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Rapid Fingerprint Analysis of Flos Carthami by Ultra-performance Liquid Chromatography and Similarity Evaluation

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Abstract

Flos Carthami (FC), the dried flower of *Carthamus tinctorius* L., has widely been used for the treatment of cardiovascular diseases in China. A rapid fingerprint analysis method of FC was established by ultra-performance liquid chromatography (UPLC) for the quality control. The analysis time was shortened by 45 min, from 75 min on conventional high-performance liquid chromatography (HPLC) to 30 min on UPLC. Ten batches of FC samples from various sources were analyzed by the UPLC method. Coexisting peaks in FC chromatograms were chosen to calculate their relative retention time (RRT) and relative peak area (RPA). Thirteen characteristic peaks were illustrated on UPLC fingerprints. In the 24-h stability test, the relative standard deviation (RSD) for the RRT and RPA was less than 0.08% and 4.41%, respectively. The RSD for precision of the RRT and RPA was less than 0.05% and 4.26%, respectively. And the RSD for repeatability was less than 0.05% for the RRT and 3.91% for the RPA. The similarities of the ten batches of FC were ranged from 0.938 to 0.995. Compared to the HPLC method, UPLC provided shorter analysis time, higher resolution and better separation performance, which is feasible for the quality control of FC.

Keywords: Flos Carthami; *Carthamus tinctorius*; UPLC; Chromatographic fingerprint; Similarity

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Introduction

Traditional Chinese Medicine (TCM) had always been regarded as one of the Chinese traditional treasures, due to its long history and rich clinical experience. With the development of globalization, Chinese herbal medicine is gaining more and more attention for its unique curative in many countries. However, the quality control of TCM is becoming a challenging task (1). Chinese herbal medicine has a variety of complex components, and its efficacy may result from the combined action of its diversified active ingredients (2, 3). Moreover, the components, quality, and clinical efficacy can vary, depending on growing environment, climate conditions, harvest seasons, processing methods and storage time (2, 4). Adulteration or selling counterfeit TCMs phenomenon is seen occasionally in different parts of the world. This situation is not only adds difficulty to the identification of Chinese medicinal materials, also caused the decline in the quality of TCM. Thus, it is essential to develop a type of quality assessment system that comprehensively analyze the complex components of TCM. Chromatographic fingerprint is an effective identification method for the quality control of TCM (5-8).

Modern analytical techniques, such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and liquid chromatograph mass spectrometer (LC-MS) have been widely used in TCM fingerprint identification. Among them, TLC, a traditional method, has been used as an essential identification method of TCM in the China pharmacopoeia (9). It is fast and easy to operate, but with poor specificity and sensitivity (10). HPLC is the most widely-used fingerprint method with high precision, sensitivity and repeatability. However, conventional HPLC suffers the disadvantage of long analysis time, low resolution and large solvent consumption (11, 12). CE is a suitable analytical tool in studies of many secondary plant metabolites, such as scutellaria, coptidis (13), aconitum (14), as parsley and thyme (15). The advantages of CE method include high separation efficiency, fast analysis speed and low operating cost (16, 17); however, there are still limitations for the application of CE (18). LC-MS is becoming a strong tool in Chinese medicine modernization research with the advantages of high sensitivity, superior separation performance and high specificity, nevertheless, the cost is relatively high (19). Therefore, fast separation with high resolution is expected in the quality control of TCM. Recently,

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ultra-performance liquid chromatography (UPLC) have been gaining popularity in the rapid profiling of TCM due to their faster analysis and better separation performance (20, 21).

Flos Carthami (FC, Honghua in Chinese) is the dried flower of *Carthamus tinctorius* L. (Asteraceae), which has been widely used in TCM for the treatment of cardiovascular, cerebrovascular, gynecological diseases and high cholesterol (22-24). The primary components in FC include hydroxysafflor yellow A, carthamin, safflor yellow A, kaempferol, apigenin, quercetin, adenosine and rutin (25). Despite of the wide application of FC in clinical practice, only few studies have been published with regard to the establishment of a convenient and feasible analytical method for quality control of FC. In those previous reports, FC has been analyzed by TLC (10), HPLC (11, 12), and CE (26). Nevertheless, those methods either only analyzed single or few ingredients or had some deficiencies in methodology. In 2003, the first fingerprints analysis of FC was reported using CE method (26). Although relatively large chemical information was provided by this analysis, a few peaks were identified or characterized. An HPLC analysis of FC using diode array detector was developed three years later, in which 10 components were separated within a 70 min elution range, and identified by their UV profile and comparison with standard compounds (11). This method was more stable and consistent compared to the previously reported CE method, however, it took a relatively long time to complete the analysis, and less chemical information was displayed. Therefore, a more convenient and feasible analytical method using ultra-high performance liquid chromatography (UPLC) was developed and validated in this study for the first time to establish a rapid chromatographic fingerprint analysis of FC as well as qualitative and quantitative evaluation of 13 components of FC simultaneously.

Experimental

Materials and reagents

Ten batches of FC samples were produced in Xinjiang and Gansu in mainland China, were provided by School of Chinese Medicine, Hong Kong Baptist University. The authentication of the samples was identified by Dr. YI Tao according to the morphological features (9). Reference compound hydroxysafflor yellow A was purchased from Phytomarker Ltd. (Tianjin, China). All the samples were stored in a cool and dry room.

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UPLC and HPLC-grade acetonitrile, methanol and formic acid were obtained from Lab-scan (Bangkok, Thailand). Deionized water was produced from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Other chemicals and reagents were of analytical grade.

Apparatus

An 1875 HTAG TRU-SWEEP ultrasonic cleaner (Crest, New York, USA), 5810 centrifugal machine (Eppendorf, Hamburg, Germany), pulverizing machine (RT-04, Fargo, Taiwan) and Adventurer electronic scales (OHAUS, USA) were prepared in this experiment.

UPLC was carried out by a Waters ACQUITY system comprising a vacuum degasser, quaternary pump, autosampler, column oven and ultraviolet detector. Empower Pro chromatographic workstation was applied to data collection and processing. An HSS C_{18} column (2.1 mm × 100 mm, 1.8 μm) was used for sample separation.

HPLC analysis was performed on an Agilent 1100 series high-performance liquid chromatography method with a diode array detection (HPLC-DAD) system with a vacuum degasser, binary pump, column oven, autosampler, Agilent Chemstation chromatographic workstation and DAD (Agilent, Palo Alto, CA, U.S.A.), which was used for acquiring chromatograms and UV spectra. An Alltima C_{18} column (4.6 mm × 250 mm，5 μm) with a compatible guard C_{18} column (4.6 mm × 7.5 mm, 5 μm) was used.

Chromatographic conditions

The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B) was used as gradient elution for separation with UPLC. The procedure was performed with a gradient program of 0—2% (B) at 0—2 min, 2—10% at 2—14 min, 10%—18% at 14—22 min and 18—25% at 22—30 min. The detection wavelength was set at 270 nm and the injection volume was 2.0 μL. The flow rate was 0.3 mL/min, and the column temperature was maintained at 40 ºC during the separation.

The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B) was used as gradient elution for separation with conventional HPLC. The procedure was performed with a gradient program of 15—20% (B) at 0—10 min, 20—53% at 10—40 min, and 53—100% at 40—60 min. The detection wavelength was set at 270 nm and the injection volume was 10.0 μL. The flow rate was 1.0 mL/min, and the column temperature was set at 30 ºC.

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Preparation of standard solution

Hydroxysafflor yellow A (HSYA) (0.50 mg) was immersed in 1 mL of 50% methanol. The solution was stored at 4 ^{o}C before use.

Preparation of sample solution

UPLC sample solution

The ten batches of FC samples were crushed with a grinder. The FC powder was passed through a 20-mesh sieve, vacuum drying to constant weight at room temperature. Accurately weighed FC powder of 0.10 g was extracted with 10 mL of 50% methanol by means of ultra-sonication (270 W) at room temperature for 30 min, and then the mixture was centrifuged (4,000 rpm) for 10 min. The supernatant solution was transferred into a 25-mL volumetric flask, and the extraction was repeated 2 times, constant volume to 25 mL with methanol. The final solution was allowed to stand for 30 min, filtered through a membrane with a pore size of 0.22 μm and injected with volume of 1μL. The filtrate was kept under 4°C for UPLC analysis.

HPLC sample solution

Accurately weighed FC powder of 0.10 g was extracted with 40 mL of 25% methanol by means of ultra-sonication (270 W) at room temperature for 30 min, and then the mixture was centrifuged (4,000 rpm) for 10 min. The supernatant solution was transferred into a 50-mL volumetric flask. The extraction was constant volume to 50 mL with methanol. The final solution was allowed to stand for 30 min, and filtered through a membrane with a pore size of 0.22 μm and injected with volume of 1μL. The filtrate was kept under 4°C for HPLC analysis.

Validation of UPLC method

The method was validated for precision, stability and repeatability. The analytical precision was determined by injecting the same FC sample solution for six times in one day. The stability was analyzed by the same FC sample solution at 0, 2, 4, 8, 16 and 24 h. The repeatability was evaluated by analyzing six independently prepared sample solution from the same batch of FC. Similarity evaluation was conducted in ten batches of FC acquired in various sources. Coexisting peaks in FC chromatograms were chosen to calculate their relative retention time (RRT) and relative peak area (RPA).

Data analysis

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The data analysis was processed by Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine software (Version 2004 A), which was recommended by the State Food and Drug Administration (SFDA) of China. This software was used to calculate the correlation coefficients of the chromatographic profiles of ten FC samples, and to generate the simulative mean chromatogram (SMC). The similarities of different chromatographic fingerprints were compared with the SMC.

Results and Discussion

The optimization of extraction conditions

The extraction efficiency of FC powder extracted with different solvents were compared, including methanol, 50% methanol, ethanol, 50% ethanol, and H_2O. The results demonstrated that FC powder extracted with 50% methanol exhibited the largest number of chromatographic peaks and the largest peak area, therefore the 50% methanol was chosen as the extraction solvent in this study. Ultrasonic extraction and reflux extraction showed comparable efficiency for the extraction. Taking into account the ultrasound is easier for operation, it was employed as the extraction method in the experiment. The extraction time were also investigated, including 15, 30, 45 and 60 min. The result shows that 30 min is sufficient for the full extraction.

The selection of detection wavelength

The photo-diode array (PDA) detector for ultraviolet wavelength scanning shows relatively higher signal-to-noise ratio for the targeting peaks under the wavelength of 270 nm, which was chosen as the detection wavelength.

The selection of mobile phase

The different composition of mobile phase including methanol-water, methanol-formic acid, acetonitrile-water and acetonitrile-formic acid were compared. The results demonstrated that 0.1% acetonitrile-formic acid exhibited satisfactory resolution, moderate retention time and smooth baseline, which was adopted as the mobile phase in this study.

The selection of chromatographic column

Compared with ACQUITY BEH C_{18} column and Amide column, HSS C_{18} column (2.1 mm × 100 mm, 1.8 μm) was more suitable for separating the constituents with medium polarity and high polarity, and produced a high column efficiency and chromatographic profile.

Identification of the reference peak

Ten batches of FC samples collected from various regions were evaluated by the established methods and thirteen coexisting peaks were found in all the analyzed samples. Peak 4 (hydroxysafflor yellow A) was assigned as the reference peak to conduct method validation since it had a relatively moderate retention time and peak area.

Comparison of HPLC and UPLC fingerprints

The chromatograms of the conventional HPLC and UPLC method were both presented in Figure 1. Peak 1' in Figure 1(A) is hydroxysafflor yellow A identified using standard. Other peaks in Figure 1(B) are tentatively identified as (**1**) guanosine, (**2**) 6-hydroxykaempferol 3,6-di-O-*β*-D-glucoside-7-O-β-D-glucuronide, (**3**) 6-hydroxykaempferol 3,6,7-tri-O-*β*-D-glucoside, (**4**) hydroxysafflor yellow A, (**5**) 6-hydroxykaempferol 3-O-*β*-rutinoside-6-O-β-D-glucoside, (**6**) 6-hydroxykaempferol 3,6-di-O-*β*-D-glucoside, (**7**) 6-hydroxyapigenin 6-O-glucoside-7-O-glucuronide, (**8**) anhydrosafflor yellow B, (**9**) unknown, (**10**) kaempferol 3-O-β-rutinoside, (**11**) rutin, (**12**) quercetin, (**13**) kaempferol by comparing their UV spectra with literature data (12, 25). The UV spectra of the identified compounds were listed in the Supplementary Information.

For HPLC, the analysis time for the fingerprint of FC was 75 min and the flow rate was 1.0 mL/min. By contrast, UPLC cost less time and used a much slower flow rate, which were 30 min and 0.3 mL/min, respectively. In the previous reports and the present study on FC fingerprints analysis, HPLC method suffers the disadvantage of long analysis time and large solvent consumption (11, 12). In contrast, the present UPLC method was time-saving and solvent-saving. Moreover, much more peaks were resolved in Figure 1 (B) using UPLC as compared to that in Figure 1 (A) using conventional HPLC. These figures indicate that UPLC have higher sensitivity and resolution than that of HPLC.

*Insert **Figure 1.** Here*

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Stability, precision and repeatability

A comprehensive validation of the present method was established, including stability, precision and repeatability, and the results are listed in Table 1. In the 24-h stability test, the same sample was stored at 40℃, and analyzed at 0, 2, 4, 8, 16 and 24h, respectively. The relative standard deviation (RSD) of relative retention time (RRT), which was calculated as the ratio of the retention time of the individual peak to that of the reference peak, was less than 0.08%. Then the relative peak area (RPA), which was calculated as the ratio of the retention peak area of the individual peak to that of the reference peak, was less than 4.41%. The results indicated that the sample solution was found to be stable within 24h. The precision of the method was evaluated by injecting six times with the same sample in the instrument. With RSD less than 0.05% for RRT and 4.26% for RPA, the method could be considered reliable. Six independently extracted FC samples were analyzed, and the repeatability for the RRT and RPA were less than 0.05% and 3.91%, which showed high reproducibility of the method. These data illustrated that the UPLC method was suitable for the qualitative and quantitative analyses of FC.

*Insert **Table I** here*

Similarity analysis of ten batches of FC

Ten FC samples acquired from various sources were analyzed with the established methods and the UPLC fingerprint profiles were obtained. Thirteen coexisting fingerprint peaks were found in all the analyzed samples.

The RRT and RPA of these thirteen peaks were calculated using the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004 A), and the results were summarized in Table 2 and Table 3, respectively. The RSD values of RRT fluctuated slightly in the range of 0.04–0.12%, while the RSD values of RPA fluctuated moderately from 10.05% to 43.09%. The RSD values for the RRT were less than 0.12%, demonstrating that the fingerprint analysis was precise. On the other hand, the RSD values for the RPA were much larger, illustrating that the processing methods had affected the contents of the compounds in the FC samples. Overall, although the contents varied, this fingerprint analysis by UPLC was still feasible and repeatable.

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The chromatographic fingerprints of ten FC samples were shown in Figure 2, and the results of the similarity analysis were listed in Table 4. The similarity results of the ten samples remained stable in the range of 0.938–0.995, which demonstrates that FC from various sources were acceptably similar.

*Insert **Table II** and **Table III** here*

*Insert **Figure 2.** here*

*Insert **Table IV** here*

Conclusion

A UPLC method for the profiling of Flos Carthami has been established and validated. Ten batches of raw material samples of FC from various cultivation locations were evaluated and thirteen coexisting peaks were found in ten samples. Ten bitches of these samples were analyzed simultaneously, and the developed method exhibited good stability, precision and repeatability. Compared to the conventional HPLC method, our established UPLC method provides shorter analysis time, reduced solvent consumption, higher resolution and better separation performance for fingerprint analysis. Base on the present study, this method laid a solid foundation for quality control of FC, and will serve as a valuable reference for quality evaluation and standardization of Chinese herbal medicine.

Acknowledgements

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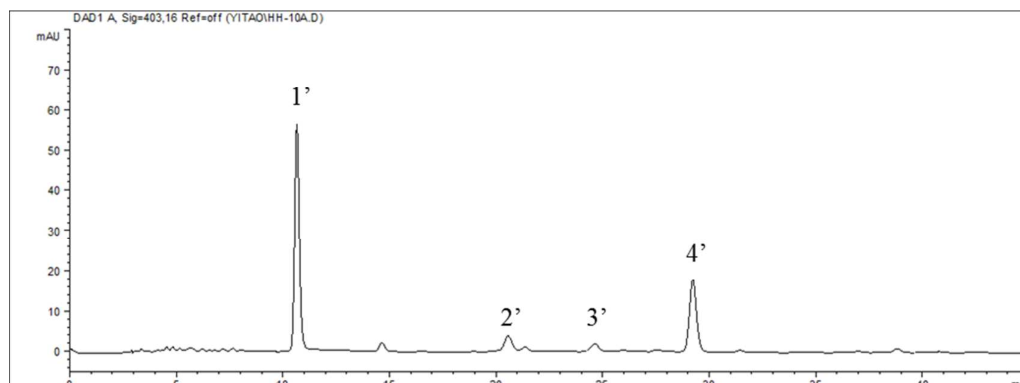
Legends for Figures

Figure 1. Chromatograms of Flos Carthami on (**A**) conventional HPLC and (**B**) UPLC at 270 nm. Peak 1' in Figure 1(A) is hydroxysafflor yellow A identified using standard. Other peaks in Figure 1(B) are tentatively identified as (**1**) guanosine, (**2**) 6-hydroxykaempferol 3,6-di-O-*β*-D-glucoside-7-O-β-D-glucuronide, (**3**) 6-hydroxykaempferol 3,6,7-tri-O-*β*-D-glucoside, (**4**) hydroxysafflor yellow A, (**5**) 6-hydroxykaempferol 3-O-*β*-rutinoside-6-O-β-D-glucoside, (**6**) 6-hydroxykaempferol 3,6-di-O-*β*-D-glucoside, (**7**) 6-hydroxyapigenin 6-O-glucoside-7-O-glucuronide, (**8**) anhydrosafflor yellow B, (**9**) unknown, (**10**) kaempferol 3-O-β-rutinoside, (**11**) rutin, (**12**) quercetin, (**13**) kaempferol by comparing their UV spectra with literature data (12, 20).

A



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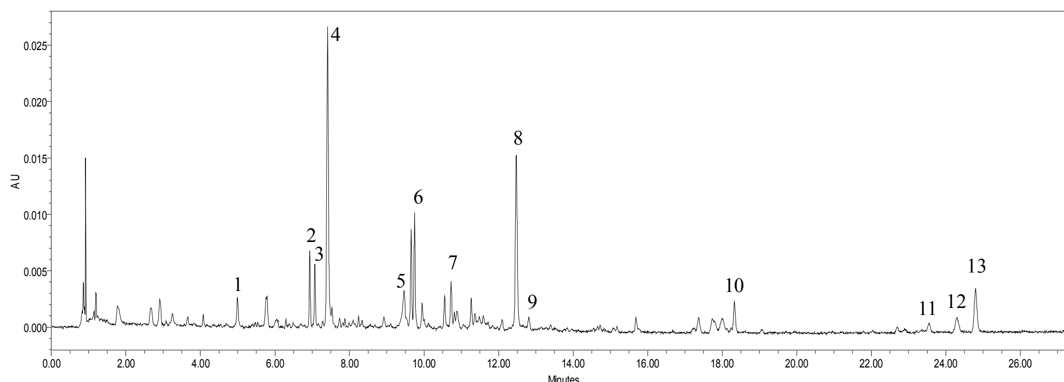

Figure 1. Chromatograms of Flos Carthami on (**A**) conventional HPLC; and (**B**) UPLC at 270 nm. Peak 1’ in **A** is identified as hydroxysafflor yellow A. other peaks in **B** are tentatively identified as (**1**) guanosine, (**2**) 6-hydroxykaempferol 3,6-di-O-*β*-D-glucoside-7-O-β-D-glucuronide, (**3**) 6-hydroxykaempferol 3,6,7-tri-O-*β*-D-glucoside, (**4**) hydroxysafflor yellow A, (**5**) 6-hydroxykaempferol 3-O-*β*-rutinoside-6-O-β-D-glucoside, (**6**) 6-hydroxykaempferol 3,6-di-O-*β*-D-glucoside, (**7**) 6-hydroxyapigenin 6-O-glucoside-7-O-glucuronide, (**8**) anhydrosafflor yellow B, (**9**) unknown, (**10**) kaempferol 3-O-β-rutinoside, (**11**) rutin, (**12**) quercetin, (**13**) kaempferol by comparing their UV spectra with literature data (12, 20).

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Legends for Figures

Figure 2. UPLC fingerprints of ten batches of Flos Carthami at 270 nm.

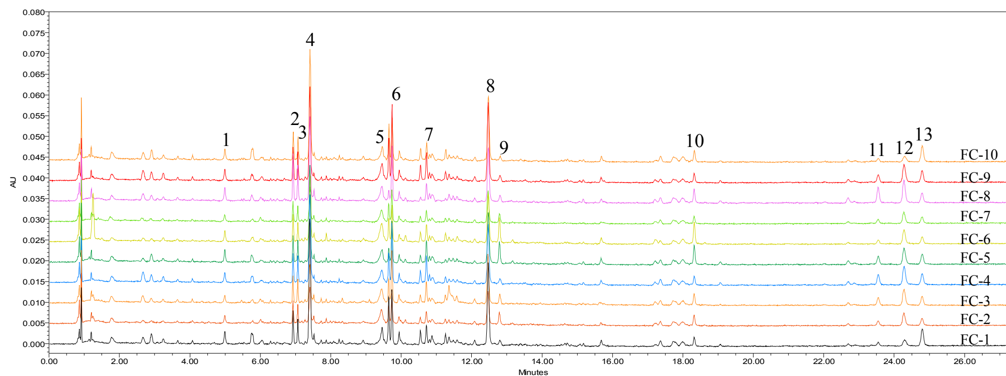

Figure 2. UPLC fingerprints of ten batches of Flos Carthami at 270 nm.

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Legends for Tables

Table I. Results of stability, precision and repeatability of common peaks in FC

Table II. RRT of common peaks in ten batches of FC samples

Table III. RPA of common peaks in ten batches of FC samples

Table IV. Similarities of ten batches of FC

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Table I. Results of stability, precision and repeatability of common peaks in FC

Peak No.	Stability (RSD, %)		Precision (RSD, %)		Repeatability (RSD, %)	
	RRT	RPA	RRT	RPA	RRT	RPA
1	0.04	2.09	0.02	2.12	0.03	2.24
2	0.03	4.03	0.02	3.91	0.01	3.70
3	0.04	4.41	0.02	2.40	0.01	3.14
4 (S, HSYA)	—	—	—	—	—	—
5	0.04	1.30	0.02	2.55	0.05	3.27
6	0.05	2.48	0.03	3.64	0.01	1.51
7	0.04	3.63	0.03	4.01	0.01	2.88
8	0.04	3.55	0.03	4.26	0.02	1.95
9	0.04	1.50	0.03	2.27	0.02	3.91
10	0.07	2.16	0.04	2.19	0.02	2.74
11	0.08	4.29	0.04	3.70	0.04	2.81
12	0.08	1.66	0.04	3.21	0.02	2.32
13	0.08	3.61	0.05	0.63	0.03	0.87

Table II. RRT of common peaks in ten batches of FC samples

Peak No.	RRT										Mean	RSD (%)
	1	2	3	4	5	6	7	8	9	10		
1	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.06
2	0.94	0.94	0.94	0.94	0.93	0.93	0.94	0.94	0.94	0.94	0.94	0.05
3	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.05
4 (S,HSYA)	1	1	1	1	1	1	1	1	1	1	1	—
5	1.27	1.28	1.28	1.28	1.27	1.27	1.28	1.28	1.28	1.28	1.28	0.12
6	1.31	1.31	1.32	1.32	1.31	1.31	1.31	1.31	1.32	1.31	1.31	0.04
7	1.45	1.45	1.45	1.45	1.45	1.45	1.45	1.45	1.45	1.45	1.45	0.05
8	1.68	1.68	1.68	1.68	1.68	1.68	1.68	1.68	1.68	1.68	1.68	0.06
9	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	0.08
10	2.47	2.47	2.47	2.47	2.47	2.47	2.47	2.47	2.48	2.47	2.47	0.04
11	3.18	3.18	3.18	3.18	3.18	3.18	3.18	3.18	3.18	3.18	3.18	0.05
12	3.28	3.28	3.28	3.28	3.28	3.28	3.28	3.28	3.28	3.28	3.28	0.06
13	3.35	3.35	3.35	3.35	3.35	3.35	3.35	3.35	3.35	3.35	3.35	0.04

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Table III. RPA of common peaks in ten batches of FC samples

Peak No.	RPA										Mean	RSD (%)
	1	2	3	4	5	6	7	8	9	10		
1	0.21	0.15	0.10	0.10	0.14	0.16	0.15	0.10	0.12	0.17	0.14	25.89
2	0.41	0.43	0.32	0.37	0.44	0.42	0.37	0.43	0.36	0.36	0.39	10.05
3	0.31	0.37	0.18	0.13	0.16	0.30	0.26	0.19	0.11	0.13	0.21	42.57
4 (S, HSYA)	1	1	1	1	1	1	1	1	1	1	1	—
5	0.53	0.45	0.49	0.55	0.51	0.46	0.31	0.23	0.35	0.27	0.42	27.81
6	0.59	0.50	0.48	0.31	0.36	0.50	0.51	0.58	0.49	0.47	0.48	18.28
7	0.27	0.40	0.23	0.18	0.20	0.26	0.16	0.23	0.11	0.12	0.21	39.60
8	0.94	0.83	0.82	0.63	0.83	0.67	0.78	1.00	0.77	0.67	0.79	14.64
9	0.36	0.24	0.36	0.22	0.32	0.28	0.29	0.36	0.24	0.24	0.29	19.29
10	0.34	0.29	0.21	0.19	0.31	0.23	0.17	0.10	0.17	0.10	0.21	39.30
11	0.17	0.27	0.18	0.12	0.08	0.17	0.18	0.11	0.07	0.09	0.14	43.09
12	0.22	0.28	0.18	0.29	0.24	0.25	0.28	0.21	0.28	0.17	0.24	18.22
13	0.32	0.34	0.19	0.15	0.14	0.29	0.19	0.18	0.20	0.25	0.22	30.62

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Table IV. Similarities of ten batches of FC

Batch	Source and year of harvest	Similarity
FC-1	Hami, Xinjiang, China (2011)	0.945
FC-2	Yili, Xinjiang, China (2012)	0.938
FC-3	Huocheng, Xinjiang, China (2011)	0.995
FC-4	Yumen, Gansu, China (2011)	0.988
FC-5	Yumen, Gansu, China (2012)	0.972
FC-6	Jiuquan, Gansu, China (2011)	0.959
FC-7	Guazhou, Gansu, China (2012)	0.982
FC-8	Yining, Xinjiang, China (2011)	0.987
FC-9	Jimusaer, Xinjiang, China (2011)	0.969
FC-10	Qitai, Xinjiang, China (2011)	0.977

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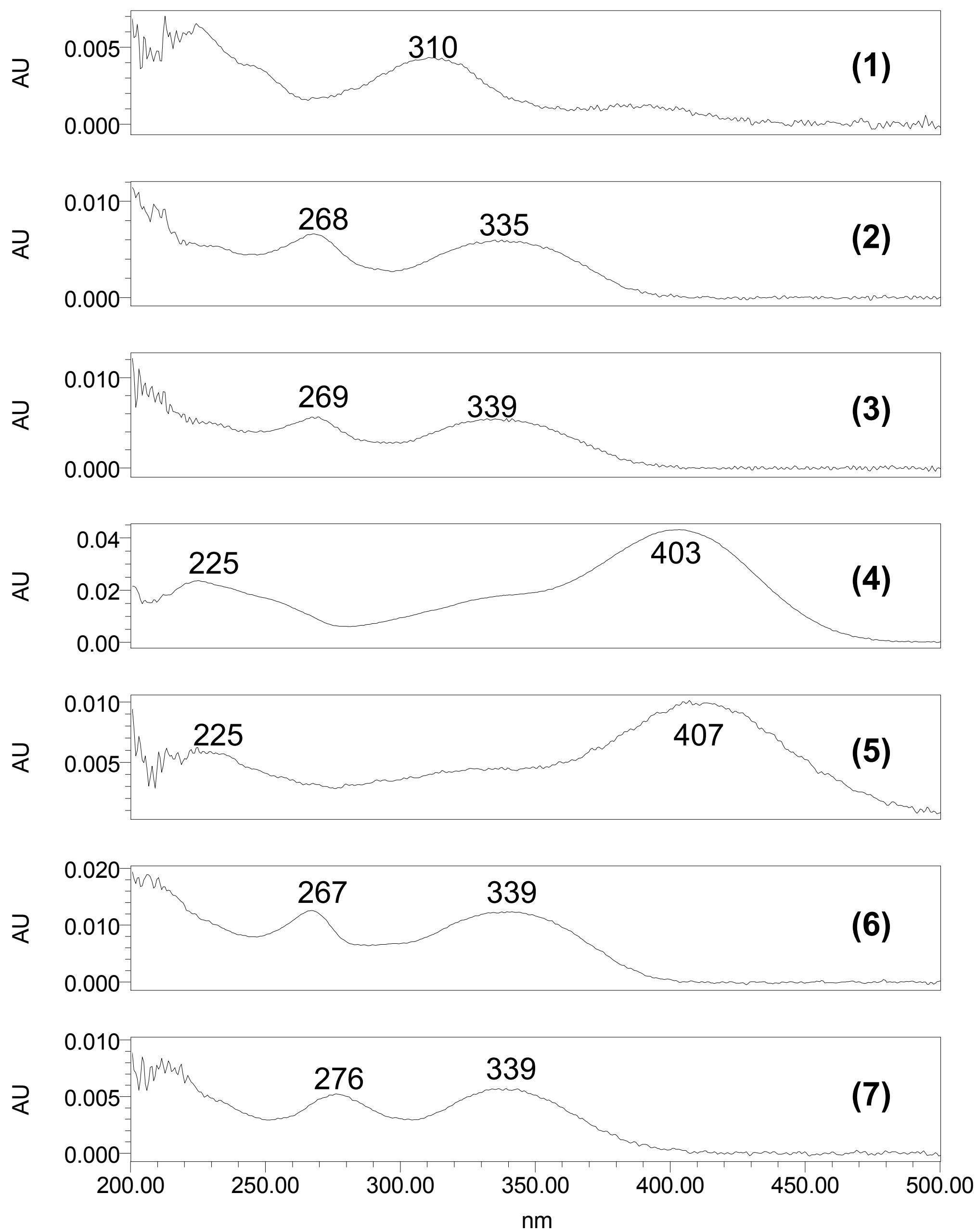
Supplementary Information



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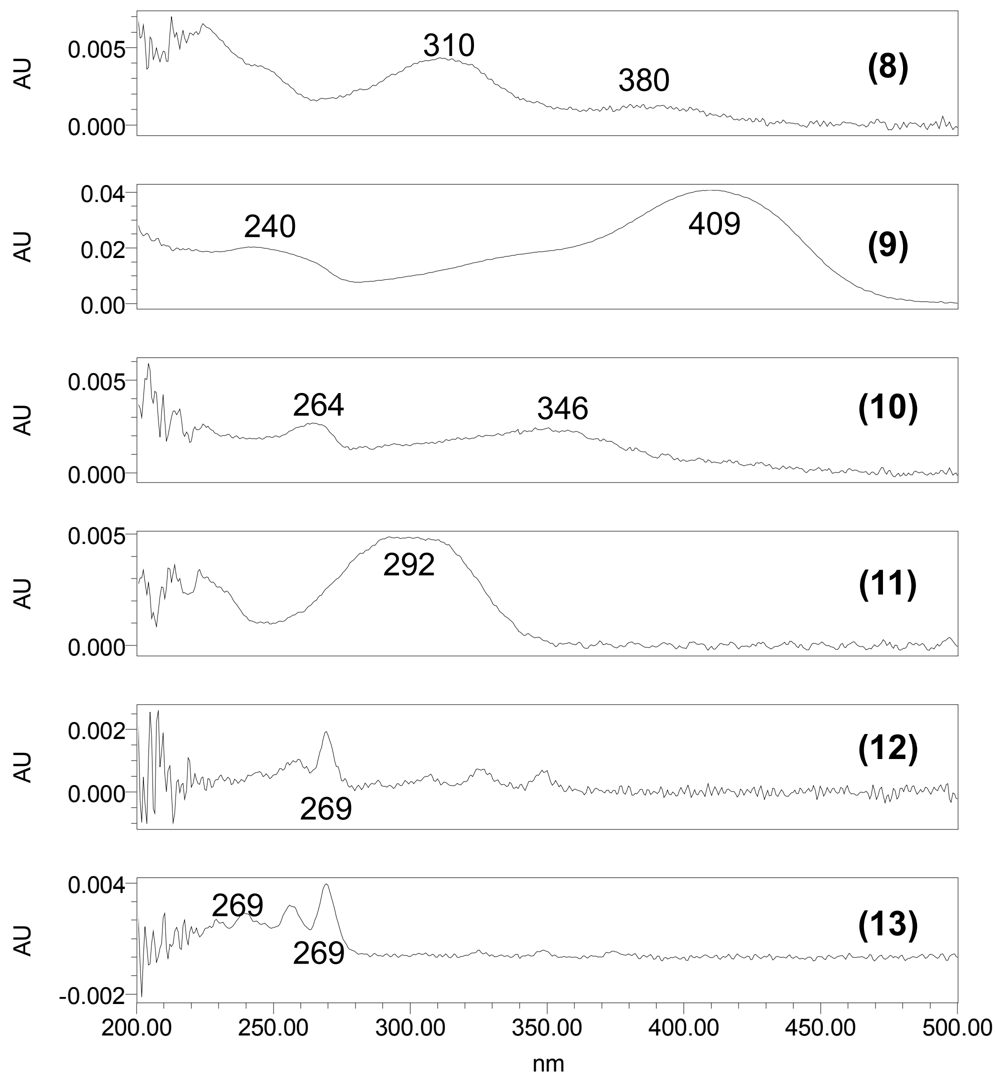

Fig. S1. The UV spectra of the identified compounds. (**1**) guanosine, (**2**) 6-hydroxykaempferol 3,6-di-O-*β*-D-glucoside-7-O-β-D-glucuronide, (**3**) 6-hydroxykaempferol 3,6,7-tri-O-*β*-D-glucoside, (**4**) hydroxysafflor yellow A, (**5**) 6-hydroxykaempferol 3-O-*β*-rutinoside-6-O-β-D-glucoside, (**6**) 6-hydroxykaempferol 3,6-di-O-*β*-D-glucoside, (**7**) 6-hydroxyapigenin 6-O-glucoside-7-O-glucuronide, (**8**) anhydrosafflor yellow B, (**9**) unknown, (**10**) kaempferol 3-O-β-rutinoside, (**11**) rutin, (**12**) quercetin, (**13**) kaempferol.