Simultaneous Determination of Seven Flavonoids in Aerial Parts of *Artemisia frigida* by HPLC

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Abstract: **Objective** To establish an HPLC method for the determination of seven flavonoids from the aerial part of *Artemisia frigida*. **Methods** Hypersil ODS-2 (300 mm × 4.6 mm, 5 μm) column was used, with acetonitril-0.2% phosphoric acid (gradient elution) as a mobile phase, and the detection wavelength was at 283 nm with flow rate at 1 mL/min. **Results** All calibration curves showed good linear regression (r > 0.9990) within the tested range. All average recovery was more than 98.00% and RSD was less than 3.0% (n = 6). **Conclusion** The method is steady and with good repeatability, and could be used to determine the content of flavonoids in *A. frigida* from different areas.

Key words: *Artemisia frigida*; Compositae; flavonoids; HPLC; simultaneous determination

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Introduction

Traditional mongolian medicine (TMM), one of the important parts in Chinese pharmaceutical treasure house, has played an important role in the prevention and treatment of diseases in China for thousands of years. The aerial part of *Artemisia frigida* Willd. (Compositae), a commonly used TMM herbal medicine with the Mongolian name "Agei", is distributed in Inner Mongolia, Qinghai, and Gansu Provinces in China. It could be used to treat joint swelling, renal heat, abnormal menstruation, and sore carbuncle, and is also one of the components of "artificial holy water" (Bai, 1986).

Sesquiterpenoids (Liu and Mabry, 1981a), coumarins (Greger, Hofer, and Robian, 1983), and flavonoids (Liu and Mabry, 1981b; 1981c; Konovalova and Sheichenko, 1991) have been found in the aerial part of *A. frigida*. We also isolated two new flavonoid glycosides, 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone-7-*O*-β-*D*-glucuronyl-(1→2)-*O*-β-*D*-glucuronide (**1**) and 5,7-dihydroxy-3′,4′,5′-trimethoxy flavone 7-*O*-β-*D*-glucuronide (**2**), along with five known flavonoids, 5,7-dihydroxy-3′,4′-dimethoxy flavone-7-*O*-β-*D*-glucuronide (**3**), chrysoeriol-4′-*O*-β-*D*-glucoside (**4**), 5,7,3′-trihydroxy-4′-methoxy flavone (**5**), 5,3′-dihydroxy-6,7,4′-trimethoxy flavone (**6**), and 5,7,3′-trihydroxy-6,4′-dimethoxy flavone (**7**) from the EtOAc-soluble fraction (Fig. 1) and flavonoids (Huang, Ma, and Chen, 2000). The seven flavonoids exhibited the significant pharmacological activities including anti-inflammation, analgesia, and anti-oxidant, which are consistent with the clinic use of *A. frigida* in Mongolian clinic.

Flavonoids were the major bioactive constituents of *A. frigida*. To compare the contents of major bioactive constituents in the aerial part of *A. frigida* from different habitats, it is essential to develop an effective quantitative analysis method. Unfortunately, only a few analytical methods for the quality control of *A. frigida* have been developed, such as GC-MS for essential oils of the herb (Yang, Li, and Xiao, 2006; Tang, Xin, and Zhang, 2007) and a few reports on determination the total flavonoids in the aerial part of *A. frigida*. (Zhang *et al*, 2008). No literatures have been given on accurate quality evaluation of *A. frigida*. Here an HPLC simultaneous quantification of flavonoids in *A. frigida* is developed.

Materials and methods

Reference substances and reagents

The reference substances of compounds **1**—**7** were

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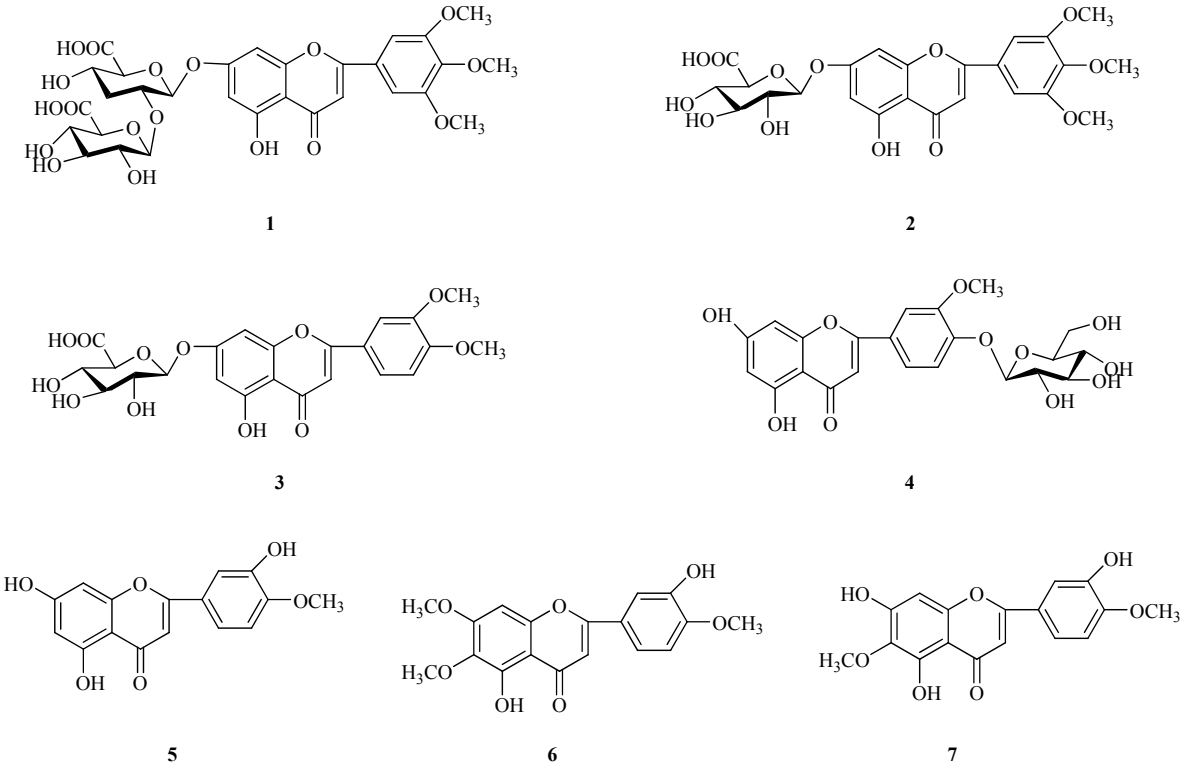

Fig. 1 Chemical structures of seven flavonoids from *A. frigida*

isolated previously from the aerial part of *A. frigida* by Wang *et al* (2009; 2010), and the structures were elucidated based on ^{1}H-NMR and ^{13}C-NMR spectral analyses (Li, Wang, and Liu, 2002; Li, Zhang, and Xiao, 1995; Rushdi *et al*, 2003). The purities of these reference compounds were determined to be above 98.0% by normalization of the peak areas detected by HPLC-DAD.

Acetonitrile and methanol of HPLC grade were from Guangfu Fine Chemical Institute (Tianjin, China). Ultrapure water was purified by Milli-Q instrument (Millipore, USA). Chloroform, anhydrous methanol, anhydrous ethanol, and phosphoric acid were of analytical grade from Yuwang Industry Co., Ltd. (Dezhou, China). D-101 macroporous adsorption resin was purchased from Bailian Fine Chemical Co., Ltd. (Tianjin, China).

Plant materials

Ten dried samples from the aerial part of *Artemisia frigida* Willd were collected in different habitats and identified by Prof. Buhebateer (College of Mongolian Medicine and Pharmacy, Inner Mongolia University for Nationalities, China). The collection habitats and time of these samples were shown in Table 1. No literature was reported during the past decades on the optimal collection time and processing method for *A. frigida*. In this experiment, the ten samples were mainly collected from June to August, dried in an oven at 30 ℃ until a constant weight, and comminuted for further extraction and separation. A voucher specimen (AFW-10-04-28) has been deposited in the Laboratory of Phytochemistry in College of Traditional Mongolian Medicine, Inner Mongolia University for Nationalities, China.

Table 1 *A. frigida* samples collected from different habitats

Sample No.	Habitats	Collected time
1	Xinjiang	July, 2008
2	Ximeng*	August, 2009
3	Ameng*	August, 2007
4	Humeng*	July, 2008
5	Tongliao*	June, 2009
6	Chifeng*	July, 2009
7	Qinghai	June, 2008
8	Huhehaote*	June, 2008
9	Bameng*	August, 2008
10	Xinganmeng*	August, 2008

* Inner Mongolia in China

Apparatus and chromatographic conditions

The LC system consists of a Shimadzu LC10-Atvp

Pump, Shimadzu SPD-M10Avp Photodiode Array Detector, Shimadzu SCL-10Avp software for data processing, and a Shimadzu DGU-12A Online Deaerator (Japan).

The chromatographic separation was performed on Hypersil ODS-2 analytical column (300 mm × 4.6 mm, 5 μm). The mobile phase was acetonitrile (A) and 0.2% phosphoric acid (B) with the gradient elution procedure: 0－10 min, 15% A; 10－25 min, 25% A; 25－35 min, 45% A; 35－40 min, 85% A. The flow rate was 1.0 mL/min and the column temperature was maintained at 30 ℃. The detection wavelength was 283 nm, the injection volume was 20 μL, and the chromatogram in 40 min was recorded in individual analysis.

Sample preparation

The stock solutions of compounds **1** － **7** in acetonitrile were prepared at the concentration of 1 mg/mL, respectively. Then, the stock solutions were diluted to proper concentration ranges for the establishment of calibration curves.

The optimized solvent and extraction procedures were applied for the preparation of sample solutions. Powdered samples (0.1 g) were extracted with 20 mL methanol in an ultrasonic bath at room temperature for 30 min. The extract was filtered and 10 mL filtrate was put on the D-101 macroporous adsorption resin column chromatography (20 g). After eluting with 30 mL ethanol-water (20:80), 100 mL ethanol-water (75:25) eluate was concentrated in vacuum to dry. The residue was then dissolved in 10 mL acetonitrile. The solutions were filtered through a 0.45 μm nylon syringe filter (Millex-HN, Ireland) before LC analysis.

Results and discussion

Optimization of extraction procedure

Three extraction solvents were selected and the separation procedure on D-101 resin was optimized as follows. Sample 5 (0.1 g) collected in Tongliao were extracted by ultrasonicator for 30 min with 20 mL of chloroform, chloroform-methanol (1:1), and methanol, respectively. Each of the extracts was filtered and separated by the method described above. The chromatograms of seven bioactive components in the methanol extracts with different eluates on D-101 resin and in the different solvent extracts with 100 mL ethanol-water (75:25) eluate on D-101 resin were shown in Fig. 2, and the contents of seven bioactive components in the different solvent extracts with 100 mL ethanol-water (75:25) eluate on D-101 resin were shown in Table 2.

Fig. 2 and Table 2 showed that the total content of seven reference substances in the extraction yield

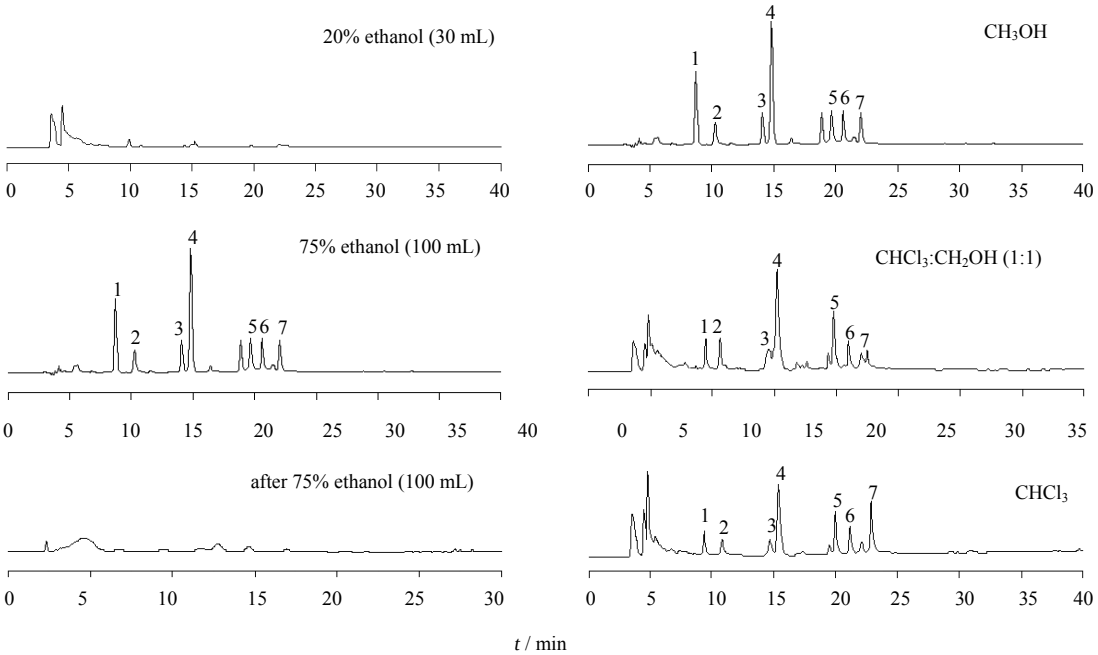

Fig. 2 LC chromatograms of seven bioactive components

Table 2 Determination of seven compounds in different solvent extracts (μg·g^{-1})

Solvents	**1**	**2**	**3**	**4**	**5**	**6**	**7**	Total
CH_3OH	3.225±0.029	1.010±0.011	1.229±0.021	6.569±0.015	1.113±0.014	0.992±0.010	1.104±0.013	15.242
$CHCl_3$-CH_3OH	1.087±0.018	1.128±0.008	0.811±0.006	6.477±0.024	1.305±0.012	0.876±0.006	0.603±0.002	12.287
$CHCl_3$	0.358±0.004	0.225±0.003	0.182±0.002	1.216±0.014	0.503±0.008	0.398±0.003	1.209±0.012	4.091

with methanol was more than those with chloroform-methanol (1:1) and chloroform, and its extract gave sharp and detached peaks after separation by D-101 resin. The seven reference substances were successfully eluted from D-101 resin by 100 mL ethanol-water (75:25) following 20% ethanol and were separated from other impurities. Therefore, methanol was selected as extraction solvent. Compared to Soxhlet extraction and refluxing extraction, the ultrasonic treatment procedure was found to be more effective and simple for seven bioactive components and so selected as the optimum extraction method. In order to investigate the volume of solvent, about 0.1 g of dried sample 5 was extracted with 5, 10, 15, 20, and 25 mL methanol, and the results showed that 20 and 25 mL methanol had the highest extraction yield with almost equal extraction capacities. Therefore, 20 mL was selected as the solvent volume. The influence of extracting time on the efficiency of the extraction was also observed. The results showed that the highest amounts of seven bioactive components were obtained with the extracting time of 30 min.

Optimization of chromatographic conditions

The selection of LC conditions was guided by the requirements for obtaining chromatograms with better resolution of adjacent peaks within a short time. In this study, mobile phase, the gradient mode and the operating conditions were optimized through several trials to achieve good resolution and symmetric peak shapes of seven reference compounds. Preliminary researches indicated that better separation and results were obtained using a mobile phase of water and acetonitrile rather than water and methanol. Therefore, in this work, water and acetonitrile were chosen as the mobile phase. Acid contained in the mobile phase, which could suppress the ionization of phenolic hydroxyl and carboxyl groups, was beneficial for good retention and separation of the seven reference compounds, and so 0.2% phosphoric acid was selected for the aqueous phase. Further studies found that many interfering compounds were present in the plant material. Thus, gradient elution program was carried out to separate these compounds in samples. The optimum eluting procedure was described above. The effect of temperature on the separation was investigated within the range of 25－40 ℃ and 30 ℃ was found to be the optimal temperature. The most suitable flow rate was found to be 1.0 mL/min. On the basis of UV-Vis spectra of the seven compounds recorded by DAD detection in the range of 190－800 nm, experimental results indicated that seven compounds had the same UV absorption peaks at about 283 nm, so 283 nm was selected for monitoring. The chromatograms of seven compounds and sample 5 are shown in Fig. 3.

System suitability test

The system suitability, performed using six replicate injections of the standard solution, showed that the mean values of theoretical plate number for the analytes were 200 876 (**1**), 178 343 (**2**), 104 326 (**3**), 196 133 (**4**), 142 323 (**5**), 232 247 (**6**), and 163 277 (**7**), respectively. The resolution values were all more than 1.5 and the relative standard deviations (RSD) of peak areas ranged from 0.67% to 2.15%.

Calibration curves

A series of concentration levels were prepared and chromatography was carried out in triplicate for calibration and linear analysis of each standard compound. The calibration curves were constructed by peak-area of the analytes (*Y*) against concentration of the calibration standards (*X*). The concentration of analytes in unknown samples was determined by interpolation from the calibration curve. Under the chromatographic conditions adopted in this study, all calibration curves exhibited good linearity ($r > 0.9991$) in a relatively wide concentration range (Table 3).

Limits of detection and quantification

The stock solutions of the seven reference compounds were further diluted to a series of concentration with acetonitril to test the limit of detection

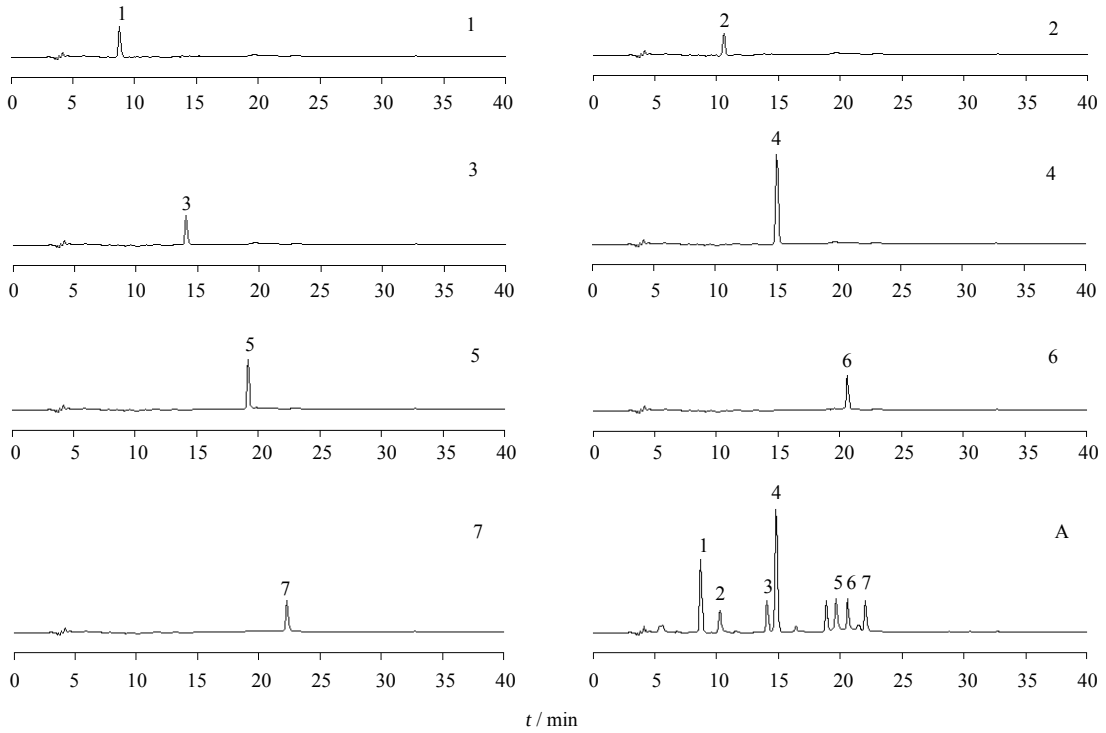

Fig. 3 Typical LC chromatograms of seven reference substances and sample solution

1－7: reference substances solution A: sample 5

Table 3 Calibration curves for seven compounds in *A. frigida*

Compounds	Calibration curves	*r*	Linear range / (μg·mL^{-1})	LOD / (μg·mL^{-1})	LOQ / (μg·mL^{-1})
1	$Y = 2.003 \times 10^6X + 8767$	0.9998	1.00－10.00	0.04	0.13
2	$Y = 2.195 \times 10^6X + 11\,653$	0.9992	0.50－10.00	0.06	0.20
3	$Y = 2.281 \times 10^6X - 12\,964$	0.9991	0.50－10.00	0.03	0.10
4	$Y = 1.229 \times 10^5X - 4444$	0.9991	5.00－80.00	0.18	0.60
5	$Y = 2.527 \times 10^5X - 3111$	0.9999	2.00－40.00	0.12	0.40
6	$Y = 3.702 \times 10^6X + 12\,567$	0.9995	1.00－30.00	0.04	0.13
7	$Y = 1.890 \times 10^6X + 6898$	0.9993	1.00－30.00	0.08	0.27

(LOD) and the limit of quantification (LOQ). The LOD and LOQ values under the present chromatographic conditions were determined at signal-to-noise (S/N) ratio of 3 and 10, respectively. LOD and LOQ for each reference compound were listed in detail in Table 3.

Precision and stability

The intra-day precision (presented as RSD) was determined for calibration sample by analyzing the five replicates on the same day, while the inter-day values were carried out over consecutive 5 d. In order to evaluate the repeatability of the developed assay, five different working solutions prepared from sample 5 were analyzed. The RSD was taken as a measure of precision. The sample stability test was performed with one sample at 0, 3, 6, 12, 24, and 48 h within 2 d. During this period, the solution was stored at room temperature. Variations were expressed by RSD (Table 4).

Table 4 showed the results of the tests of precision and stability of the seven compounds. It indicated that the RSD values of the overall intra- and inter-day variations were less than 2.56% for all seven compounds. Further, validation studies of this method proved that this assay had good reproducibility with RSD less than 2.01% for all the analyses and the sample solutions were stable during 48 h at room temperature with RSD less than 1.44%.

Table 4 Precision and stability of seven compounds

Compounds	RSD / %			
	Intra-day (n = 5)	Inter-day (n = 5)	Precision (n = 5)	Stability (n = 6)
1	1.88	2.05	1.25	1.42
2	2.41	2.56	1.38	1.36
3	2.02	2.54	2.01	1.44
4	1.32	1.22	1.54	1.04
5	0.87	1.08	0.74	0.88
6	0.58	1.12	0.12	0.28
7	2.08	2.26	1.54	1.36

Accuracy

Accuracy was determined by recovery test with an appropriate amount of herb sample spiked with three different (low, medium, and high) quantities of authentic standards. Each sample was analyzed in triplicate. The results showed that the developed analytical method had good accuracy with the overall recovery from 97.26% to 98.92% for the compounds concerned (Table 5). Therefore, the HPLC method was precise, accurate, and sensitive enough for simultaneously quantitative evaluation of seven compounds from the aerial part of *A. frigida*.

Method performance of sample assays

Ten samples of *A. frigida* from different habitats were extracted and separated as described above. Extraction solution (20 μL) was subjected to the determination under the selected chromatographic conditions. The results showed that compound **4** was the most abundant among seven compounds in most samples, but with great variations (3.862－12.97 mg/g) (Table 6). The content of total flavonoids in the aerial parts of *A. frigida* also showed high variations (9.235－23.27 mg/g). The higher degree of variability in the flavonoid content (total and individuals) in samples from different geographical locations could be due to various factors, such as geographical source, climate, harvest time, storage condition, and ages of the plant.

Table 5 Recoveries for seven analytes in *A. frigida* (n = 3)

Compounds	Original	Spiked	Found	Recoveries	RSD / %
1	0.1624	0.30	0.4574	98.33	0.97
		0.20	0.3555	96.55	
		0.10	0.2593	96.90	
2	0.0506	0.08	0.1300	99.25	0.92
		0.04	0.0896	97.50	
		0.02	0.0702	98.00	
3	0.0617	0.08	0.1414	99.62	1.10
		0.04	0.1013	99.00	
		0.02	0.0812	97.50	
4	0.3293	0.60	0.9291	99.97	1.68
		0.30	0.6238	98.17	
		0.15	0.4743	96.67	
5	0.0556	0.08	0.1347	98.88	1.47
		0.04	0.0943	96.75	
		0.02	0.0755	99.50	
6	0.0497	0.08	0.1295	99.75	1.25
		0.04	0.0895	99.50	
		0.02	0.0692	97.50	
7	0.0551	0.08	0.1344	99.13	1.21
		0.04	0.0947	99.00	
		0.02	0.0745	97.00	

Conclusion

A new analytical method for simultaneously quantitative evaluation of seven compounds from the aerial part of *A. frigida* was established to be precise and accurate.

Table 6 Determination of seven compounds in aerial part of *A. frigida* from ten different samples ($\bar{X} \pm s$, n = 5, μg·g^{-1})

Sample No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	—	1.092 ± 0.012	1.561 ± 0.021	7.012 ± 0.068	3.545 ± 0.052	1.123 ± 0.020	1.108 ± 0.016
2	0.815 ± 0.011	0.207 ± 0.004	0.798 ± 0.011	3.927 ± 0.034	2.427 ± 0.041	1.456 ± 0.035	0.663 ± 0.012
3	1.786 ± 0.023	0.893 ± 0.012	1.108 ± 0.020	8.829 ± 0.076	1.348 ± 0.024	0.978 ± 0.016	1.027 ± 0.018
4	—	0.903 ± 0.015	1.117 ± 0.024	12.86 ± 0.101	3.308 ± 0.062	3.566 ± 0.071	1.203 ± 0.022
5	3.247 ± 0.044	1.012 ± 0.014	1.233 ± 0.019	6.586 ± 0.072	1.112 ± 0.018	0.993 ± 0.019	1.102 ± 0.018
6	1.183 ± 0.015	0.877 ± 0.011	1.204 ± 0.026	9.475 ± 0.098	6.812 ± 0.087	1.078 ± 0.023	4.385 ± 0.098
7	—	0.898 ± 0.012	1.085 ± 0.016	12.97 ± 0.121	3.367 ± 0.074	3.827 ± 0.088	1.121 ± 0.017
8	—	0.655 ± 0.007	1.022 ± 0.011	8.799 ± 0.086	1.502 ± 0.039	1.024 ± 0.019	1.107 ± 0.017
9	—	0.289 ± 0.003	0.802 ± 0.010	3.862 ± 0.042	2.412 ± 0.051	1.285 ± 0.028	0.585 ± 0.009
10	0.824 ± 0.012	0.883 ± 0.014	1.101 ± 0.013	9.448 ± 0.102	6.522 ± 0.102	1.103 ± 0.018	4.402 ± 0.100

References

Bai QY, 1986. *Chinese Medicine. Encyclopaedia-Mongolian Medicine.* Inner Mongolia Science and Technology Press: Chifeng.

Greger H, Hofer P, Robian W, 1983. Types of sesquiterpene-coumarin ethers from *Achillea ochroleuca* and *Artemisia tripartita*. *Phytochemistry* 22: 1997-2003.

Huang HS, Ma CG, Chen ZW, 2000. The progress of the pharmacological effect study of flavonoids. *Chin J Chin Mater Med* 25: 589-591.

Konovalova OA, Sheichenko VI, 1991. Chemical composition of *Artemisia frigida*. *Chem Nat Compd* 27: 127-128.

Li HX, Wang Z, Liu YZ, 2002. Progress in studies on chemical constituents and pharmacological effects of Punicaceae. *Chin Tradit Herb Drugs* 33: 765-768.

Li WK, Zhang RY, Xiao PG, 1995. Studies on the chemical constituents of *Seseli yunnannense* Franch. *Chin Tradit Herb Drugs* 26: 568-570.

Liu YL, Mabry TJ, 1981a. Sesquiterpene lactones from *Artemisia frigida*. *J Nat Prod* 44: 722-728.

Liu YL, Mabry TJ, 1981b. Flavonoids from *Artemisia frigida*. *Phytochemistry* 20: 1389-1395.

Liu YL, Mabry TJ, 1981c. Two methylated flavones from *Artemisia frigida*. *Phytochemistry* 20: 309-311.

Rushdi AI, Ritter G, Grimalt JO, Simoneit BRT, 2003. Hydrous pyrolysis of cholesterol under various conditions. *Org Geochem* 34: 799-812.

Tang L, Xin K, Zhang W, 2007. GC-MS analysis on essential oil from traditional Mongolian medical herb *Artemisia frigida*. *Acta Chin Med Pharm* 35: 36-37.

Wang QH, Wang JH, Eerdunbagen Tana, 2009. Study on chemical constituent of *Artemisia frigida* Willd. *Chin Tradit Herb Drugs* 40(10): 1540-1543.

Wang QH, Ao WLJ, Wang XL, Bao XH, Wang JH, 2010. Two new flavonoid glycosides glycosides from *Artemisia frigida* Willd. *J Asian Nat Prod Res* 12: 950-954.

Yang LQ, Li CZ, Xiao DH, 2006. Analysis on essential oil from a traditional Mongolian medical herb *Artemisia frigida*. *J Inn Mon Univ Nat* 21: 275-278.

Zhang W, Tang L, Xin K, Cui J, 2008. Determination of total flavonoids in raw and charred Mongolian drug Agei. *Lishizhen Med Mater Med Res* 19: 2952-2953.