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A New Approach to Develop a Standardized Method for Simultaneous Analysis of Astragaloside IV and Formononetin in Radix Astragali by High-Performance Thin-Layer Chromatography

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Key Words

Radix Astragali
Quality evaluation
Astragaloside IV
Formononetin
High-performance thin-layer chromatography

Summary

A new high-performance thin-layer chromatographic (HPTLC) method has been developed for the simultaneous estimation of astragaloside IV and formononetin in Radix Astragali. Samples were employed to degrease the materials by petroleum ether (boiling point: 60–90°C) and extracted by methanol, and then were alkalized and extracted with *n*-butanol saturated with water. Separation was achieved on HPTLC plates using petroleum ether (boiling point: 60–90°C) and *n*-butanol saturated with water–glacial acetic acid as the mobile phase, the results of which were compared with HPLC. The well-resolved peaks for astragaloside IV and formononetin were observed at R_F values 0.43 ± 0.02 and 0.75 ± 0.02, respectively. The calibration curves were found linear with a wide range of concentration 1.01–10.10 μg μL^{-1} with good correlation coefficient for astragaloside IV and formononetin. The method was validated for linearity, precision, reproducibility, accuracy, and limits of detection and quantification. This simple, rapid, sensitive, economic, and reliable HPTLC method is suitable for the routine quantitative analysis and quality control of traditional Chinese medicines (TCMs) such as Radix Astragali, which can be applied for the quality control of saponins and flavonoids in other plants or extracts.

1 Introduction

Radix Astragali, the dried root of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao or *A. membranaceus* (Fisch.) Bge., is known as Huangqi in China and has been widely used for the treatment of various cardiovascular diseases in East Asia [1–3]. It is one of the most widely used tonic herbs in many Chinese formulas, which has played an important role in clinical therapy and has been attracting more and more attentions for its complementary therapeutic effects to western medicines with low toxicity and few or even no complication [4]. The plant has attracted ever-increasing attention in a variety of pharmacological studies and clinical practices, including hepatoprotective, antioxidative, anti-inflammatory, anticancer, and immunological properties [5, 6], along with cardiotonic and antiaging activities [7, 8].

The chief ingredients in Radix Astragali are mainly saponins and isoflavonoids. Astragaloside IV (AS-IV, **Figure 1**), a major active saponin compound from Radix Astragali, is commonly used as standardization for the quality control of the herb and related preparations in the Pharmacopeia of the People's Republic of China. It has a broad range of pharmacological properties, such as anti-inflammation [9–11], inhibiting oxidative stress [12], promoting the discharge of pus and the growth of new tissues [13, 14], ameliorating renal fibrosis [15], and the ability to reduce infarct size as well as to improve postischemic heart function [14, 16].

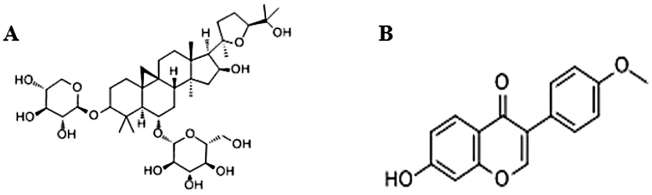

Figure 1

The structures of AS-IV (A) and FN (B).

Formononetin (FN, Figure 1), one type of isoflavonoids purified from *Astragalus membranaceus,* has been long used for treating carcinomas in China. It always can be used as a marker compound for the quality evaluation of Radix Astragali. The recent pharmacological results indicated that formononetin had the ability to vascular relaxation action [17], neuroprotective effects *in vitro* [18], and antioxidative and estrogenic effects [19, 20].

Normally, isoflavonoids and triterpene saponins are analyzed separately by thin-layer chromatography or high-performance liquid chromatography (HPLC–ultraviolet [UV] or HPLC–

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evaporative light scattering detector [ELSD]) [21, 22]. However, simultaneous determination of the two compounds would be a better strategy for the comprehensive quality evaluation. Unfortunately, the UV and ELSD signals contain complementary information; the UV detection strongly reveals UV-absorbing compounds and compounds below the detection limit of the ELSD such as isoflavonoids in Radix Astragali, while ELSD reveals non- or poorly UV-absorbing compounds such as saponins; the mentioned methods could only employ AS-IV and FN separately. If you want to simultaneously analyze AS-IV and FN by high-performance liquid chromatography, it should be coupled with diode array and evaporative light scattering detectors, which is expensive and time-consuming. However, based on literature researches, no studies have been conducted on the simultaneous detection of AS-IV and FN by high-performance thin-layer chromatography (HPTLC). Therefore, this research was aimed to develop a sensitive, rapid, and validated HPTLC method for the analysis of the two bioactive components in Radix Astragali, which may provide a basis for its quality evaluation.

2 Experimental

2.1 Materials and Methods

The instrument used in analysis was a CAMAG HPTLC system, comprising of Reprostar 3 digital documentation system, TLC Scanner 3, in absorbance mode, using the deuterium light source, and equipped with winCATS 1.4.2 software, twin-trough glass chamber (10 × 10 cm), TLC plate heater III (all CAMAG, Muttenz, Switzerland). Silica gel H efficient precast TLC plates (Yantai Xinde Chemical Co., Ltd., Yantai, China), silica gel G plates (Qingdao Haiyang Institute of Chemical Industry, China), and silica gel H plates (Yantai Xinde Chemical Co., Ltd., Yantai, China) were used as the stationary phase; the plates were developed in a CAMAG twin-trough glass chamber by ascending method. Water was purified by a Milli-Q System (Millipore Corporation, Bedford, MA, USA). All samples and mobile phases were prepared using analytical grade reagents. Standard solutions of AS-IV and FN were prepared using absolute methanol for the TLC–densitometry method and in the mobile phase for the HPLC method.

The plant of Astragalus species for the proposed study was collected from Lejialaopu, Hefei, Anhui, and authenticated by Professor Lihua Li, Department of The First Affiliated Hospital of Anhui University of Chinese Medicine. The standard compounds of AS-IV and FN were purchased from the National Institute for Control of Pharmaceutical and Biological Products (NICPBP, Beijing China).

2.2 Sample Preparation

The dried roots were powdered into pieces by a mill, accurately weighed (10 g), then wrapped with gauze, and defatted by Soxhlet extractor with 100 mL petroleum ether (boiling point: 60–90°C) for 30 min. The herb residue was added to a round-bottomed flask containing 60 mL of methanol, and the mixture was heated under reflux for 3 h. The methanol solution was filtered and evaporated to dryness. The residue was dissolved in 20 mL water and extracted with *n*-butanol saturated with water 3 times (30 mL, 30 mL, 20 mL); the pooled butanol extracts were basified with ammonia solution (2 × 20 mL). Discarding ammonia extracts, the butanol extracts were evaporated to dryness and dissolved in methanol and then made up to exactly 5 mL with methanol using a volumetric flask.

2.3 High-Performance Thin-Layer Chromatography (HPTLC)

HPTLC analysis was performed on 5 × 10 cm TLC glass plates coated with 200-µm layer thickness of silica gel H. Sample glass with an autosampler and three lanes were applied per plate with a 5-mm band width, 1 cm between bands, and 1 cm from the bottom of the plate. The plates were developed to a dis-

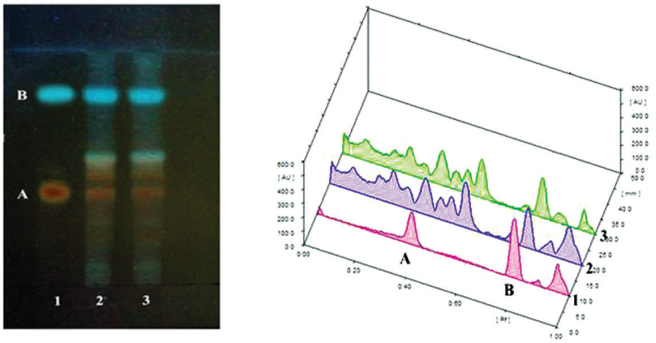

Figure 2

HPTLC separation of AS-IV and FN (track 1, mixture of AS-IV and FN standards; A, standard of AS-IV; B, standard of FN; tracks 2 and 3, sample extracts).

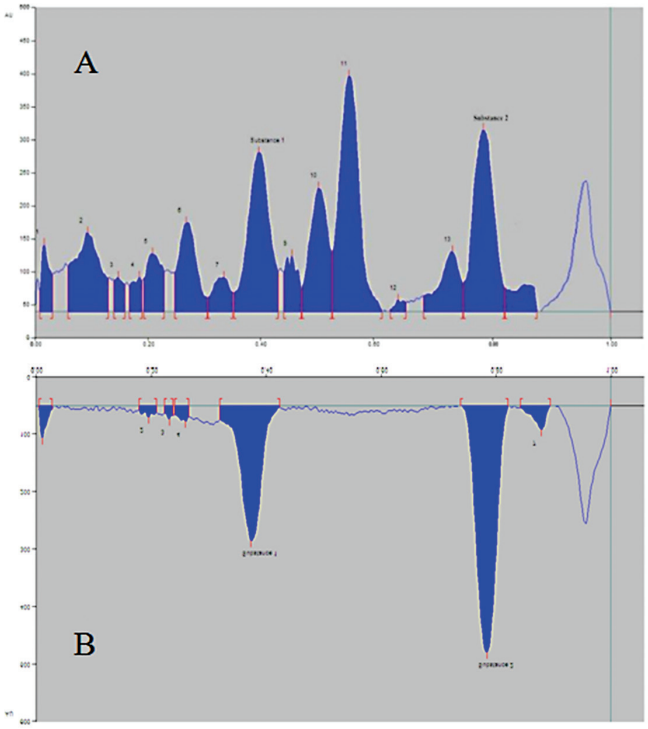

Figure 3

Single-track scan chromatograms of Radix Astragali (A); single-track scan chromatograms of a mixture of AS-IV and FN standards (B).

tance of 8 cm in a (10 cm × 10 cm) twin-through glass chamber which had been presaturated with mobile phase vapors (petroleum ether–*n*-butanol saturated with water–glacial acetic acid 3.5:2:4, *v/v*). The optimized chamber-saturation time was 15 min at room temperature. Development plates were air-dried; AS-IV points on the plate were derivatized by spraying 10% sulfuric acid ethanol solution with a CAMAG TLC Sprayer, while the FN points should be protected from derivatization. Then, it was heated to 105°C until the color of the AS-IV spots was clear, viewed in a CAMAG UV cabinet, and scanned at 254 nm by means of a CAMAG TLC Scanner 3 after full wavelength scanning from 200 to 400 nm **(Figures 2 and 3).** The mobilities of the compounds are expressed as retention factor (R_F) values (R_F = distance moved by compound / distance moved by solvent front).

2.4 High-Performance Liquid Chromatography (HPLC)

Analyses were performed on an Agilent Series 1260 liquid chromatograph (Agilent Technologies, Palo Alto, CA), consisting of a dual pump, an auto sampler, a DAD, an HP ChemStation software (Agilent Technologies, USA), and a ZORBAX ODS C_{18} column (4.6 mm × 250 mm I.D., 5 m). The column temperature was kept constant at 25°C and the mobile phase flow rate was 1 mL min^{-1}. The solvent system consisted of (A) acetonitrile and (B) water, using a ratio of 40:60 in 25 min **(Figures 4 and 5).** AS-IV and FN were detected on a DAD detector at 245 nm.

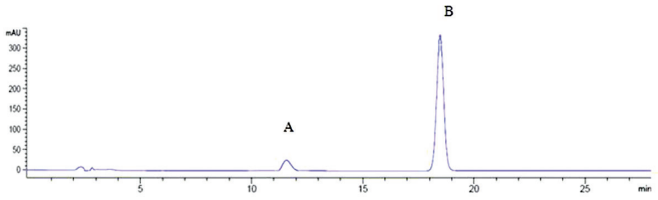

Figure 4

HPLC chromatogram of AS-IV and FN (A, standard of AS-IV; B, standard of formononetin).

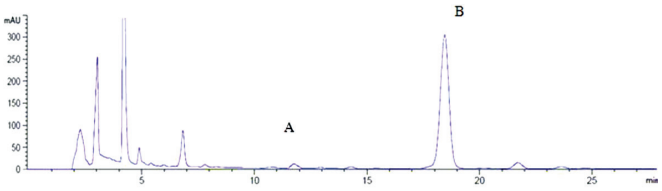

Figure 5

HPLC chromatogram of Radix Astragali (A, AS-IV; B, formononetin).

2.5 Method Validation [24–27]

The optimized HPTLC method was validated for linearity, limits of detection and quantification, precision, reproducibility, specificity, and accuracy.

2.5.1 Calibration Curves and Limits of Detection and Quantification

The calibration curve area *versus* concentration was found to be linear in the range of 1.01–10.10 μg μL^{-1} of astragaloside IV and formononetin. Sample solutions of 1, 2, 4, 6, 8, and 10 μL were spotted on HPTLC plates to obtain 1.01, 2.02, 4.04, 6.06, 8.08, and 10.1 μg per spot, respectively. The linear regression data for the calibration curve for the two samples, astragaloside IV and formononetin, showed a good linear relationship over the concentration with respect to peak area, as shown in **Table 1.** The limit of detection (LOD) and limit of quantification (LOQ) were evaluated on the basis of signal-to-noise ratios of 3 and 10, respectively.

Table 1

Analytical characteristics of the validated HPTLC method for the quantitation of AS-IV and FN.

Parameter	AS-IV	FN
R_F value	0.43 ± 0.02	0.75 ± 0.02
Densitometric linear relationship		
Working concentration range	1.01 μg μL^{-1} – 10.10 μg μL^{-1}	1.01 μg μL^{-1} – 10.10 μg μL^{-1}
Regression equation	1165.3x + 23.047	3448.9x + 74.736
Correlation coefficient (r^2)	0.992	0.996
Precision (RSD, %)	0.36	0.19
Reproducibility (RSD, %)	1.26	0.95
Stability (RSD, %)	0.18	0.16
Average recovery (%)	97.9	98.6

2.5.2 Precision

The precision of the method was assessed by six replicate analysis of the same concentration of the standard solution and expressed as relative standard deviation (RSD).

2.5.3 Reproducibility and Stability

To test the reproducibility of the assay, six independently prepared samples of Radix Astragali extracts in parallel were prepared and analyzed. The stability of the samples was analyzed every 2 h within 24 h at room temperature. Variations were expressed as RSD.

2.5.4 Specificity

The specificity of the method was determined by analysis of standards and test samples. The identity of the sample spots from the samples was confirmed by comparison of their R_F and spectrums with those from standards **(Figure 6).**

2.5.5 Accuracy

Recovery was assessed by the method of standard additions. An accurately known amount of the standard solutions was added to the known sample with three concentrations of the drug, corresponding to 80, 100, and 120%, and then, extraction and analysis were done as described above (Table 1).

2.5.6 Sample Analysis

The method was subsequently applied for the simultaneous determination of the two compounds in sample. The sample was analyzed using the optimized extraction and hydrolysis method under optimized TLC and HPLC conditions. The results are shown in **Table 2.**

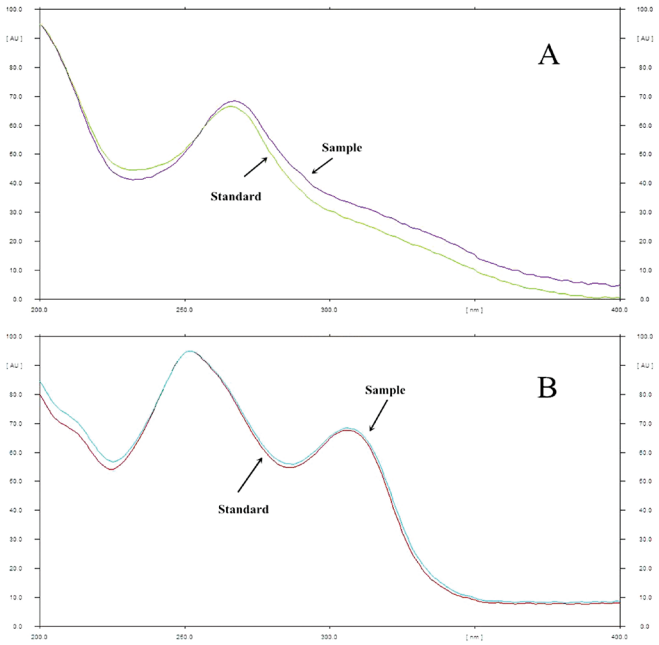

Figure 6

Comparison of the UV spectra of the respective compounds of AS-IV (A) and FN (B) in sample and standard.

Table 2

Assay for AS-IV and FN in samples.

	AS-IV		FN	
	Found (n = 3) (mg g^{-1})	RSD (%)	Found (n = 3) (mg g^{-1})	RSD (%)
Sample	1.114	1.10	0.296	0.98

3 Results and Discussion

According to the scanning profile and single-track scan chromatograms, two spots in the sample of Radix Astragali have a similar color and shape to the standards of AS-IV and FN, and the retention factors (R_F) of the two spots are 0.43 and 0.75, respectively, which are also the same as the standards (Figures 2 and 3).

3.1 Method Optimization

It is well known that the application of method optimization plays a vital role in the separation of the objective, such as the mobile phase, the different extraction methods, the species of TLC plates, etc. In this present study, several different methods were tested in order to improve the degree of separation and image sharpness.

3.1.1 Extraction Method Optimization

In order to obtain quantitative extraction, the purification method and the extraction time were optimized. Basifying with ammonia solution [21] was compared with solid-phase extraction [22]. Eventually, results revealed that the samples were employed to degrease the materials by petroleum ether (boiling point: 60–90°C) and extracted by methanol and then were extracted with *n*-butanol saturated with water and alkalized with ammonia solution, which could obtain clearly spots and better separation.

3.1.2 HPTLC Method Optimization

In addition, mobile phase and plate species were also optimized, as shown in **Table 3** and **Figure 7** (A and B as high polarity mobile phase; C as low polarity phase; D, E, F, and G as medium polarity phase; H, I, and J as plate species).

Table 3

Results of method optimization.

Methods	Mobile phase	Ratio	TLC plate	R_F		Result
				AS-IV	FN	
A	Chloroform, methanol, water	8:1.5:0.5	Silica gel G plate	0.62	0.89	Spots unclear and on the high side
B	Chloroform, methanol	9:1		0.06	0.61	
C	Petroleum ether, ethyl acetate	1:1		0	0.40	Spots unclear, AS-IV spots on downside
D	Petroleum ether, ethyl acetate, glacial acetic acid	5:2:3		0.31	0.77	Sample spots unclear, and positions not reasonable
E	Petroleum ether, *n*-butanol saturated with water, glacial acetic acid	2:4:4		0.54	0.86	Spot positions reasonablebut unclear
F		3:2:4		0.41	0.74	Sample spots unclear, eluent stratification
G	Petroleum ether, *n*-butanol, glacial acetic acid	3:2:4		0	0.31	
H	Petroleum ether, *n*-butanol saturated with water, glacial acetic acid	3.5:2:4	Silica gel G plate	0.42	0.75	Spot positions reasonable but unclear
I		3.5:2:4	Silica gel H plate	0.44	0.76	
J		3.5:2:4	Silica gel H efficient plate	0.43	0.75	Spots clear with higher separation

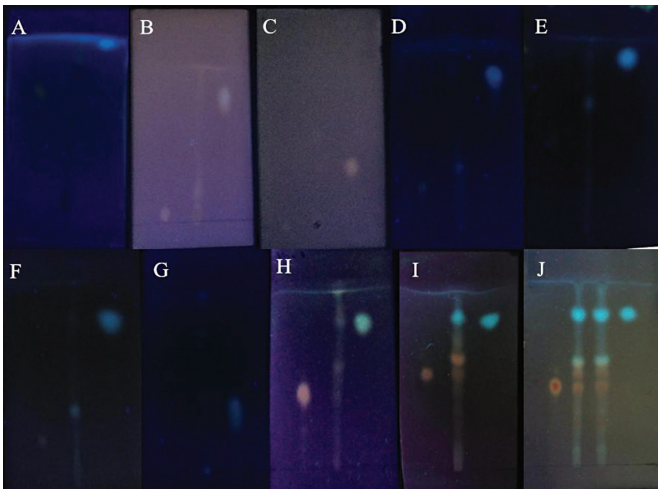

Figure 7

Optimization of the mobile phase, species of TLC plates as listed in Table 3. On the last plate, spots clear with higher separation.

A polar solvent like chloroform–methanol–water allowed the migration of the two standards in HPTLC, but both spots were on the high side. A gradient elution was found to be useful at the separation of the objective; more than ten different methods were studied in order to improve the degree of separation, to adjust the spots to an appropriate level, and to improve image sharpness. The results showed that images were unable to be perfect when just investigating the polarity of the mobile phase except for the type of chromatography plate. Basing on the results, we chose petroleum ether–*n*-butanol saturated with water–glacial acetic acid (3.5:2:4) as the best mobile phase and silica gel H efficient precast plate as the stationary phase with 5 μL sample volume (Table 3, Figure 7).

Due to the fact that AS-IV has the secondary UV absorption near 260 nm, while FN owns a strong UV absorption at 252 nm, we selected 254 nm as the detection wavelength. AS-IV was measured after chromogenic agent spraying, and there would be losses of the peak area in both the sample and the reference, which could make less influence on quantitative determination. In **Table 4,** both HPLC and HPTLC method have shown linearity for FN over the entire working range (1.01−10.10 μg μL^{-1}). However, the HPLC method could not show good linearity for AS-IV, while the linear range of the HPTLC method was 1.01−10.10 μg μL^{-1} for AS-IV; the calibration values for the HPLC and HPTLC methods were compared by analyzing a solution of mixed standards at known concentrations.

Table 4

Validation parameters of HPTLC and HPLC methods used for FN quantification.

Parameter	HPTLC	HPLC
Precision (RSD, %)	0.19	0.11
Reproducibility (RSD, %)	0.95	0.58
Stability (RSD, %)	0.16	0.09
Average recovery (%)	98.6	99.5
Analysis time (min)	15	25
Analysis cost (RMB)	10	120

4 Conclusion

Compared to HPLC, HPTLC offered a rapid, economic, and sensitive method for detection, which avoided the use of a plurality of detectors and could be more stable for the determination of Astragaloside because of its good linearity, precision, specificity, and accuracy. In addition, HPTLC analysis greatly simplifies the preprocessing of samples and eliminates the steps of chromatographic separation detection, which improves effiiency and decreases the experiment costs. Therefore, HPTLC offers significant advantages in that it has the ability to simultaneously run multiple samples, thus, using less solvent and saving time and cost. This work demonstrates that the proposed HPTLC–densitometry methods are satisfactory for the quantification of AS-IV and FN and could provide a reference for the quality control of Astragale.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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