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Determination of flavonoids in *Semen Cuscutae* by RP-HPLC

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Abstract

Flavonoids contents in 40 samples of *Semen Cuscutae* collected from areas all around China were investigated. Five principal flavonoids, quercetin 3-*O*-β-D-galactoside-7-*O*-β-D-glucoside, quercetin 3-*O*-β-D-apiofuranosyl-(1→2)-β-D-galactoside, hyperoside, quercetin and kaempferol were analyzed simultaneously by using a reversed phase liquid chromatograph system with 0.025 M phosphoric acid–methanol as mobile phase. The recovery of the method was 97.0–102.9%, and all the flavonoids showed good linearity ($r \geq 0.9990$) in a relatively wide concentration range. The results indicated that contents of flavonoids in *S. Cuscutae* varied significantly from species to species, locality to locality, and parasiting host to host. *Cuscuta australis* contained a much higher content of kaempferol than *C. chinensis*, and few flavonoids were detected in *C. japonica*. The quality of *S. Cuscutae* can be evaluated according to the contents of flavonoids.

Keywords: *Semen Cuscutae*; Flavonoids; High performance liquid chromatography; Quality control; Chinese material medicine

1. Introduction

Semen Cuscutae is a well-known Chinese material medicine recorded first in the famous Shen Nong's Herbal as an upper grade drug. It has been used in China to treat impotence and seminal emission as a tonic for thousands of years. Modern pharmacological experiments suggest that it has such biological activities as improving sexual function, regulating the body's endocrine and immune system, and antioxidation [1–3].

The pharmacopoeia of People's Republic of China (Version 2000) specifies *S. Cuscutae* as the dried ripe seeds of *Cuscuta chinensis* Lam. (Family Convolvulaceae) [4]. However, this species is now rarely seen in the herb market according to the results of market investigation held lately by the author. *C. australis* R. Br. was found to be the predominant source of this drug. And *C. japonica* Choisy. was also used as *S. Cuscutae* in Southwest China. In addition, many fakes were found in the crude drug samples, which seriously influenced

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the drug's quality. Hence, quality control of this vegetable drug is in great demand. Unfortunately, few studies on the quantitative determination of chemical constituents in *S. Cuscutae* have been reported so far. In previous papers, total flavonoids or total polysaccharides were determined by colorimetry, but the results could not reflect the drug's quality exactly and rapidly [5].

Flavonoids are the main biologically active constituents in *S. Cuscutae*. Their contents can be an important index in quality evaluation of this crude drug. However, no report has been made on the determination of flavonols and their glycosides in this drug by High Performance Liquid Chromatography (HPLC) before. By preparative column chromatography, we have isolated a series of flavonoids from *S. Cuscutae*, and their chemical structures were identified by modern spectral techniques (UV, NMR and MS). Of these compounds, quercetin 3-*O*-β-D-galactoside-7-*O*-β-D-glucoside and quercetin 3-*O*-β-D-apiofuranosyl-(1→2)-β-D-galactoside are flavonol diglycosides rarely seen in the plant kingdom [6]. Their co-occurrence can be considered as an important characteristic of *C. chinensis* and *C. australis* with chemotaxonomic value. In addition, quercetin, kaempferol and hyperoside have been reported to exhibit various pharmacological activities which to some extent might elucidate the mechanism of clinical effects of this commonly-used Chinese medicine [7,8]. Therefore, quantitative analysis of these constituents is of great significance for the crude drug's quality control.

In this paper, the five major flavonoids in 40 *S. Cuscutae* samples were determined simultaneously by reversed phase liquid chromatography. Their contents in *S. Cuscutae* could be used to evaluate the crude drug's quality. Factors that influenced flavonoids contents were also discussed. In addition, a simple, rapid and accurate analysis method was presented.

2. Materials and methods

2.1. HPLC apparatus and conditions

An HP 1100 liquid chromatograph system consisting of a quaternary solvent delivery system, an autosampler and photodiode array detector coupled with an analytical workstation was used.

The column was a Hypersil-ODS (5 μm, 125 × 4.6 mm I.D.) packing. The eluents were A (aqueous 0.025 M phosphoric acid) and B (100% methanol). The initial mixture was A–B (82:18, v/v) for 3 min; linearly gradient to (55:45, v/v) in 11 min; and then hold for 3 min. The flow rate was 1 ml min^{-1}. The analysis was monitored at 360 nm and absorption spectra of the compounds were recorded between 200 and 400 nm. The column temperature was 40 °C, and the sample injection volume was 10 μl. The compounds were identified by comparing their t_R values and UV spectra with those of the standards.

2.2. Materials

Crude drug samples were purchased from drug stores all around China, or cultivated and collected by the author. All the samples were identified by Professor Yuning Yan (Beijing University of Chinese Medicine, Beijing, People's Republic of China). Voucher specimens were deposited at School of Pharmacognosy, Beijing University of Chinese Medicine.

2.3. Reagents and standards

Quercetin 3-*O*-β-D-galactoside-7-*O*-β-D-glucoside (**1**), quercetin 3-*O*-β-D-apiofuranosyl-(1→2)-β-D-galactoside (**2**), hyperoside (**3**), quercetin (**4**) and kaempferol (**5**) were all isolated by the author from the seeds of *C. chinensis* Lam. (see Fig. 1), and their

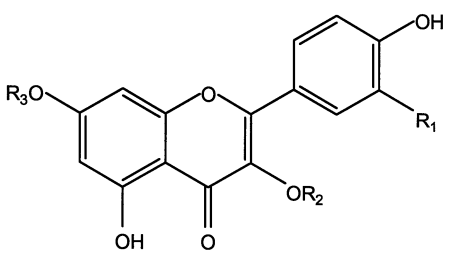

Fig. 1. Chemical structures of **1**–**5**. Quercetin 3-O-β-D-galactoside-7-O-β-D-glucoside (**1**). R_1 = OH; R_2 = gal; R_3 = glc. Quercetin 3-O-β-D-apiofuranosyl-(1→2)-β-D-galactoside (**2**). R_1 = OH; R_2 = api(1,2)-gal; R_3 = H. Hyperoside (**3**). R_1 = OH; R_2 = gal; R_3 = H. Quercetin (**4**). R_1 = OH; R_2 = R_3 = H. Kaempferol (**5**). R_1 = R_2 = R_3 = H. gal = galactosyl residue; glc = glucosyl residue; api = apiofuranosyl residue.

structures were fully characterized by chemical and spectroscopical methods (UV, IR, NMR, 2D-NMR, MS). Purity analysis suggested that their purity were above 98%.

Methanol and phosphoric acid were of analytical grade. The water used was of HPLC quality, purified in a Milli-Q Water Purification system (Millipore, MA, USA). The solvents were filtered through 0.45 μm membranes and degassed in an ultrasonic bath before use.

2.4. Sample preparation

Powdered samples (60 mesh, 1 g) were suspended in 80% methanol (40 ml) and extracted in an ultrasonic bath for 1 h. After filtration, the filtrate was transferred to a 50 ml volumetric flask and diluted with 80% methanol to volume. The obtained solution was filtered through a membrane filter (0.45 μm pore size) prior to injection.

2.5. Linearity studies

For standard solutions, reference compounds **1** (9.3 mg), **2** (11.3 mg), **3** (15.0 mg), **4** (4.0 mg) and **5** (9.1 mg) were dissolved in methanol and diluted with the same solvent to give eight different concentrations. Each concentration was analyzed five times using the same HPLC condition as described above.

3. Results

3.1. Extraction

In order to obtain quantitative extraction, variables involved in the procedure such as solvent and extraction time were optimized. Pure and aqueous methanol were tried as the extraction solvent. The best solvent was found to be 80% methanol which allowed extraction of all the flavonoids in high yields, while pure methanol could not extract the flavonol glycosides efficiently. According to the literature, an ultrasonic bath at room temperature was a suitable extraction system for flavonoids [9]. Hence the ultrasonic bath extraction was chosen as a preferred method. Extraction time was also investigated. Powdered seeds (1.0 g) were extracted with 40 ml 80% MeOH for 30, 60, 90, 120 min, respectively. The results suggested that all the flavonoids were almost completely extracted within 60 min. After the extraction, the residue was further extracted with 80% MeOH for another 60 min, and nearly no flavonoid was detected by TLC and HPLC. Therefore, later samples were extracted for 60 min.

3.2. Optimization of separation conditions

The choice of the experimental conditions was guided by the need to obtain chromatograms with better resolution of adjacent peaks within a short analysis time, especially when numerous samples were to be analyzed.

In the beginning, various mixtures of water and methanol were used as mobile phase but separation was not satisfactory. Phosphoric acid was then added to reduce the ionization of phenol groups, so that the flavonols and their glycosides could be separated much better [10–12]. It was also suggested that separation was improved when column temperature was increased to 40 °C.

Since the polarity, solubility and other characteristics of the five flavonoids differ greatly from each other, 40–50 min was needed for a single run with isocratic HPLC system. Finally gradient elution was carried out so as to ensure that each run of analysis completes within a short time. Fig. 2 showed chromatograms of mixed standards and the samples. The five flavonoids could be completely separated within 15 min.

The chromatograms were recorded at 255 and 360 nm, respectively, according to the UV absorption maxima of the flavonols. However, baseline separation of **1** and **2** could not be obtained at 255 nm. Therefore, 360 nm was chosen as the detection wavelength.

3.3. Regression equations

Linear regression analysis for each of the five flavonoids was performed by the external standard method. The calculated results were given in Table 1, where a, b and r were the coefficients of the regression equation $y = ax + b$, x referred to the concentration of the flavonoid

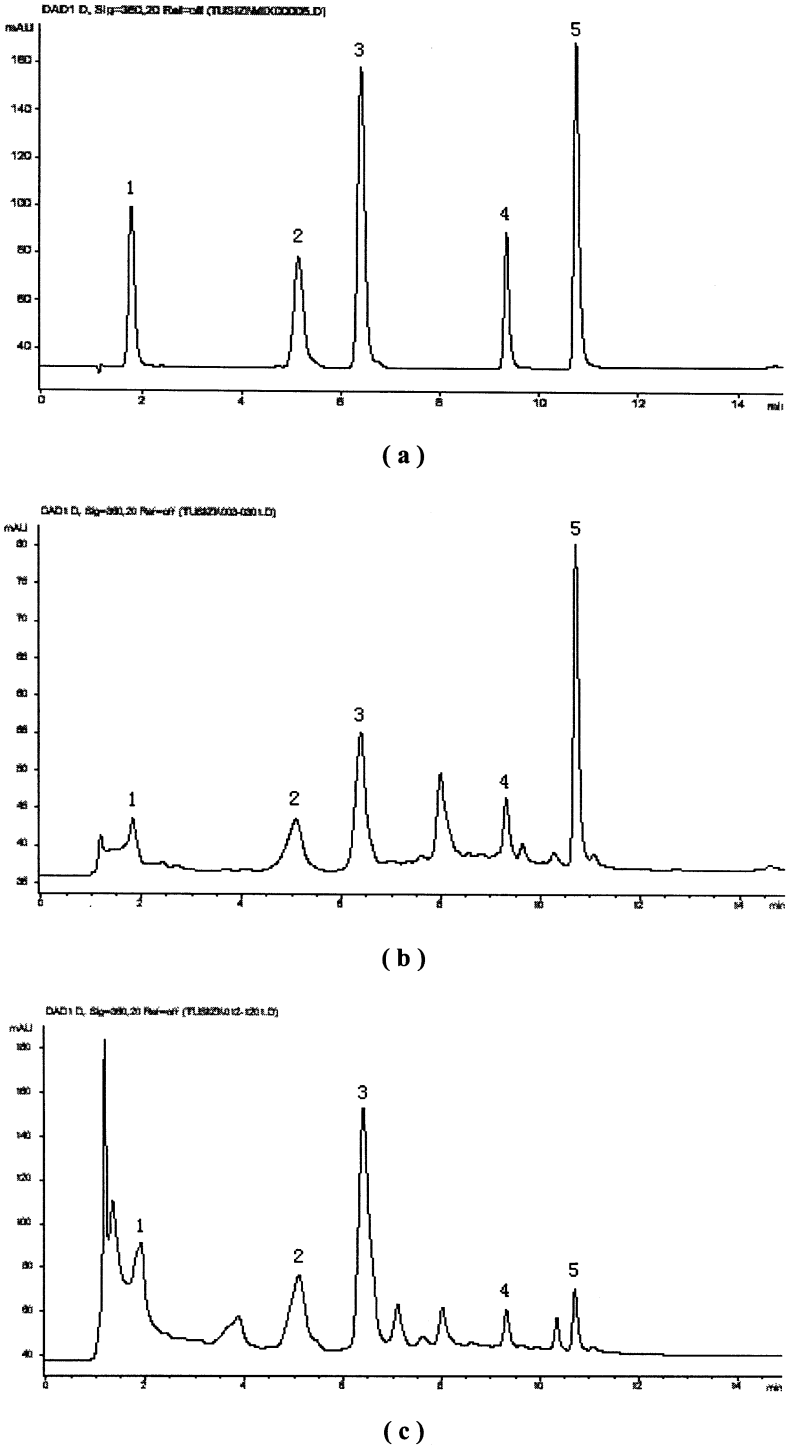

Fig. 2. HPLC chromatograms of (a) standard mixture, (b) *Cuscuta australis* and (c) *C. chinensis*. Conditions: mobile phase, 0.025 M phosphoric acid-methanol; flow rate, 1 ml min^{-1}; detection wavelength, 360 nm; column temperature, 40 °C; injection volume, 10 μl. For peak identification, see Fig. 1.

Table 1
Linear relation between peak area and concentration ($n = 8$)

Standard	Regression equation	r	Linear range (μg ml^{-1})	LOD (ng ml^{-1})
1	$y = 16\,512.4x - 13.37$	0.9996	1.488–59.52	103
2	$y = 15\,598.1x - 12.95$	0.9995	1.808–72.32	90
3	$y = 22\,337.8x + 0.94$	0.9999	2.400–96.00	60
4	$y = 39\,244.9x + 0.22$	0.9990	0.640–25.60	64
5	$y = 39\,829.6x + 0.53$	0.9997	1.456–58.24	72

In the regression equation $y = ax + b$, x refers to the concentration of the flavonoid compound (μg ml^{-1}), y the peak area, and r the correlation coefficient of the equation. LOD, limit of detection. For chemical structures of the standards, see Fig. 1.

compound (μg ml^{-1}), y the peak area, and r the correlation coefficient of the equation. All the flavonoid compounds showed good linearity ($r \geq 0.9990$) in a relatively wide concentration range. The limits of detection (LOD) ranged from 60 to 103 ng ml^{-1}, detected at 360 nm.

3.4. System suitability test

The recovery assays of the five flavonoids were carried out by adding the standards to the crude drug powder, which was treated according to the procedure described in Section 2.4. The average recovery for the five flavonoids was 97.0–102.9% (see Table 2).

The precision test was carried out by injecting the same sample solution five times, and the results showed that R.S.D. (relative standard deviation) of peak area of each flavonoid was 0.51–1.21%.

For stability test, the same sample solution was analyzed every 12 h in 2 days, and the analytes were found to be rather stable within 48 h (R.S.D. $< 3\%$).

3.5. Application

A volume of 10 μl of the solution of each sample was injected into the instrument. Peaks in the obtained chromatograms were identified by comparing the retention time and on-line UV spectra with those of the standards (see Fig. 2). Retention parameters for **1**, **2**, **3**, **4** and **5** were 1.79, 5.14, 6.40, 9.34, 10.75 min, respectively. The contents of the five flavonoids in the samples were calculated and the results were shown in Table 3 with the mean values of three replicate injections.

4. Discussion

The data in Table 3 showed that contents of the five determined flavonoids in *S. Cuscutae* varied considerably from species to species, from locality to locality, and from parasiting host to host. Since flavonoids are important biologically active secondary metabolites of *Cuscuta* spp., it is indicated that quality of this drug can vary significantly. This may be one of the most predominant reasons why curative effects of Traditional Chinese Medicine are uncertain and unstable. Therefore, quality control of this commonly used vegetable drug is in great demand. Hyperoside, quercetin and kaempferol isolated from *S. Cuscutae* have various pharmacological activities, such as improving the body's immune and endocrine system, and antioxidation, which to some extent can reflect the herb's clinical effects. Quercetin 3-*O*-β-D-galactoside-7-*O*-β-D-glucoside and quercetin 3-*O*-β-D-apiofuranosyl-(1→2)-β-D-galactoside are flavonoids rarely distributed in the plant kingdom, and can be considered as characteristic constituents of *S. Cuscutae*. Hence determination of the five principal flavonoids can be of great importance for the identification and quality evaluation of this herb drug.

The five flavonoids could be detected in the seeds of *C. chinensis* and *C. australis*, while were nearly not present in those of *C. japonica*. According to phytotaxonomic system, all the three species belong to the Genus *Cuscuta* Linn. *C. australis* and *C. chinensis* are under the Subgenus *Grammica*, while *C. japonica* belongs to the Subgenus *Monogyna*. Our results can be another support of the classification of *Cuscuta* spp. In addition, HPLC profile of *C. japonica* was quite different from those of *C. australis* and *C. chinensis*. Pharmacological experiments also suggested that biological activities of *C. japonica* were inferior to those of the other species [1]. So *C. japonica* could not be used as the substitute of *S. Cuscutae* in spite of its high yields.

There were also differences between the flavonoid contents of *C. australis* and those of *C. chinensis*. The seeds or stems of *C. australis* contained a much higher amount of kaempferol than those of *C. chinensis*. This character can be an important evidence in the identification of the two similar species. In previous papers, methods such as TLC, GC, HPCE and UV spectrum were tried to distinguish the seeds of the two species, but no satisfactory results were obtained [13–15]. However, results of this research suggested that they could be identified rapidly and easily by the proposed HPLC procedure.

In the stems of *Cuscuta* plants (Samples Number 35–40), flavonoid glycosides could hardly be detected. HPLC profiles of the stems were also quite different from those of the seeds, indicating that they contained different chemical constituents. Stems of *C. australis* contained a high level of kaempferol, and could be utilized as a new resource of this bioactive compound.

Cuscuta spp. are parasiting plants, and their chemical constituents are closely related to the hosts. For example, *Cuscuta* plants contain very few alkaloids themselves, but a large amount of alkaloids were detected in those plants parasiting on alkaloid-containing hosts [16,17]. Flavonoids can be synthesized by *Cuscuta* spp., while the influence of hosts on the flavonoid contents has not been studied yet. In this experiment, the original plants of Samples Number 33 and 34 were cultivated in the same plot of land and grew in the same environmental conditions, except that they parasited on *Achyranthes bidentata* Bl. and *Perilla frutescens* (L.) Britt., respectively. It was shown that the contents of flavonoids in these samples were quite different. Hence a further study on the relation between contents of flavonoids and hosts is needed.

Adulterant is another factor that seriously influences the drug's quality. Small gravels and seeds of other plants, which were difficult to be distinguished from the true drug because of their similar appearance were frequently found in the vegetable drug purchased from drug stores. For example, Samples Number 9 and 14 were completely of small stones, and seeds of *Brassica campestris* L. and other *Brassica* spp. (Family Cruciferae) were also found in some samples such as Number 26 and 27. As a result, none of the five flavonoid compounds were detected in these samples.

Table 2
Recovery of the flavonoids **1**–**5** ($n = 5$)

Compound (%)	Initial amount (mg)	Added amount (mg)	Detected amount (mg)	Recovery (%)	R.S.D.
1	0.2112	0.2381	0.4529	101.50	2.13
2	0.1829	0.2893	0.4808	102.97	2.53
3	0.5807	0.3840	0.9679	100.83	2.11
4	0.1252	0.1024	0.2245	97.01	1.83
5	0.6546	0.2330	0.8891	100.64	3.27

Table 3
Contents of flavonoids in *S. Cuscutae* samples (mg g^{-1}, $n = 3$)

Peak number	Species	Collection place	Collection date	**1**	**2**	**3**	**4**	**5**	Total
1	*C. australis*	Beijing	1999.4	0.8426	1.6903	1.6399	0.0169	0.3328	4.5225
2	*C. chinensis*	Anguo, Hebei	1990.9	0.7848	0.3839	1.4359	0.0995	0.0200	2.7241
3	*C. australis*	Ningxia	1997.1	0.2095	0.2090	0.6810	0.0331	1.0000	2.1326
4	*C. australis*	Xi'an, Shaanxi	1997.1	0.1276	0.1348	0.3782	0.0336	0.3526	1.0268
5	*C. australis*	Beijing	1997.4	0.1792	0.2396	0.3725	0.0324	0.2871	1.1108
6	*C. australis*	Xining, Qinghai	1997.5	0.2694	0.5372	0.7280	0.0224*	0.5967	2.1537
7	*C. australis*	Shanghai	1997.1	0.1987	0.2823	0.5071	0.0265*	0.1983	1.2129
8	*C. australis*	Beijing	1997.4	0.2572	n.d.	0.1187*	0.0239*	0.3529	0.7527
9	fake	Anguo, Hebei	1998.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	*C. australis*	Zhuji, Zhejiang	1997.1	0.2067	n.d.	n.d.	0.0266	0.2707	0.5040
11	*C. australis*	Dawa, Liaoning	1997.6	0.2112	0.1829	0.5807	0.1252	0.6546	1.7546
12	*C. australis*	Dalian, Liaoning	1997.1	0.1137	0.1102	0.0459*	n.d.	0.0796	0.3494
13	*C. australis*	Hangzhou, Zhejiang	1997.4	0.1779	0.2172	0.1352	0.0336	0.4951	1.0590
14	fake	Inner Mongolia	1997.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15	*C. australis*	Zhengzhou, Henan	1997.5	0.2634	0.6256	0.5168	n.d.	0.1295	1.5353
16	*C. australis*	Hefei, Anhui	1997.3	0.0721	0.1042	0.1275	n.d.	0.0965	0.4003
17	*C. australis*	Beijing	1997.4	0.1019	0.1517	0.1797	n.d.	0.1027	0.5360
18	*C. australis*	Huanggang, Hubei	1997.1	0.1208	n.d.	n.d.	n.d.	n.d.	0.1208
19	*C. australis*	Sanmenxia, Henan	1997.2	0.1137	0.1395	0.4740	0.0448	0.4021	1.1741
20	*C. australis*	Datong, Shanxi	1997.1	n.d.	n.d.	0.2051	n.d.	0.5622	0.7673
21	*C. australis*	Jilin	1997.1	0.0944	n.d.	0.0553*	n.d.	n.d.	0.1497
22	*C. australis*	Qingdao, Shandong	1997.3	n.d.	n.d.	0.0438*	n.d.	0.1233	0.1671
23	*C. japonica*	Guizhou	1998.9	n.d.	n.d.	0.2631	n.d.	n.d.	0.2631
24	*C. japonica*	Lianhuachi, Sichuan	1998.6	n.d.	n.d.	0.2094	n.d.	n.d.	0.2094
25	*C. japonica*	Chongqing	1999.3	n.d.	n.d.	0.2321	n.d.	n.d.	0.2321
26	*Brassica campestris* L.	Shenyang, Liaoning	1997.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
27	fake	Zhangshu, Jiangxi	1997.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
28	*C. chinensis*	Anguo, Hebei	1997.9	0.4605	0.3605	0.6820	0.0366	n.d.	1.5396
29	*C. chinensis*	Anguo, Hebei	1997.9	0.3280	0.5646	1.1126	n.d.	0.0316*	2.0368
30	*C. chinensis*	Beijing	1999.9	0.4031	n.d.	0.7144	0.0386	0.0339*	1.1900
31	*C. chinensis*	Beijing	1997.9	0.7244	n.d.	0.6308	n.d.	n.d.	1.3552
32	*C. chinensis*	Beijing	1997.10	1.5605	n.d.	0.9127	0.0279	0.0501*	2.5512
33	*C. australis*	Beijing	1997.9	0.4684	n.d.	n.d.	0.2643	4.4490*	5.1817
34	*C. australis*	Beijing	1997.9	0.4955	0.8363	1.1060	n.d.	1.8441	4.2819
35	*C. australis*	Beijing	1997.9	n.d.	n.d.	n.d.	0.2043	3.2572	3.4615
36	*C. australis*	Beijing	1997.9	n.d.	n.d.	n.d.	0.1113	2.1417	2.2530
37	*C. chinensis*	Beijing	1997.9	n.d.	n.d.	0.9262	0.0543	n.d.	0.9805
38	*C. chinensis*	Anguo, Hebei	1997.9	n.d.	n.d.	1.0793	0.0696*	0.0589	0.2078
39	*C. chinensis*	Anguo, Hebei	1997.9	n.d.	n.d.	0.6671	0.0170*	0.0520	0.7361
40	*C. japonica*	Sichuan	1998.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Notes: Samples Number 1–27 were purchased from drug stores. Samples Number 28–32 and Number 37–40 were collected from the fields and Number 33–36 were cultivated by the author. Samples Number 35–40 were stems of *Cuscuta* spp., while the other samples were seeds. Samples Number 28 and 39 (parasiting on *Chenopodium album* L.), Number 29 and 38 (parasiting on *Salsona collina* Pall.), Number 33 and 35 (parasiting on *Achyranthes bidentata* Bl.), Number 34 and 36 (parasiting on *Perilla frutescens* (L.) Britt.) were seeds and stems of the same plant, respectively. The original plants of Samples Number 31 and 32 parasited on *Humulus scandens* (Lour.) Merr. and *Chrysanthemum morifolium* Ramat., respectively. n.d., not detected. *, Out of calibration range.

5. Conclusions

This was the first report of simultaneous determination of the major flavonoids in *S. Cuscutae*. A simple, rapid and accurate approach was presented. The data obtained by analyzing 40 samples suggested that contents of flavonoids in *S. Cuscutae* collected from different areas varied significantly. The five falvonoids were detected in *C. australis* and *C. chinensis*, while nearly not present in *C. japonica*. Therefore, It was proposed by the authors that *C. japonica* should not be considered as a source of *S. Cuscutae*. *C. australis* contained a much higher amount of kaempferol than *C. chinensis*. This character could be used to distinguish the two similar species. Parasiting host was also found to be a factor which influenced flavonoid contents in *S. Cuscutae*. The HPLC method developed here represented an excellent technique for quality control of this vegetable drug.

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