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Qualitative and quantitative analysis of flavonoids in the leaves of *Isatis indigatica* Fort. by ultra-performance liquid chromatography with PDA and electrospray ionization tandem mass spectrometry detection

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ABSTRACT

A novel method based on ultra-performance liquid chromatography with photo diode array and electrospray ionization tandem mass spectrometry detection (UPLC-PDA-ESI-MS/MS) was developed for the qualitative and quantitative analysis of flavonoids in the leaves of *Isatis indigotica* Fort. (Daqingye). The separation was carried out on an Acquity UPLC BEH C_{18} column with 0.1% formic acid and methanol as the mobile phase under gradient conditions. Eight flavone *C*-glucosides were identified and their mass spectrometric fragmentation patterns were studied. Among them, the fragmentation pathways of three flavone 6-*C*-diglucosides with the rare 1 → 3 interglycosidic linkage were investigated for the first time. In addition, a quantitative analytical method for six flavone *C*-glucosides in Daqingye by UPLC-ESI-MS/MS was established and applied for the determination of commercial Daqingye samples from different resources.

1. Introduction

Flavonoids are very common and widespread secondary plant metabolites in herbal medicine. Most flavonoids in plant cells are present as glycosides. Flavonoids glycosides are mainly divided into two categories: *C*-glycosides and *O*-glycosides, according to the site of sugar substitution on the flavonoid skeleton through hydroxyl groups or directly to carbon atoms in ring A, respectively. Flavonoids are reported to have diverse pharmacological activities, such as antioxidative [1,2], antimicrobial [3], anti-inflammatory [1], immunoregulatory [4] and the potential for prevention and therapy against cancer [5,6].

Isatis indigotica Fort. is a biennial herbaceous plant species distributed widely in China. The leaves of *I. indigotica* are recorded as "Daqingye" in Chinese in current China Pharmacopeia [7]. Daqingye is a commonly used traditional Chinese medicine (TCM) with long history as antibacterial [8], antiviral [9], antiendotoxin [10], immunoregulatory [11] and antitumor [12]. As reported previously, Daqingye mainly contains alkaloids [13], organic acids [13,14], nucleosides [15] and lignanoids [13]. Isovitexin and 6-β-D-glucopyranosyldiosmetin are the only reported flavonoids separated from Daqingye [13,14]. In our study on chemical constituents in Daqingye, we obtained eight flavone *C*-glucosides: three flavone *C*-monoglucosides, four flavone *C*-diglucosides and one flavone *O*-glucosyl-*C*-glucoside [16]. The study on flavonoids in Daqingye will contribute to its quality control and unlocking the secret of pharmacological activities of Daqingye.

With the soft ionization source such as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), MS hyphenated with chromatographic techniques has become a powerful approach in the identification, quantification and structural confirmation of natural products from biological material. And this approach is greatly reinforced by the introduction of UPLC technology. LC-APCI-MS and LC-ESI-MS methods have been developed for detection and analysis of alkaloids in Daqingye [17,18]. However, to the best of our knowledge, the qualitative and quantitative study on flavonoids in Daqingye has not been reported yet. In this paper, a UPLC-PDA-ESI-MS/MS-based analysis on flavonoids in Daqingye was described and six flavonoids were determined quantitatively in Daqingye samples.

2. Experimental

2.1. Reagents and materials

HPLC-grade methanol, acetonitrile and formic acid were purchased from Fisher Scientific International Inc. (Pittsburgh, PA,

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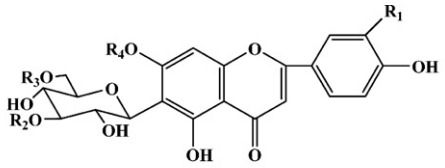


Saponarin (**1**): R_1=H, R_2= H, R_3=H, R_4= glucosyl, MW 594
Isovitexin 6"-**O**-glucopyranoside (**2**): R_1=H, R_2=H, R_3=glucosyl, R_4=H, MW 594
Isoorientin (**3**): R_1=OH, R_2=H, R_3=H, R_4=H, MW 448
Isoorientin 3"-O-glucopyranoside (**4**): R_1=OH, R_2=glucosyl, R_3=H, R_4=H, MW 610
Isovitexin (**5**): R_1=H, R_2=H, R_3=H, R_4=H, MW 432
Isovitexin 3"-**O**-glucopyranoside (**6**): R_1=H, R_2=glucosyl, R_3=H, R_4=H, MW 594
Isoscoparin (**7**): R_1=OCH_3, R_2=H, R_3=H, R_4=H, MW 462
Isoscoparin 3"-**O**-glucopyranoside (**8**): R_1=OCH_3, R_2=glucosyl, R_3=H, R_4=H, MW 624

Fig. 1. Structures of the flavonoids isolated from Daqingye.

USA). All other reagents were of analytical grade obtained from Jiangsu Hanbon Sci. & Tech. Co., Ltd. (Jiangsu, China).

Isoorientin, isoorientin 3″-*O*-glucopyranoside, isovitexin, isovitexin 3″-*O*-glucopyranoside, isoscoparin, isoscoparin 3″-*O*-glucopyranoside, isovitexin 6″-*O*-glucopyranoside and saponarin were isolated in our laboratory (over 95% purity by HPLC). The aqueous EtOH extract of Daqingye was suspended in water and partitioned with chloroform. The water layer was subjected to column chromatography on macroporous resin, silica gel, Sephadex LH-20 and preparative HPLC to yield these flavonoids. Their structures (Fig. 1) were identified by spectral analysis [16,19].

Dried leaves of *I. indigotica* were collected from Anhui, Hebei, Liaoning and Shandong Province, China. Voucher specimens of these collections were identified and deposited at School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, China.

2.2. Apparatus and operating conditions

An Acquity ultra-performance liquid chromatograph (UPLC) with photo diode array (PDA) detector coupled with a triple quadrupole tandem mass spectrometer (Micromass® Quattro micro™ API, Waters Corp., Milford, MA, USA) with electrospray ionization interface was employed. An Acquity UPLC BEH C_{18} column (1.7 μm, 100 mm × 2.1 mm i.d., Waters Corp.) was used for the analysis. The column temperature was maintained at 40 °C. The UPLC mobile phase consisted of 0.1% formic acid in water as solvent A and MeOH as solvent B. The gradient for identification was: 0 min 100% A, 10.0 min 75% A, 18.0 min 47% A, and 20.0 min 5% A. The gradient for quantitative analysis was: 0 min 80% A, 8.0 min 56% A. The flow rate was 0.25 ml/min and the injection volume was 5 μl. The UV spectra by PDA were recorded between 190 and 400 nm.

MS detection was performed directly after PDA measurements without stream splitting. The ESI source was optimized as follows: negative and positive ionization mode, scan spectra from *m*/*z* 100 to

Table 1
The MRM transition in negative mode, cone voltage and collision energy for the determination of six flavonoids in Daqingye

Compounds	Transition	Cone voltage (V)	Collision energy (eV)
Isoorientin	447 → 327	40	20
Isoorientin 3″-*O*-glucopyranoside	609 → 339	65	40
Isovitexin	431 → 311	40	20
isovitexin 3″-*O*-glucopyranoside	593 → 323	50	40
Isoscoparin	461 → 341	65	40
Isoscoparin 3″-*O*-glucopyranoside	623 → 353	65	35

Table 2
Quasi-molecular ions and on-line UV spectral data of compounds in Daqingye sample by UPLC-PDA-MS analysis

Compound no.	t_R (min)	$[M+H]^+$ (*m*/*z*)	$[M-H]^-$ (*m*/*z*)	UV λ_{max} (nm)	Identification
1	10.20	595, 617[M+Na], 633[M+K]	593	213,271,326	Saponarin
2	11.85	595,617[M+Na],633[M+K]	593	214,269,335	Isovitexin 6″-*O*-glucopyronoside
3	12.38	449[M+Na],471[M+K]	447	228,269,345	Isoorientin
4	12.67	611,633[M+Na]	609	237,270,334	Isoorientin 3″-*O*-glycopyronoside
5	13.64	433,445[M+Na]	431	214,269,336	Isovitexin
6	13.81	595	593	227,269,334	Isovitexin 3″-*O*-glycopyronoside
7	14.04	463,485[M+Na]	461	213,270,347	Isoscoparin
8	14.16	625,647[M+Na]	623	213,270,347	Isoscoparin 3″-*O*-glycopyronoside
9	6.58	439[M+Na]	415,831[2M−H]	239,324	Unknown
10	7.69	439[M+Na],455[M+K]	415	237,326	Unknown
11	8.02	617	615	238,327	Unknown
12	8.67	571[M+Na],587[M+K]	547	220,326	Unknown
13	9.02	439[M+Na],455[M+K]	415	237,328	Unknown
14	10.50	757,779[M+Na]	755	214,271,324	Unknown
15	10.63	757,779[M+Na]	755	213,271,324	Unknown
16	10.87	625,647[M+Na]	623	214,270,326	Unknown
17	10.96	777[M+Na]	753	238,324	Unknown
18	11.25	787,809[M+Na]	785	239,271,333	Unknown
19	11.56	645[M+Na]	621	232,320	Unknown
20	12.17	645[M+Na],661[M+K]	621	237,325	Unknown
21	12.31	625,647[M+Na],663[M+K]	623	229,270,331	Unknown
22	12.45	801,823[M+Na]	799	231,271,328	Unknown
23	12.89	831,853[M+Na]	829	239,270,334	Unknown
24	14.46	777[M+Na]	753	239,327	Unknown
25	14.79	777[M+Na]	753	240,329	Unknown
26	15.31	983[M+Na]	959	239,327	Unknown
27	15.43	615	613	240,328	Unknown
28	17.15	983[M+Na]	959	240,328	Unknown

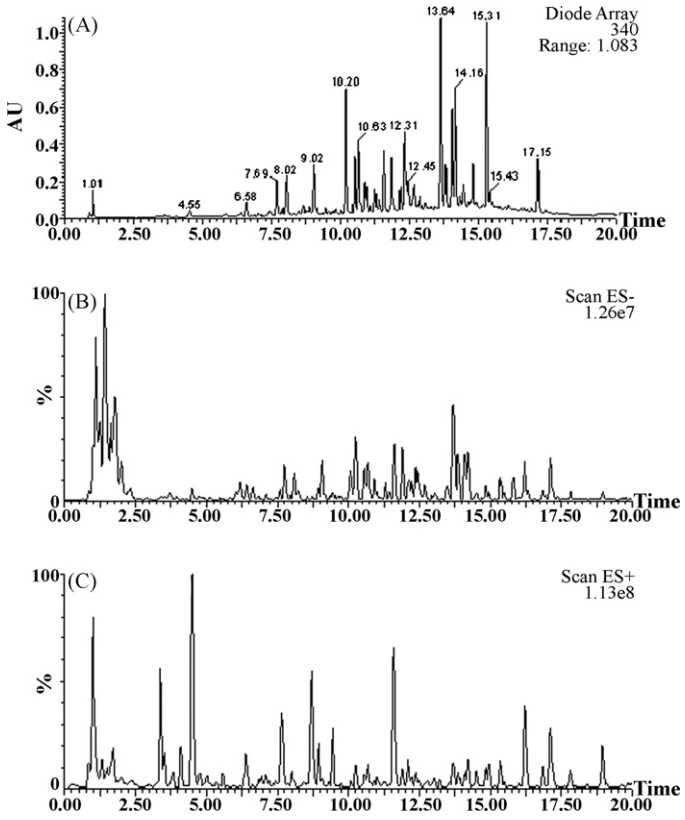


Fig. 2. The ultra-performance liquid chromatogram at 340 nm (A) and the TIC chromatograms in negative (B) and positive (C) ionization mode of Daqingye sample.

1000, capillary voltage 3.0 kV, cone voltage 40 V, source temperature 115 °C and desolvation temperature 350 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 400 and 40 l/h, respectively.

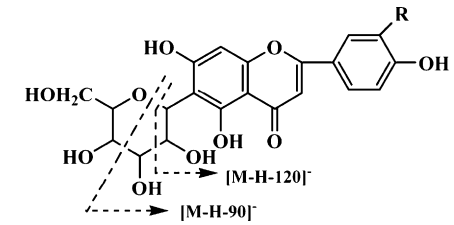
For quantitative determination of the six flavonoids, the MS detection was operated in negative ESI mode with multiple reaction monitoring (MRM). Argon was used as the collision gas at a pressure of 2.50×10^{-3} mbar. The MRM transition, capillary voltage, cone voltage and collision energy used for each compound are summarized in Table 1. And other MS parameters were the same as those for identification.

2.3. Preparation of standard solutions

Stock solutions of isoorientin (200 μg/ml), isoorientin 3″-*O*-glucopyranoside (280 μg/ml), isovitexin (240 μg/ml), isovitexin 3″-*O*-glucopyranoside (350 μg/ml), isoscoparin (290 μg/ml) and isoscoparin 3″-*O*-glucopyranoside (410 μg/ml) were prepared in methanol, respectively. A set of standard solutions were prepared by appropriate dilution of the stock solution with methanol, containing 0.040–4.0 μg/ml of isoorientin, 0.056–5.6 μg/ml of isoorientin 3″-*O*-glucopyranoside, 0.048–4.8 μg/ml of isovitexin, 0.070–7.0 μg/ml of isovitexin 3″-*O*-glucopyranoside, 0.058–5.8 μg/ml of isoscoparin and 0.082–8.2 μg/ml of isoscoparin 3″-*O*-glucopyranoside. All solutions were stored at 4 °C before analysis.

2.4. Preparation of Daqingye samples

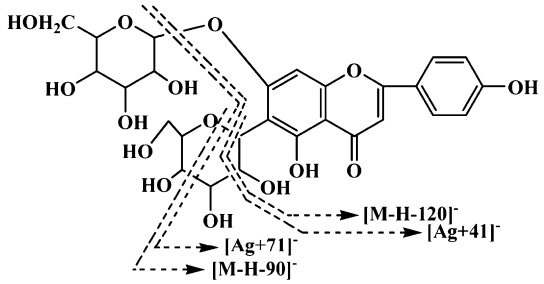
Dried powder of Daqingye (1 g) was refluxed with water or aqueous ethanol for 1.5 h. The extraction was repeated twice, and the extracts were combined and condensed to dry under vacuum. The residue was reconstituted in 10 ml of water or aqueous methanol of corresponding concentration. For quantitative analysis, the extracts were further diluted 200-fold with a mixture of MeOH-water (1:1, v/v). The herbal extract was passed through a syringe filter of 0.22 μm and 5 μl of the filtrate was introduced into the UPLC-PDA-MS/MS system.



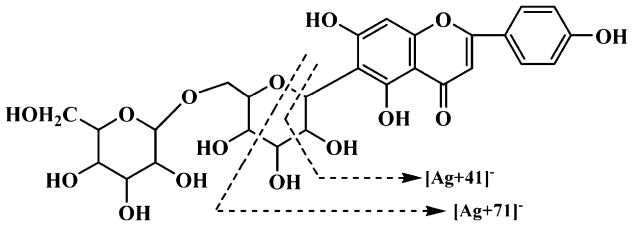
Isoorientin (**3**): R=OH

Isovitexin (**5**): R=H

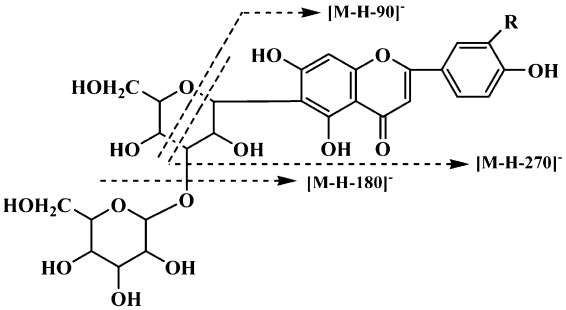
Isoscoparin (**7**): R=OCH_3



Saponarin (**1**)



Isovitexin 6”-O- glucopyranoside (**2**)



Isoorientin 3”-O-glucopyronoside (**4**): R=OH

Isovitexin 3”-O-glucopyronoside (**6**): R=H

Isoscoparin 3”-O-glucopyronoside (**8**): R=OCH_3

Fig. 3. The main fragmentation pathways and product ions for compound **1**–**8**.

Table 3
The main product ions for compound **1–8**

	Compounds	$[M-H]^-$	$[M-H-90]^-$	$[M-H-120]^-$	$[M-H-180]^-$	$[M-H-270]^-$	$[Ag+71]^-$	$[Ag+41]^-$
1	Saponarin	593	503	473			341	311
2	Isovitexin 6″-*O*-glucopyranoside	593					341	311
3	Isoorientin	447	357	327				
4	Isoorientin 3″-*O*-glucopyranoside	609	519		429	339		
5	Isovitexin	431	341	311				
6	Isovitexin 3″-*O*-glucopyranoside	593	503		413	323		
7	Isoscoparin	461	371	341				
8	Isoscoparin 3″-*O*-glucopyranoside	623	533		443	353		

Ag: aglycon.

3. Results and discussion

3.1. Qualitative study of flavonoids in Daqingye samples by UPLC-PDA-MS/MS

The optimized UPLC conditions were achieved by trying several mobile phase systems of acetonitrile–water, MeOH–water with or without formic acid and various gradient elution processes of those solvent systems. The mobile phase, as described in Section 2.2, consisting of 0.1% aqueous formic acid–MeOH with gradient elution provided high resolution for those components in Daqingye samples. All of the components were eluted within 20 min due to the high peak capacity benefited from UPLC technique.

Water and aqueous ethanol of 30%, 50%, 70% and 95% (v/v) were investigated as the extraction solvent of Daqingye samples. The peak intensities of early eluted constituents decreased significantly with the increase of ethanol concentration in the extraction solution and some of them become too small to be detected. The eight known flavonoids were eluted during the middle period of the total gradient elution process. The sample extracted with 50% ethanol exhibited higher contents of the flavonoids than those with other tested solvents, which was further confirmed by the UPLC-ESI-MS/MS method in MRM mode. Therefore, 50% ethanol was selected as the extraction solvent for both identification and quantitative analysis of Daqingye.

The Daqingye samples and standard solutions were analyzed by UPLC-PDA-MS, respectively. The chromatogram at 340 nm and the total ion current (TIC) chromatograms in positive and negative ionization mode of a Daqingye sample are shown in Fig. 2. The quasi-molecular ions and on-line UV spectral data of 28 compounds detected in Daqingye sample are concluded in Table 2. All of those flavonoid-like compounds exhibited absorption maxima at 220–280 nm (band II) and 300–400 nm (band I) in their UV spectra, which are characteristic absorption bands of flavone skeleton. According to the deduced molecular weight by both positive and negative quasi-molecular ion peaks, several groups of isomers with similar UV spectrum were found in Daqingye, taking isomers compounds **1**, **2** and **6** as an example. Due to lack of sufficient related reports on flavonoids in Daqingye and standards available, only eight flavonoids, compounds **1–8**, were identified by comparison with the retention times, UV spectra and quasi-molecular ions of the corresponding standards.

In order to study the fragmentation patterns of these known flavonoids, collision-induced dissociation (CID) MS/MS spectra in negative ESI mode were recorded. The main fragmentation pathways and product ions for compounds **1–8** are shown in Fig. 3 and Table 3. In the MS/MS spectra of isoorientin (**3**), isovitexin (**5**) and isoscoparin (**7**), characteristic fragment ions $[M-H-120]^-$ (*m*/*z* 327, 311, 341) and $[M-H-90]^-$ (*m*/*z* 357, 341, 371) of flavone *C*-glucosides were observed [20]. For flavone *C*-monoglycosides, the fragment ion $[M-H-120]^-$ equals to ion $[Ag+41]^-$ and $[M-H-90]^-$ to $[Ag+71]^-$, which are the product ions of the internal cleavage within the single sugar at C-6 position. In the MS/MS spectra of saponarin (**1**), the presence of $[M-H-90]^-$ (*m*/*z* 503), $[M-H-120]^-$ (m/z 473), $[Ag+71]^-$ (*m*/*z* 341) and $[Ag+41]^-$ (*m*/*z* 311) ions was consistent with structure of *O*-glycosyl-*C*-glycosyl flavone [21]. Only $[Ag+71]^-$ (*m*/*z* 341) and $[Ag+41]^-$ (*m*/*z* 311) ions was found in the MS/MS spectra of isovitexin 6″-*O*-glucopyranoside (**2**) [21].

Since compounds **4**, **6** and **8** are flavone 6-*C*-diglucosides with the rare $1 \rightarrow 3$ interglycosidic linkage, there were no reports on the MS fragmentation pattern for such kind of compounds. In our study, three characteristic fragment ions were found in their MS/MS spectra (Fig. 4). The occurrence of ion $[M-H-90]^-$ (*m*/*z* 519, 503, 533) without ion $[M-H-120]^-$ indicated that the *O*-glycosylation is linked to the position 3″ of the C-6 glucose, which prevents the formation of ion $[M-H-120]^-$. The ion $[M-H-180]^-$ (*m*/*z* 429, 413,

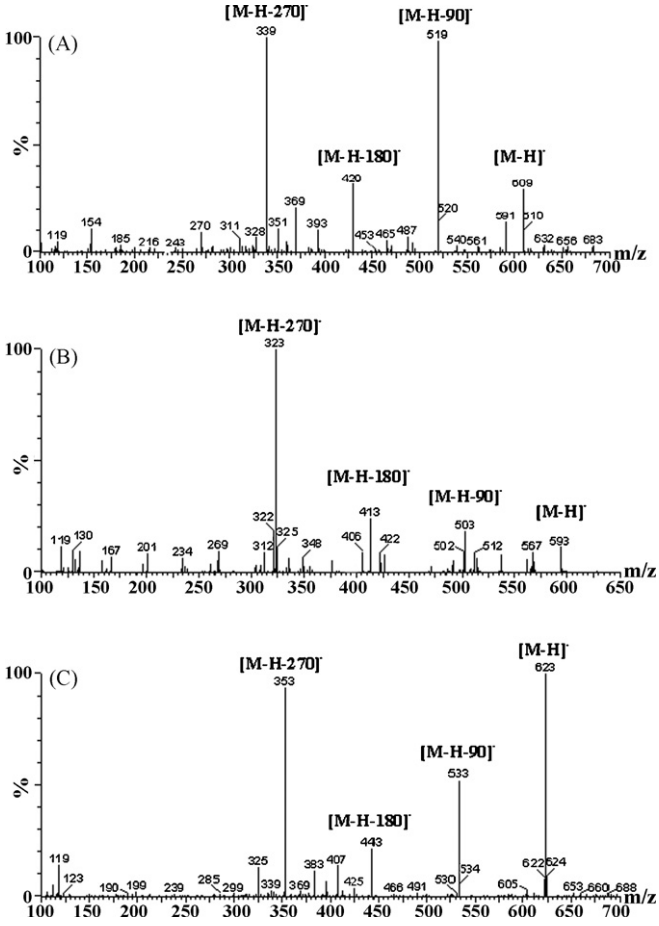


Fig. 4. The MS/MS spectra of isoorientin 3″-*O*-glucopyranoside (A), isovitexin 3″-*O*-glucopyranoside (B) and isoscoparin 3″-*O*-glucopyranoside (C).

443) was the product ion of the loss of the outer glucose. And the ion $[M-H-270]^-$ (m/z 339, 323, 353) was deduced to be the loss of both $[M-H-90]^-$ and $[M-H-180]^-$ ions.

3.2. Quantification of six flavone C-glycosides with UPLC–MS/MS system

Isoorientin, isoorientin 3″-*O*-glucopyranoside, isovitexin, isovitexin 3″-*O*-glucopyranoside, isoscoparin, and isoscoparin 3″-*O*-glucopyranoside in Daqingye samples were quantitatively determined. Since the six flavonoids assayed are three pairs of homologous compounds with same flavone aglycon and mono/diglycoside at C-6 position, it is difficult to accomplish satisfied separation by conventional HPLC. The UPLC-ESI-MS/MS method was utilized to the quantitative determination of these flavonoids. The UPLC conditions, spectrometric parameters and MRM transition were optimized as listed in Section 2.2. Typical MRM chromatograms of six flavonoids in Daqingye sample are shown in Fig. 5.

The linearities of the peak area (y) versus concentration (x) curves for these flavonoids were investigated by analyzing a set of standard solutions. Good linear relationship between the peak area and concentration was obtained for each of the six flavonoids over the tested concentration range with correlation coefficient >0.998, as listed in Table 4. The limit of detection (LOD) defined as the concentration resulting in a signal of three times the noise level is also shown in Table 4. The lowest concentration of the calibration range was considered to be the limit of quantification (LOQ), which were 40, 56, 48, 70, 58 and 82 ng/ml for the six flavonoids, respectively.

The intra-day and inter-day precisions were investigated by determining the six flavonoids in a Daqingye sample six times per day and on three consecutive days. The intra-day and inter-day precisions of the determination of the six flavonoids were less than 3.5% and 4.9%, respectively.

To evaluate the matrix effect, a Daqingye sample (from Hebei province) were extracted as described in Section 2.4 and 1 ml of the sample solution was spiked with 1 ml of standards mixture solution with known concentration, while another aliquot of 1 ml sample solution was diluted to 2 ml with 50% MeOH. The standard solution were also diluted one-fold with 50% MeOH and analyzed. The corresponding peak areas of the analytes in the standard spiked sample solutions (A) minus those in sample solutions (B) were then compared to those of the standard solutions (C). The ratio $[(A-B)/C \times 100\%]$ is defined as the matrix effect, which were 101.7%, 102.3%, 101.9%, 99.3%, 100.7% and 101.5% for the six flavonoids, respectively. The results indicated that no significant matrix effect was observed.

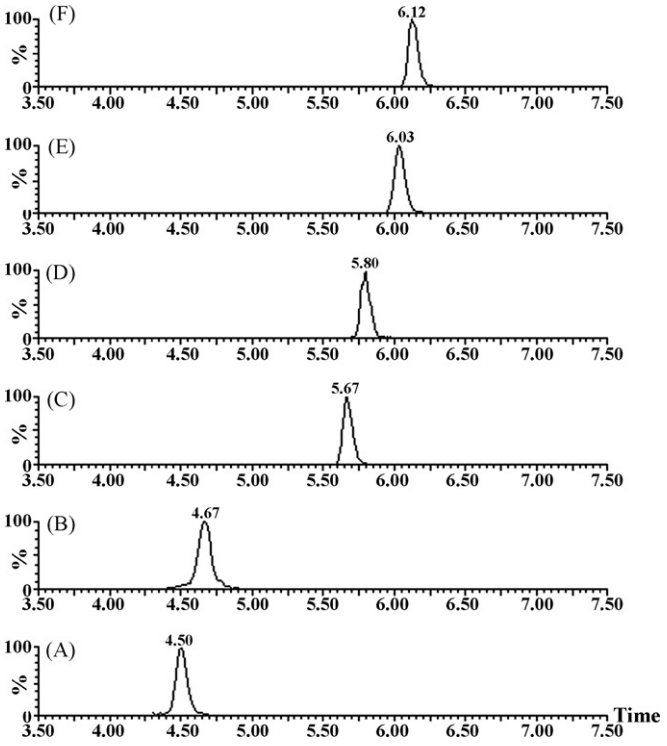


Fig. 5. Typical MRM chromatograms of six flavonoids in Daqingye samples: (A) t_R 4.50 min, isoorientin; (B) t_R 4.67 min, isoorientin 3″-*O*-glucopyranoside; (C) t_R 5.67 min, isovitexin; (D) t_R 5.80 min, isovitexin 3″-*O*-glucopyranoside; (E) t_R 6.03 min, isoscoparin; (F) t_R 6.12 min, isoscoparin 3″-*O*-glucopyranoside.

The recovery of the tested compounds was evaluated by analysis of spiked samples. The amount of the flavonoids about the same as that in the sample was spiked into 0.5 g of the Daqingye sample. The mixture was extracted and determined using the method described in Section 2. The recovery results are shown in Table 5. It turned out that the recoveries of the six flavonoids were within 96.6–100.2% and the R.S.Ds were below 4.7%.

The developed UPLC-ESI-MS/MS method was applied to the determination of six flavonoids in commercial samples of Daqingye. The content of each analyte was calculated from the corresponding calibration curve. As shown in Table 6, the contents of the six

Table 4
Linearity equations, correlation coefficients, linear ranges, and limits of detection for the six flavonoids determined

Compounds	Linear range (μg/ml)	Linearity equation	r^2	LOD (ng/ml)
Isoorientin	0.040–4.0	$y = 3130x + 40.72$	0.9996	0.9
Isoorientin 3″-*O*-glucopyranoside	0.056–5.6	$y = 500.8x - 36.40$	0.9995	4.5
Isovitexin	0.048–4.8	$y = 3177x + 101.1$	0.9986	0.8
Isovitexin 3″-*O*-glucopyranoside	0.070–7.0	$y = 2074x + 60.10$	0.9994	3.5
Isoscoparin	0.058–5.8	$y = 1133x - 11.01$	0.9994	1.0
Isoscoparin 3″-*O*-glucopyronoside	0.082–8.2	$y = 1645x + 103.1$	0.9992	4.1

Table 5
Recovery (%) of six flavonoids in Daqingye ($n = 3$)

Compounds	Amount added (mg)	Amount found (mg)	Recovery (%)	R.S.D. (%)
Isoorientin	0.130	0.126 ± 0.004	96.7	2.9
Isoorientin 3″-*O*-glucopyranoside	0.480	0.471 ± 0.009	98.1	1.9
Isovitexin	1.31	1.31 ± 0.06	96.6	4.7
Isovitexin 3″-*O*-glucopyranoside	0.890	0.863 ± 0.017	99.1	1.9
Isoscoparin	0.980	0.962 ± 0.024	98.2	2.5
Isoscoparin 3″-*O*-glucopyronoside	1.35	1.35 ± 0.03	100.2	2.1

Table 6
The contents (mg/g) of six detected flavonoids in Daqingye from different resourses

Compounds	Resourses			
	Hebei	Liaoning	Anhui	Shandong
Isoorientin	0.26	0.38	0.65	0.85
Isoorientin 3″-*O*-glucopyranoside	0.96	1.50	0.70	2.38
Isovitexin	2.61	3.27	5.25	4.31
Isovitexin 3″-*O*-glucopyranoside	1.78	1.71	0.40	0.88
Isoscoparin	1.96	2.86	4.54	4.27
Isoscoparin 3″-*O*-glucopyronoside	2.71	3.62	2.29	2.03

flavonoids in Daqingye samples from different resources varied significantly. The content of isovitexin, a flavonoid with demonstrated pharmacological activities [22,23] is the highest among the six flavonoids in those samples. The results indicated that quantitative determination of these flavonoids in Daqingye is significant in the quality evaluation of Daqingye and dosage guidance during clinical studies.

4. Conclusion

A UPLC-PDA-ESI-MS/MS method is described in this paper for the qualitative and quantitative analysis of flavonoids in Daqingye. The identification of flavonoids in Daqingye provided valuable information for the comprehension of the pharmacological efficacy of this herbal drug. The specific and sensitive UPLC–MS/MS method for quantification of six flavonoids in Daqingye can be applied as an alternative approach for the quality control of Daqingye crude drugs.

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