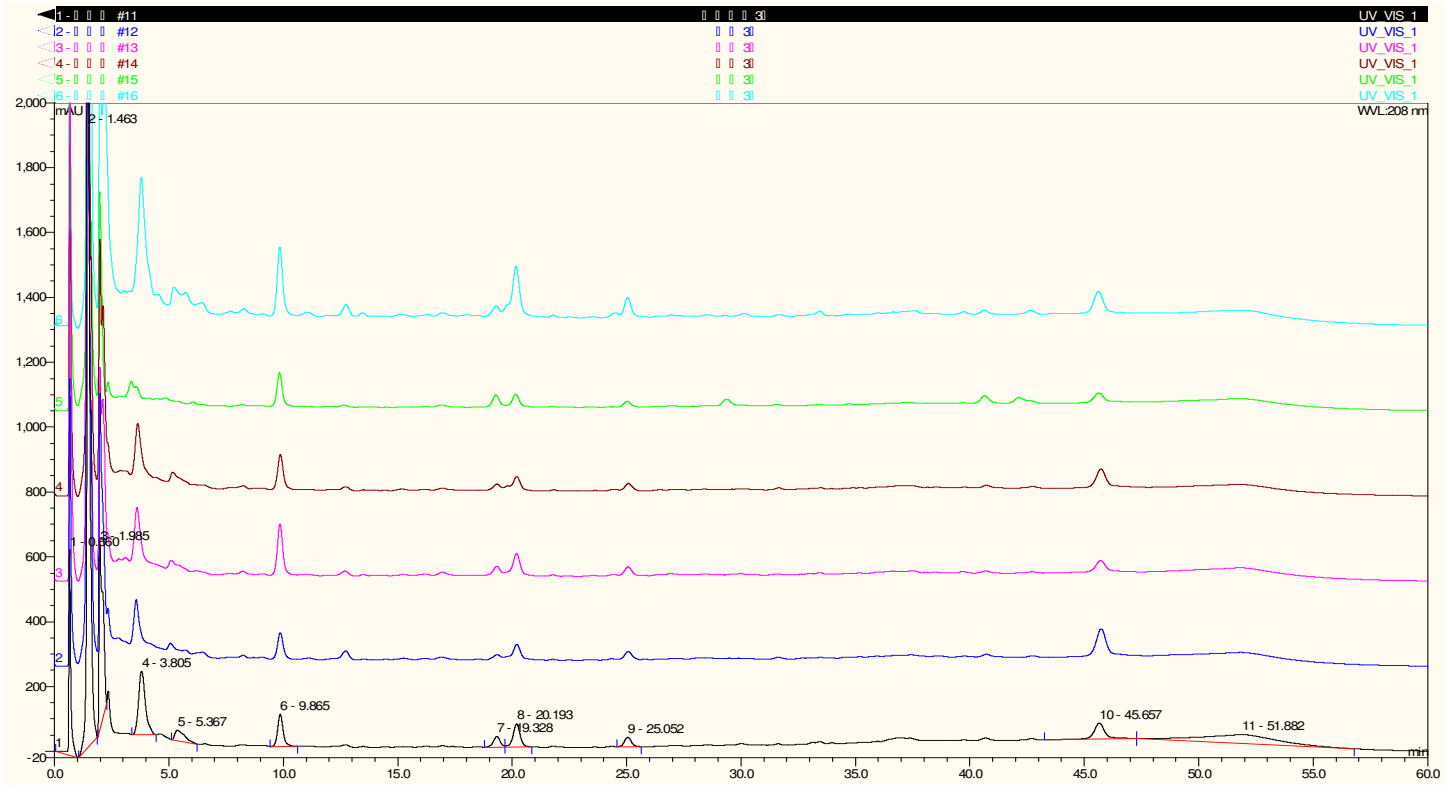

Figure 3. Three-year-old Astragalus of different producing areas.

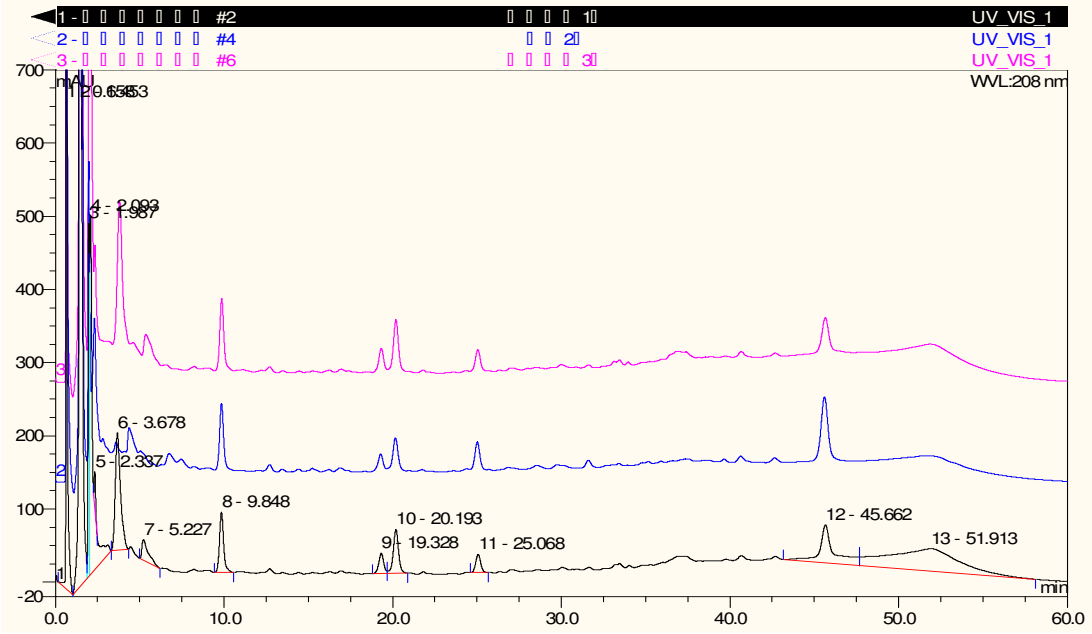

Figure 4. Inner Mongolia produced 1, 2 and 3 year old Astragalus.

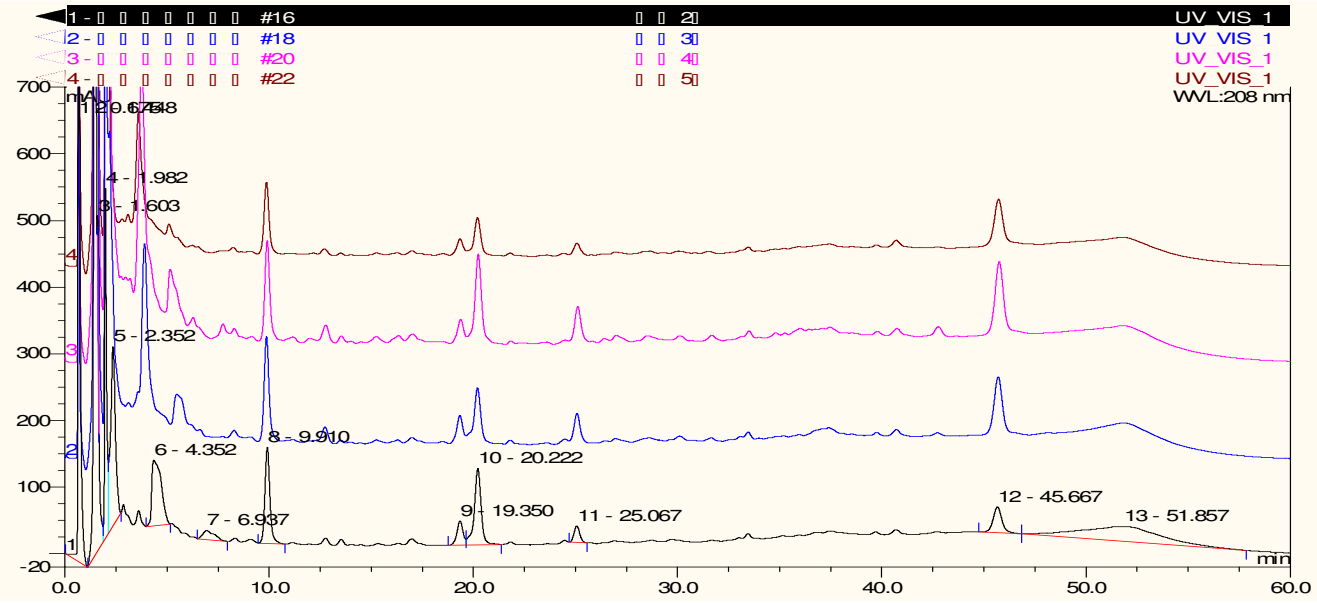

Figure 5. Ying County in Shanxi produced 2, 3, 4and 5 year old Astragalus .

peak was relatively low, but the peak height of peak 18 is the highest among six batches of three-year old Astragalus herbs. Shanxi Astragalus is characterized by the equal content of all components, and generally superior to Inner Mongolia and Gansu Astragalus. 6 batches of these herbs, the quality of Shanxi and Inner Mongolia Astragalus was excellent, followed by that of Gansu.

2. The characteristics of Shanxi local produced Astragalus fingerprints: It can be seen from three-year old Astragalus fingerprint of the same age but different producing areas (Figure 3) that the quantity and content of components of Fanzhi Astragalus was close to that of Ying County Astragalus, but the quantity and content of components of Hunyuan Astragalus was better than the former two.

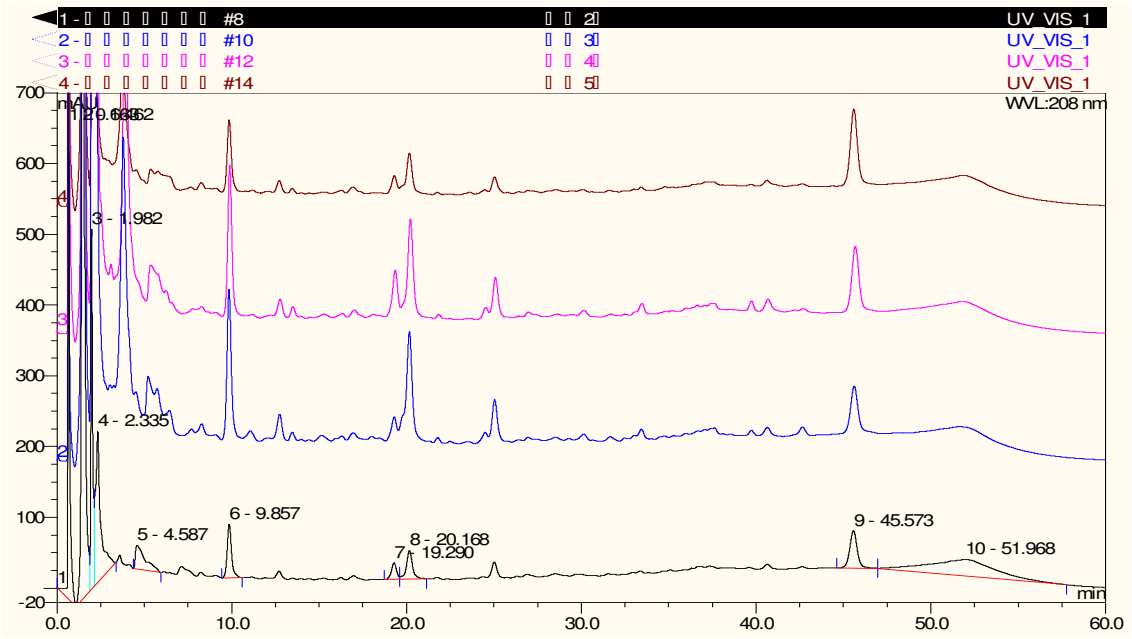

Figure 6. Hunyuan County in Shanxi produced 2, 3, 4 and 5 year old Astragalus.

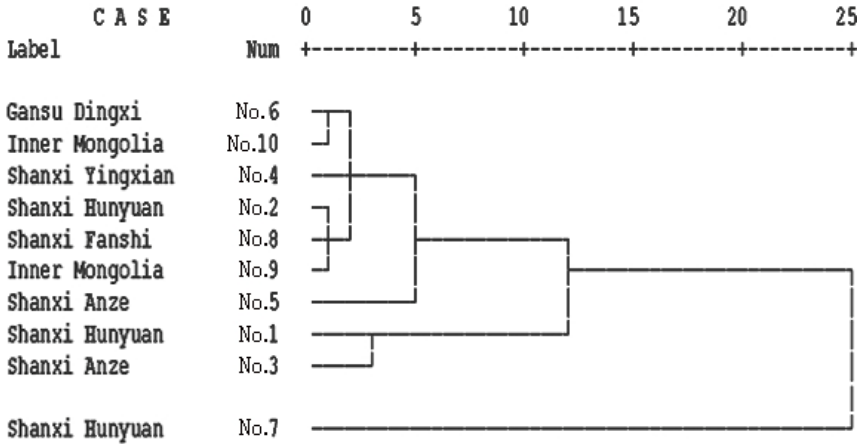

Figure 7. Cluster dendrogram of 10 batches of herbs of two years old.

Investigation of the characteristics of Astragalus fingerprint from the same producing areas but different age

The characteristics of Inner Mongolia Guyang Astragalus fingerprints of different age: It can be seen from fingerprint of the Inner Mongolia Guyang Astragalus of different ages (Figure 4) that with the growth of age, the composition and content of herbs was basically in an upward trend. However, there were exceptions. Peak heights 11 and 18 peaks in fingerprints was significantly increased in the first to second years, but sharply reduced from the second to third year.

The characteristic of Shanxi Ying County Astragalus fingerprints of different ages: It can be seen from the Shanxi Ying County Astragalus fingerprint of different ages (Figure 6) that with the growth of age, the composition and content of herbs was basically first increase and then decrease. The quantity and content of components of two-year old Astragalus was relatively low, and that of three-year and four-year old Astragalus was comparable and reached their peak, while that of five-year old Astragalus was significantly decreased

The characteristic of Shanxi Hunyuan Astragalus fingerprints of different ages: It can be seen from the

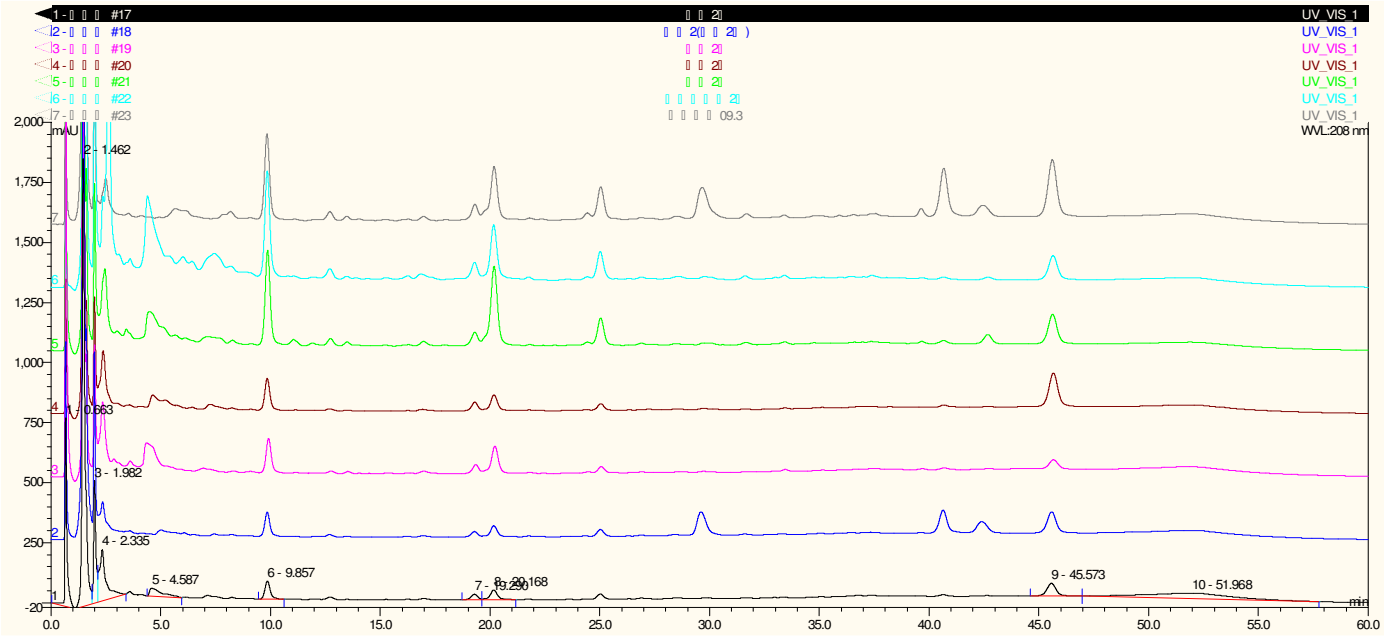

Figure 8. Two-year-old Shanxi Astragalus Fingerprints.

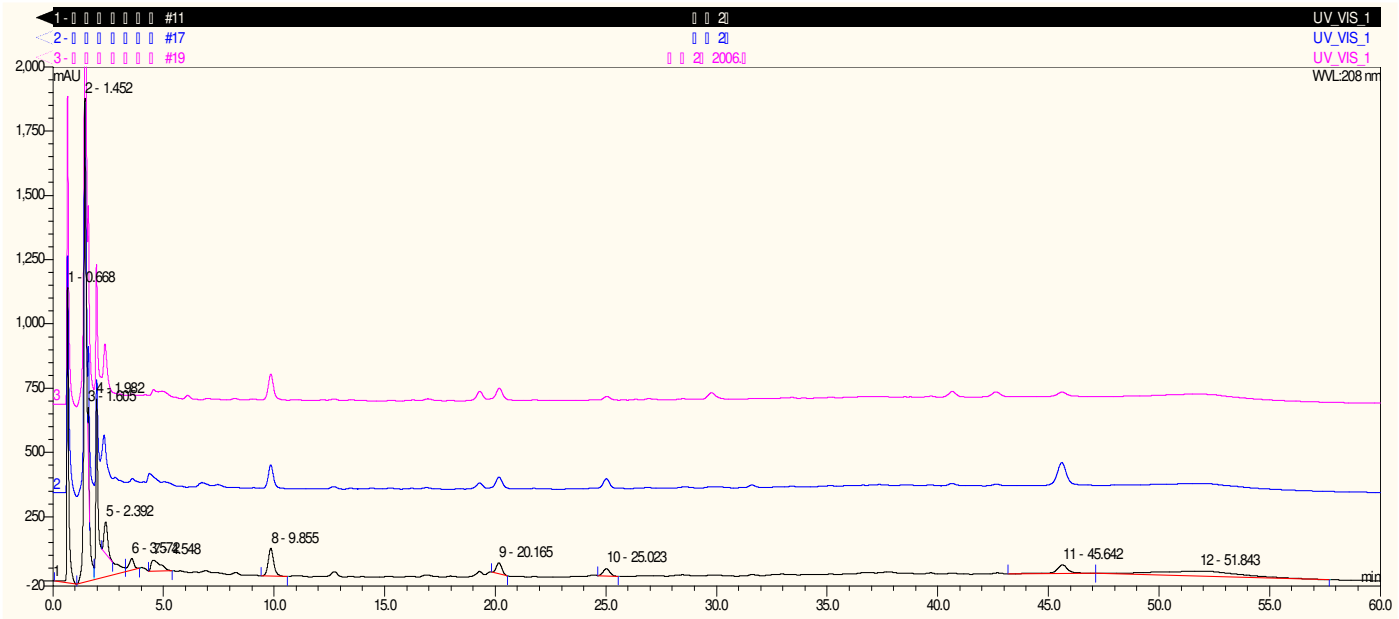

Figure 9. Two-year-old other provinces Astragalus Fingerprints

Shanxi Hunyuan Astragalus fingerprint of different ages (Figure 6) that with the growth of age, the composition and content of herbs was basically first increase and then decrease. The quantity and content of components of two-year old Astragalus was relatively low, and that of three-year and four-year old Astragalus was comparable and reached their peak, while that of five-year old Astragalus was significantly decreased. But there were exceptions, the peak height of peak 18 in the fingerprints, with the growth of years, show an increasingly high trend.

Summary of Astragalus fingerprint characteristics

Fingerprint is a kind of method to show chemical information of TCM with chromatograms, spectrograms

and other graphs by chemical analytical techniques (Liu et al., 2006; Zhao et al., 2011; Chen et al., 2011). Fingerprint analysis has been introduced and accepted by WHO as a strategy for the assessment of herbal medicines (Abbasi et al., 2010). And it is also required by the Drug Administration Bureau of China to standardize injections made from TCM and their raw materials (Hussain et al., 1990). It is therefore necessary to build up fingerprint chromatography on identifying and assessing the stability of the medicine.

According to the investigation on the characteristics of Astragalus fingerprints from different producing areas but same age, it can be indicated that among three areas which produced Astragalus, the quality of the Shanxi was the best, and the Mongolia followed, slightly less of the Gansu; besides, in the light of the further investigation on the characteristics of Astragalus fingerprint from the same producing areas but the different age, we wondrously found the Astragalus of one to four year growth, whose composition variety and content presented basically rise, and the Astragalus of four to five year growth presented basically a downward trend. All the results can be inferred from the relative fingerprints.

DISCUSSION

The choice of solvent

This paper has tried ethyl acetate and methanol extraction of the Astragalus. From a review of the results, ethyl acetate extract samples gave good separation and nice peak shapes, but the number of peaks is too small, only 12. Separation of methanol extracted samples was slightly worse than ethyl acetate, but due to its good peak shape and a large number of peaks, methanol was finally chosen to extract.

The choice of detection wavelength

Photodiode array detection was used to investigate 200 to 400 nm different detection wavelength of chromatograms. As shown, peak shape at 254 nm was the best though with few peak number, while peak shape at 208 nm was not only good but could accord with the principle of moderate peak number; so we finally choose peak shape at 208 nm as the detection wavelength. The differences of the peak number at 203 and 254 nm have something to do with the structure categories of Astragalus composition. Around 250 nm, the flavonoids of Astragalus was observed, and at 208 nm both flavonoids of Astragalus and saponin composition which was dominant was observed.

Chromatogram column selection

Two different manufacturers of chromatogram column was studied: (1) GraceSmart RP18 (5u, 250 mm × 4.6 mm, SN: 0170805685), (2) waters XTerraTM RP8 (5 µm, 3.9 mm × 150 mm, Column Part No.186000481). As the C8 column (Column 3) has a high column efficiency, good separation and symmetrical peak shape, it was chosen C8 in this experiment.

Mobile phase selection

According to references, since the studies of HPLC fingerprint of Astragalus choose acetonitrile and water (acidic water) as mobile phase, acetonitrile was chosen in this study as one of the mobile phase. Our experiment tried different gradient, water - acetonitrile and 0.1% phosphoric acid - acetonitrile mobile phase system, the results showed that 0.1% of phosphoric acid - acetonitrile gradient elution separation is better but it has poor peak shape. While in the water - acetonitrile 2 element gradient elution system, both separation and peak shape are nice, so it was chosen in this experiment mobile phase.

The choice of flow rate

Our experiment tried flow rates of 1.0, 0.8, and 0.6 ml/min. It is indicated by comparison that when the flow rate is 1.0 ml/min, the retention time of the peak is concentrated, the peak separation and the peak shape is poor. When the flow rate is 0.8 ml/min, the retention time of each peak is more even and all go backward, and the peak shape and peak separation is fairly good. When the flow rate is 0.6 ml/min, the retention time of each peak continue to go backward, the separation is fairly good, but it has poor peak shape with serious trail. Experiments demonstrated that the flow rate of 0.8 ml/min is more appropriate, therefore we choose it to do measuring.

Optimization of gradient elution program

Our experiment compared the different acetonitrile – water gradient elution programs, and experimental results showed that the gradient elution programs recently used in both the separation and the number of peaks are appropriate.

Peaks identified

Formononetin to identify

As Formononetin maximum absorption wavelength is around 250 nm, we chose to observe at 254 nm. The retention time of peaks 10 in Figure 10 is 42.415 min; the UV spectrum is shown in Figure 11. The chromatogram of Formononetin which contrast at 254 nm (Figure 12) showed that the retention time of Formononetin is 42.355 min; the UV spectrum is shown in Figure 13. As No. 10

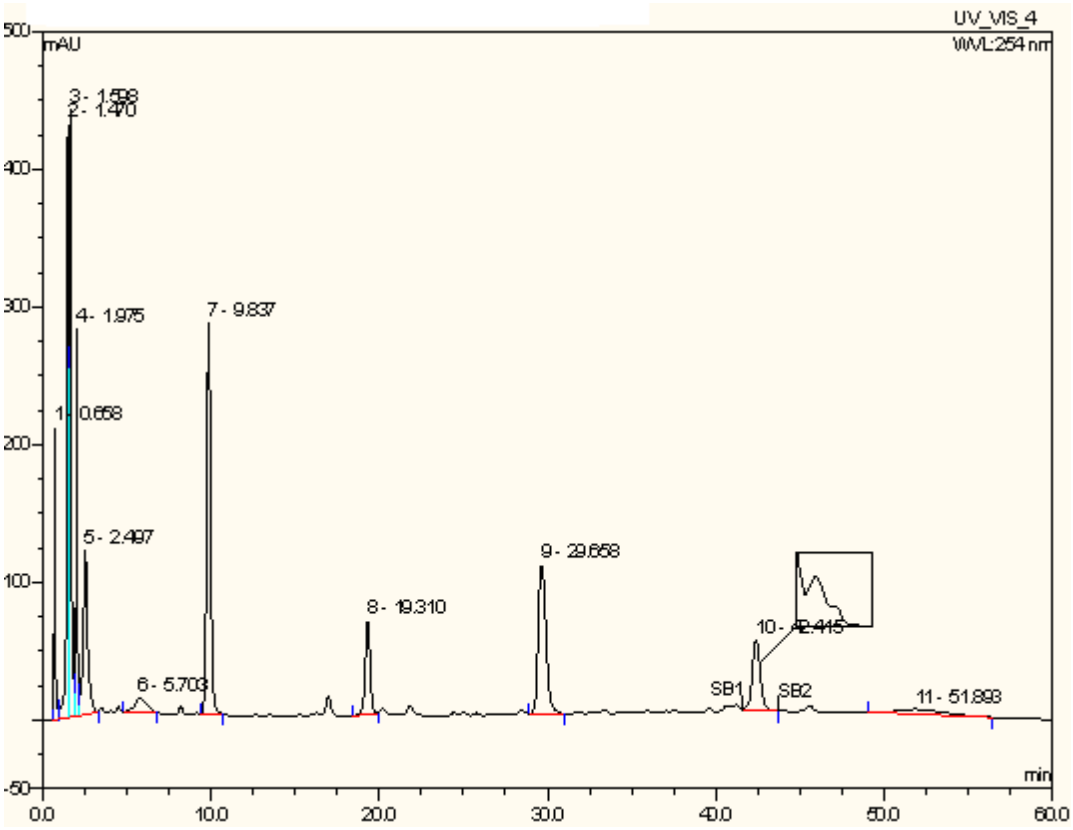

Figure 10. Sample 1 below 254 nm chromatogram

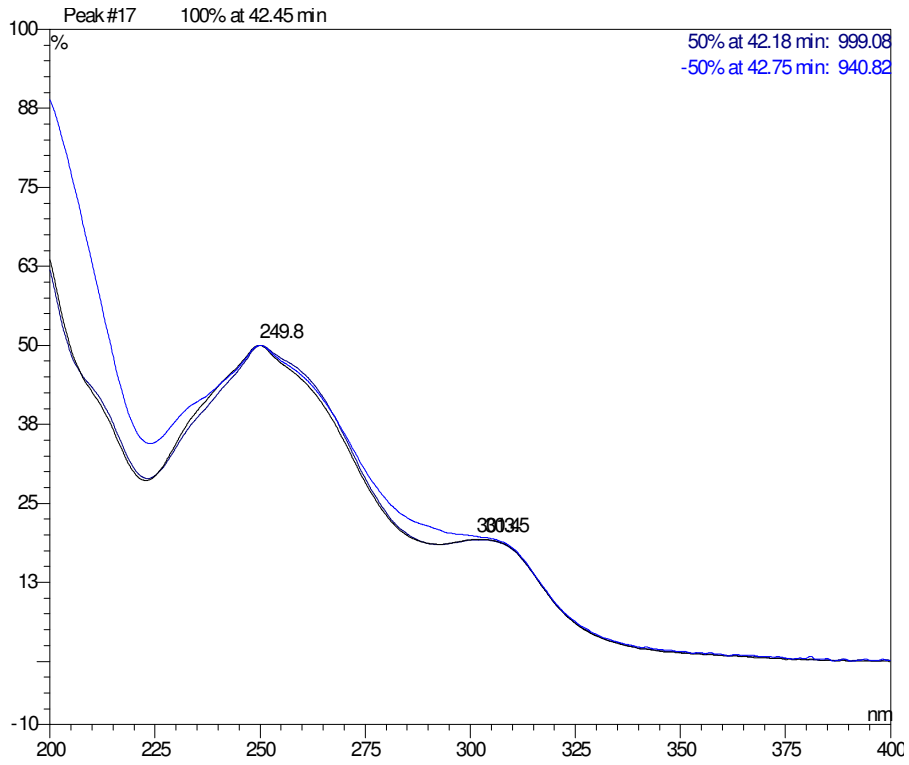

Figure 11. Spectrum of Sample 1 below 254 nm chromatogram.

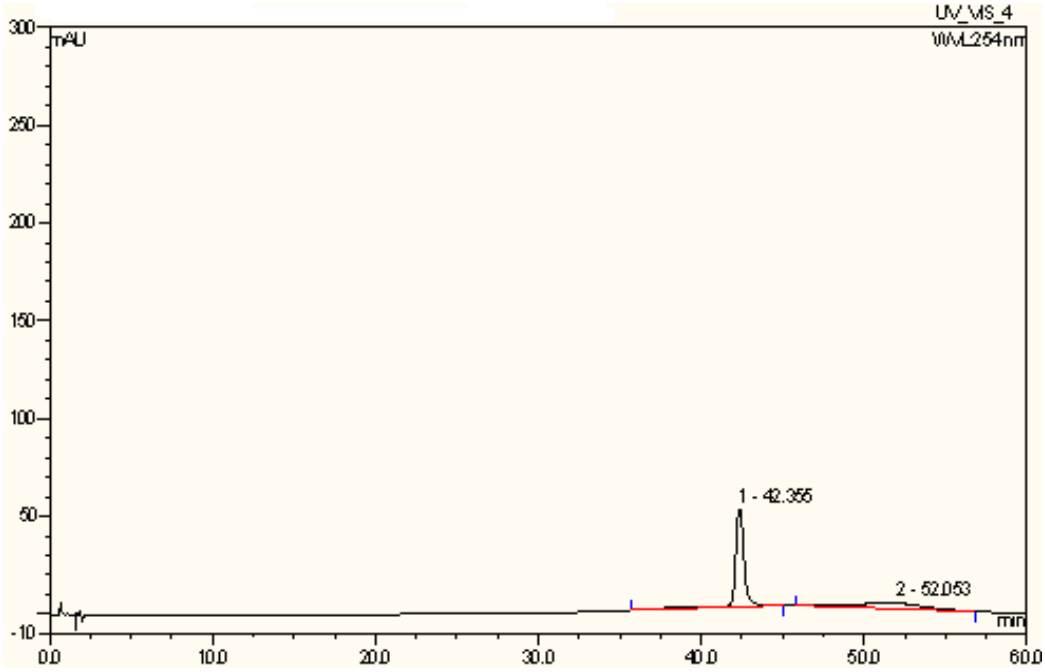

Figure 12. Formononetin contrast below 254 nm chromatogram.

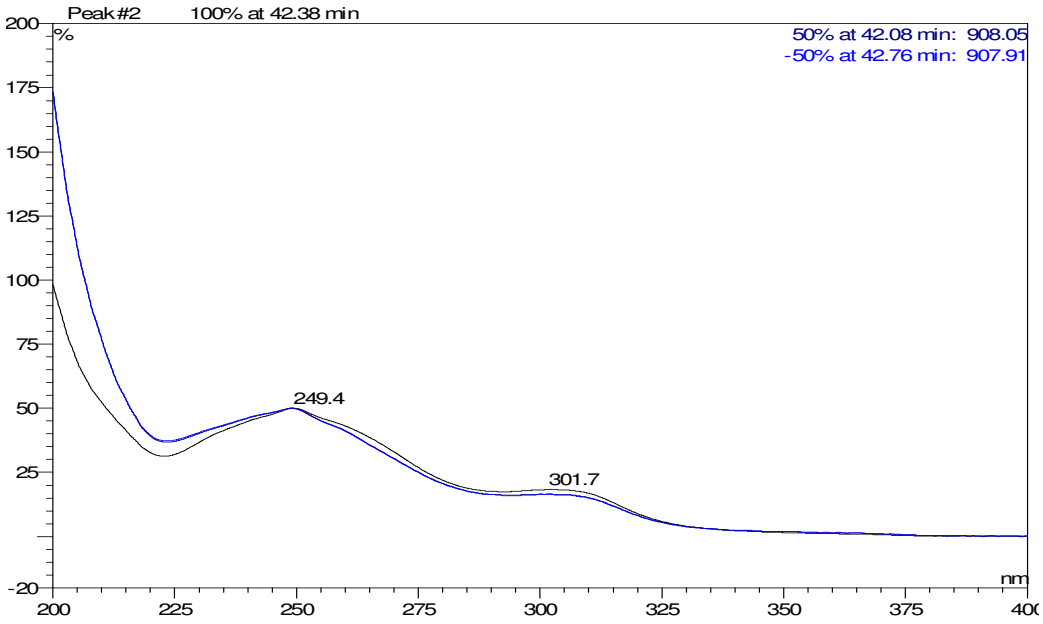

Figure 13. Formononetin contrast below 254 nm chromatogram.

peak and Formononetin contrast peaks shared the same retention time, and the UV spectra of the two shapes were similar and the maximum absorption were at 249 nm, it is concluded that under that chromatographic conditions, the peak, of which retention time shown in Figure 10 is 42.415 min (Figure 1 in 17 peak) is the Formononetin.

Identification of Astragaloside

Because the UV spectrum of Astragaloside is the terminal absorption, we choose to observe at 208 nm. The retention time of the 15 peaks in Figure 14 is 39.642 min; the UV spectrum is shown in Figure 15. Chromatogram of Astragaloside, relative to at 208 nm (Figure 16),

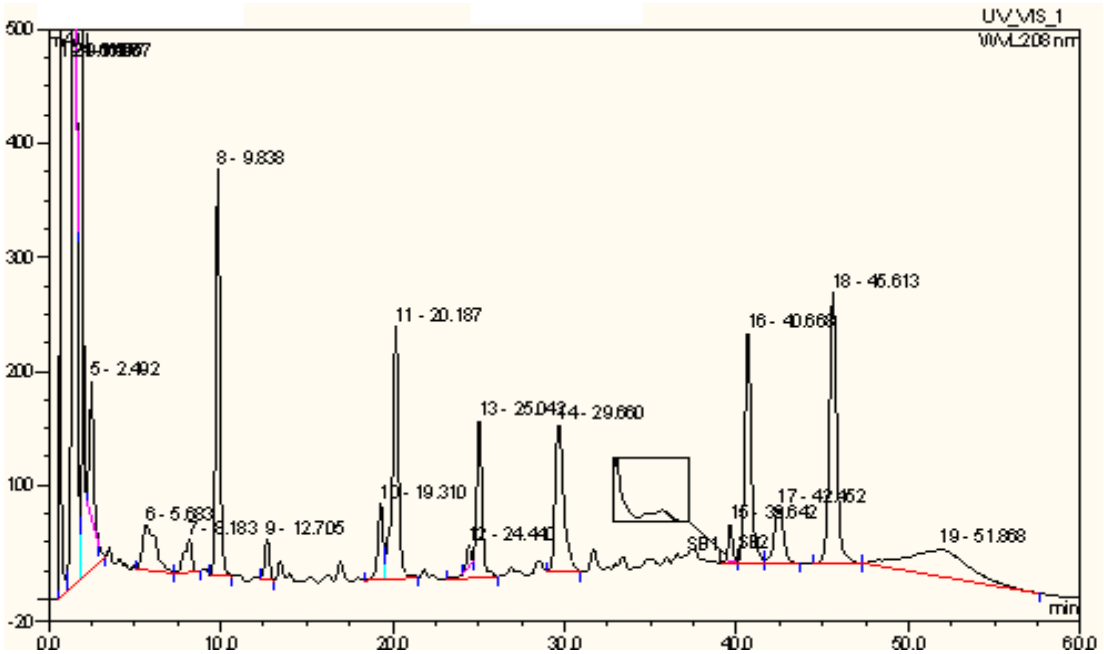

Figure 14. Sample 1 at 208 nm chromatogram.

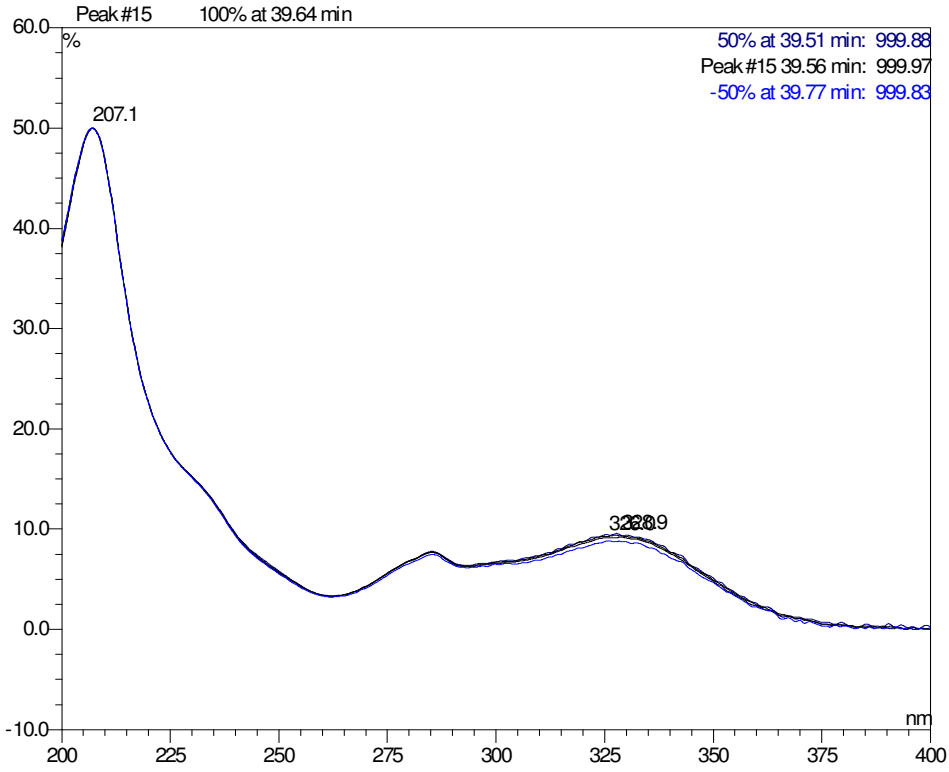

Figure 15. Sample 1 at 208 nm spectrogram.

showed the retention time Astragaloside is 39.882 min, the UV spectrum is shown in Figure 17.

Since peak 15 Astragaloside contrast peak shared the same retention time, and both UV spectrum with similar shape and terminal absorption (since in the case of terminal absorption, UV spectrum is unstable, so UV

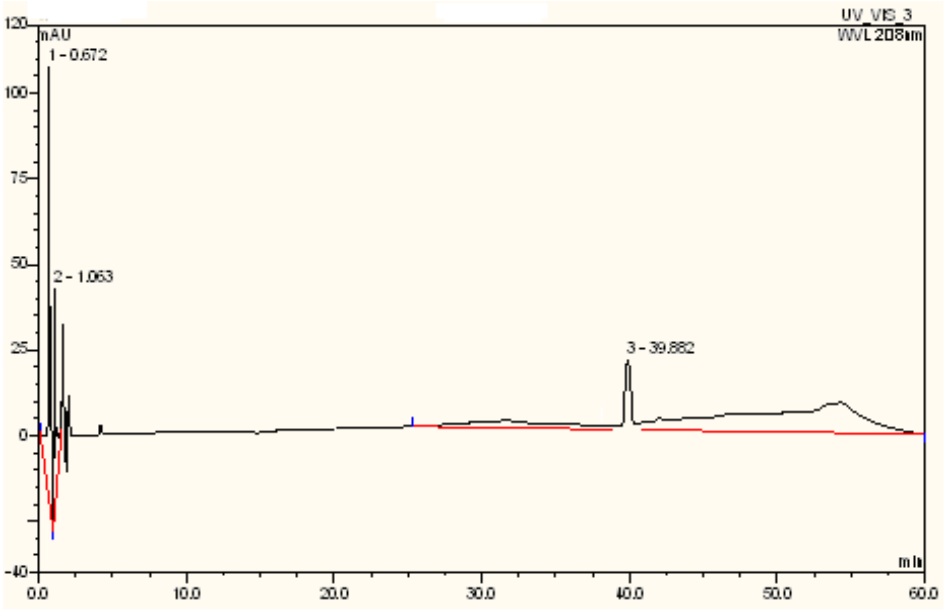

Figure 16. Astragaloside reference substance at 208 nm chromatogram.

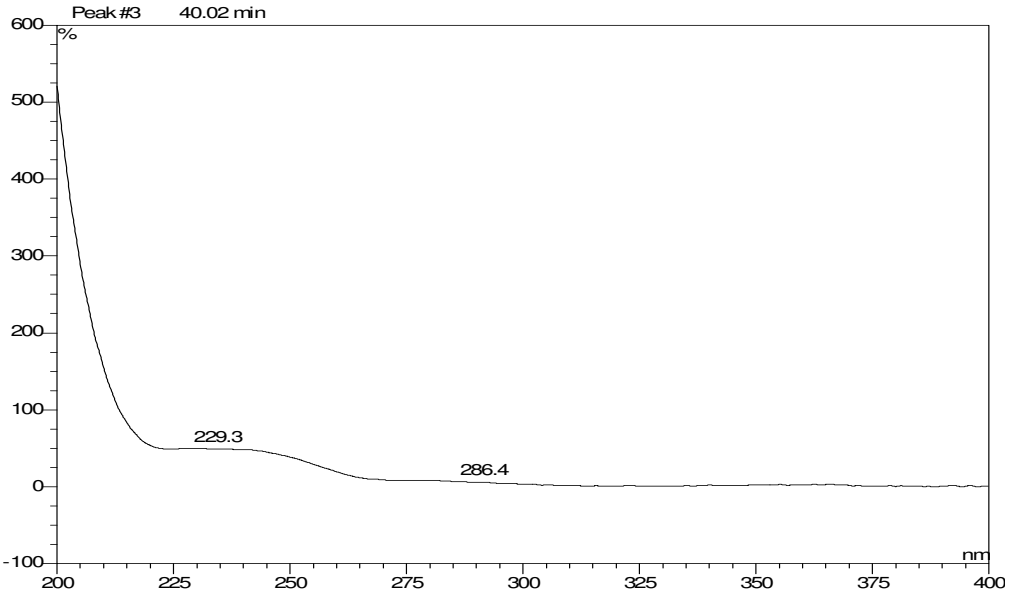

Figure 17. Astragaloside reference substance at 208 nm spectrogra

spectrum of the standard and the samples have certain differences, but can be ignored), it can be concluded that in that chromatographic conditions, the peak in Figure 14 whose retention time is 39.642 min (Figure 1, No.15 peak) is Astragaloside.

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