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ORIGINAL ARTICLE

Validation and application by HPLC for simultaneous determination of vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin, and hyperoside

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Abstract A simple, precise, and rapid high-performance liquid chromatographic method was developed and validated for the simultaneous determination of vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin, and hyperoside. The HPLC separation was performed using a Shim-pack VP-ODS C_{18} column (250 mm × 4.6 mm i.d., 5 μm) with the isocratic mobile phase consisting of tetrahydrofuran/acetonitrile/0.05% phosphoric acid solution (20:3:77, v/v/v), and the flow rate was set at 1.0 mL/min. UV detection was carried out at a wavelength of 360 nm and the whole analysis took 25 min. The method was linear in the range of 4.12–206.00 μg/mL for vitexin-2″-O-glucoside, 4.05–202.50 μg/mL for vitexin-2″-O-rhamnoside, 1.64–82.00 μg/mL for rutin, 1.74–87.00 μg/mL for vitexin, and 1.41–70.60 μg/mL for hyperoside with the correlation coefficient for each analyte more than 0.998. The limit of detection (LOD) and limit of quantitation (LOQ) were 0.6 and 2 ng for vitexin-2″-O-glucoside, 0.6 and 2 ng for vitexin-2″-O-rhamnoside, 0.3 and 1 ng for rutin, 1 and 3 ng for vitexin, and 0.5 and 2 ng for hyperoside, respectively. Intra- and inter-day precision and accuracy (RSD) were less than 3%. The developed HPLC method was successfully applied to the analysis of five flavonoids in hawthorn leaves, hawthorn fruits, and the preparations containing hawthorn leaves or fruits.

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1. Introduction

The hawthorn (*Crataegus*), a member of the Rosaceae family, has been used as medicine and food around the world for centuries. It is widely distributed in northern temperate zones, primarily in East Asia, Europe, and North America [1]. There are about 16 species in PR China, where *Crataegus pinnatifida Bge. Var Major* N. E. Br and *Crataegus pinnatifida* Bge. are the two major species, named as Shanlihong and Shanzha, respectively. Their fruits and leaves are the mainly considered

medicinal parts of the plant. Pharmacological and toxicological studies have demonstrated that the extract of hawthorn fruits and leaves has been confirmed to possess a wide range of pharmacological properties, such as improving digestion and relieving food stasis [2], anti-inflammatory [3], sedative effects [4], anti-arrhythmia [5], effectively inhibiting lipid oxidation [6], and decreasing blood pressure [7] and serum levels of cholesterol [8]. At the same time, it shows mild pharmacological action and few side effects [9], even at very high doses.

The chemical components of hawthorn fruits and leaves consist of sterols, triterpene acids, flavonoids, oligomeric proanthocyanidins, and organic acids [10]. Generally, flavonoids are considered to be the main group of active constituents in hawthorn, and the group is used for standardization and quality control in many state pharmacopoeias. At present, several approaches including high-performance liquid chromatography (HPLC) [11,12], capillary-zone electrophoresis (CZE) [13,14], and high-performance liquid chromatography equipped with electrospray ionization mass spectrometric (HPLC/ESI-MS) have been reported for the analysis of flavonoids in the hawthorn leaves and fruits. However, there are no investigation concerning the simultaneous determination of vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin, and hyperoside (Fig. 1) from hawthorn leaves and fruits, even though they are among the five important active components of hawthorn leaves and fruits. So, it is necessary to develop an analytical method for the simultaneous determination of the five flavonoids for standardization and quality control of hawthorn leaves, hawthorn fruits, and the preparations containing hawthorn leaves or fruits.

Therefore, the aim of the present study was to optimize and validate a simple, precise, and rapid HPLC with UV for the simultaneous determination of vitexin-2″-O-rhamnoside, vitexin-2″-O-glucoside, rutin, vitexin, and hyperoside in hawthorn leaves, hawthorn fruits, and the preparations containing hawthorn leaves or fruits.

2. Experimental

2.1. Chemical reagents and materials

Vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin, and hyperoside were purchased from National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). HPLC grade acetonitrile and tetrahydrofuran were from Fisher Scientific (Pittsburg, USA). Phosphoric acid, methanol, and ethanol were all products of Xi'an Analytical Instrument Factory (Xi'an, China). High purity water, prepared by a Milli-Q50 SP Reagent Water System (Millipore Corporation, MA, USA), was used throughout the study. Hawthorn leaves and fruits were purchased from the TCM Store (Xi'an, PR China) and were authenticated by associate professor Xiao-Feng Niu (School of Medicine, Xi'an Jiaotong University, China) according to morphological characteristics. Yixintong tablets (Shanxi Taisheng Pharmaceutical Co. Ltd.), Jiangzhining tablets (Shaanxi Tianyang Pharmaceutical Co. Ltd.), Baohe pills (Henan Wanxi Pharmaceutical Co. Ltd.) and Dashanzha pills (Xian Zhengda Pharmaceutical Co. Ltd.) were from Xi'an Tongrentang pharmaceutical stores (Shaanxi, Xi'an, China).

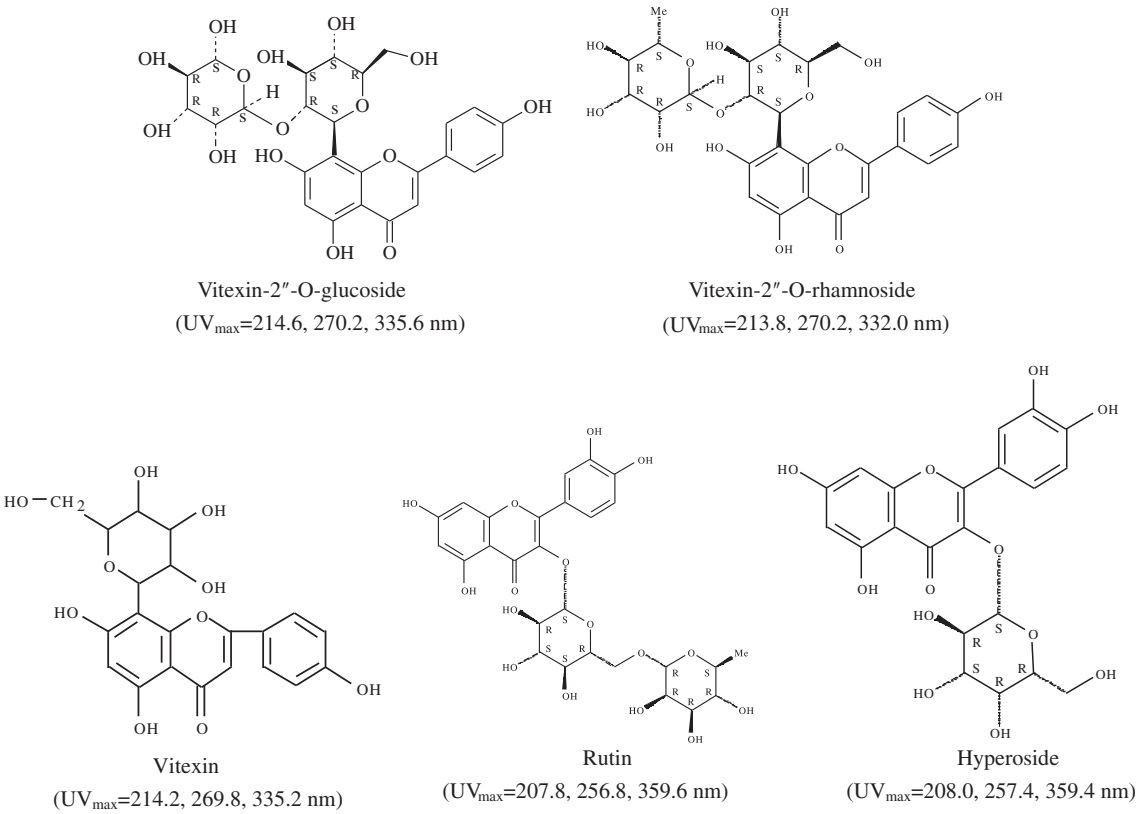

Figure 1 Chemical structures and UV_{max} of five flavonoids in Chinese hawthorn leaves and fruits.

2.2. Sample preparation

Hawthorn leaves and fruits were dried in an oven at 60 °C for 24 h. The dried materials of Hawthorn leaves and fruits were powdered by a disintegrator (HX-200A, Yongkang Hardware and Medical Instrument Plant, China) and then sieved (40–60 mesh). The sugar coats of Yixintong tablets or Jiangzhining tablets were washed off with water, and then the tablets were dried and ground into powder. Baohe pills were ground into powder. Dashanzha pills were cut into pieces.

Pulverized hawthorn leaves (1 g), hawthorn fruits (0.5 g), Yixintong tablets (0.1 g), Jiangzhining tablets (1 g), Baohe pills (1 g), and Dashanzha pills (1 g) were carefully weighed and extracted with 50 mL of ethanol–water (50:50, v/v) at 30 °C for 30 min in an ultrasonic bath (Kunshan Ultrasonic Instrument, China), the process was repeated twice with 25 mL of ethanol–water (50:50, v/v). The filtered solutions were combined and concentrated to dryness in the vacuum rotation evaporator at 40 °C. The residue was then dissolved in 25 mL of methanol. After filtration through a 0.45 μm Millipore membrane filter, aliquots of 20 μL were directly injected into the HPLC system.

2.3. Instrumentation

Chromatographic separation was performed on a Shimadzu LC-10AT$_{VP}$ pump equipped with a Shimadzu SPD-10A$_{VP}$ UV–vis detector (Shimadzu, Kyoto, Japan). Data integration was done by using LC-solution software (Shimadzu, Kyoto, Japan). Injections were carried out using a 20 μL loop.

2.4. Chromatographic conditions

The HPLC separation was performed using a Shim-pack VP-ODS C_{18} column (250 mm × 4.6 mm i.d., 5 μm) and a Shim-pack GVP-ODS C_{18} guard column (10 mm × 4.6 mm i.d., 5 μm). The isocratic mobile phase, consisting of tetrahydrofuran/ acetonitrile/0.05% phosphoric acid solution (20:3:77, v/v/v), was delivered at a flow-rate of 1.0 mL/min. Prior to use the mobile phase was filtered through 0.45 μm Millipore membrane filters and degassed by sonication in an ultrasonic bath. Detection wavelengh was set at 360 nm and the column temperature was maintained at 25 °C.

2.5. Standard solutions and calibration curves

Methanol stock solution containing vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin, and hyperoside was prepared and diluted to appropriate concentrations for the construction of calibration curves. At least six concentrations of the solution were analyzed in triplicate, and then the calibration curves were constructed by plotting the peak area versus the concentration of each analyte detected by HPLC.

2.6. The LOD and LOQ

The stock solution containing vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin, and hyperoside was diluted to a series of appropriate concentrations with methanol, and an aliquot of the diluted solutions was injected into HPLC for analysis. The LOD and LOQ under the present chromatographic conditions were determined at S/N of 3 and 10, respectively.

2.7. Precision, repeatability, and accuracy

Intra- and inter-day variations were chosen to determine the precision of the developed assay. The known concentrations of vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin, and hyperoside were tested. For intra-day variability test, the mixed standards solution was analyzed for six times within one day, while for inter-day variability test, the solution was examined in duplicates for three consecutive days. Variations were expressed as the RSD.

To confirm the repeatability, the powder of 0.5 g of hawthorn leaves and fruits was extracted, respectively, and analyzed by HPLC as mentioned above. The RSD was used as the measurement of repeatability.

Recovery test was used to evaluate the accuracy of the method. A known amount of standards was added to a certain amount (0.5 g) of hawthorn leaves and fruits, and then extracted and analyzed using the method described above. Three replicates were performed for the test. The recovery was calculated as follows:

$$\text{Recovery (\%)} = 100 \times (\text{amount found} - \text{original amount})/\text{amount spiked}.$$

2.8. Identification and quantification

Identification of the different compounds was made by comparing their retention time and UV spectra with those of pure standards. Quantification was performed on the basis of external standard method.

3. Results and discussion

3.1. Separation and identification

The purpose of this study was to develop a simple and rapid HPLC method for the simultaneous determination of five active components (vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin, and hyperoside) in hawthorn leaves, hawthorn fruits, and the preparations containing hawthorn leaves or fruits. During the method development, top priority was given for the complete separation of the five analytes of interest from other compounds existing in the extract of hawthorn leaves, hawthorn fruits and the preparations containing hawthorn leaves or fruits, and good peak shapes. When methanol (acetonitrile)–water, methanol (acetonitrile)–phosphoric acid (acetic acid)–water, methanol–acetonitrile–water, and methanol–acetonitrile–phosphoric acid (acetic acid)–water were chosen as the mobile phases in a isocratic elution, the separation or the peak shapes of the five analytes of interest existing in the extract of hawthorn leaves, hawthorn fruits and the preparations containing hawthorn leaves or fruits were not satisfactory. After trial and error, a isocratic elution of tetrahydrofuran–acetonitrile–0.5% phosphoric acid (20:3:77, v/v/v) was finally used to achieve complete separation of the five analytes of interest existing in the extract of hawthorn leaves, hawthorn fruits and the preparations containing hawthorn leaves or fruits, and good

peak shapes. Typical chromatograms corresponding to a standard mixture of the selected five flavonoids (vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin, and hyperoside), an unknown sample of hawthorn leaves, hawthorn fruits and the preparations containing hawthorn leaves or fruits, are shown in Fig. 2.

The UV_{max} of the five analytes of interest is shown in Fig. 1. The UV_{max} of vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside and vitexin is 270 nm and 335 nm, respectively, but the UV_{max} of rutin and hyperoside is 257 nm and 360 nm, respectively. Because the contents of rutin and hyperoside are much less than those of vitexin-2″-O-rhamnoside and vitexin-2″-O-glucoside, the detection was set at 360 nm. The results showed that a good sensitivity was observed by using UV at 360 nm for all the analytes.

The samples of hawthorn leaves and its preparation (Yixintong tablets) were found to contain the five analytes of interest, but the samples of hawthorn fruits and its preparation (Jiangzhining tablets, Baohe pills, and Dashanzha pills) only contained one analyte of interest (hyperoside). The eluted peaks were identified by comparing their retention time with that of the standard. The five analytes of interest were identified from the samples prepared from hawthorn leaves, hawthorn fruits and the preparations containing hawthorn leaves or fruits (Fig. 2). The retention time of vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin, and hyperoside was 8.62, 10.97, 14.18, 15.58, and 20.28 min, respectively. In addition, the eluted peaks were identified by comparing the UV spectra from SPD detector of standards and the extracts under the same chromatographic conditions.

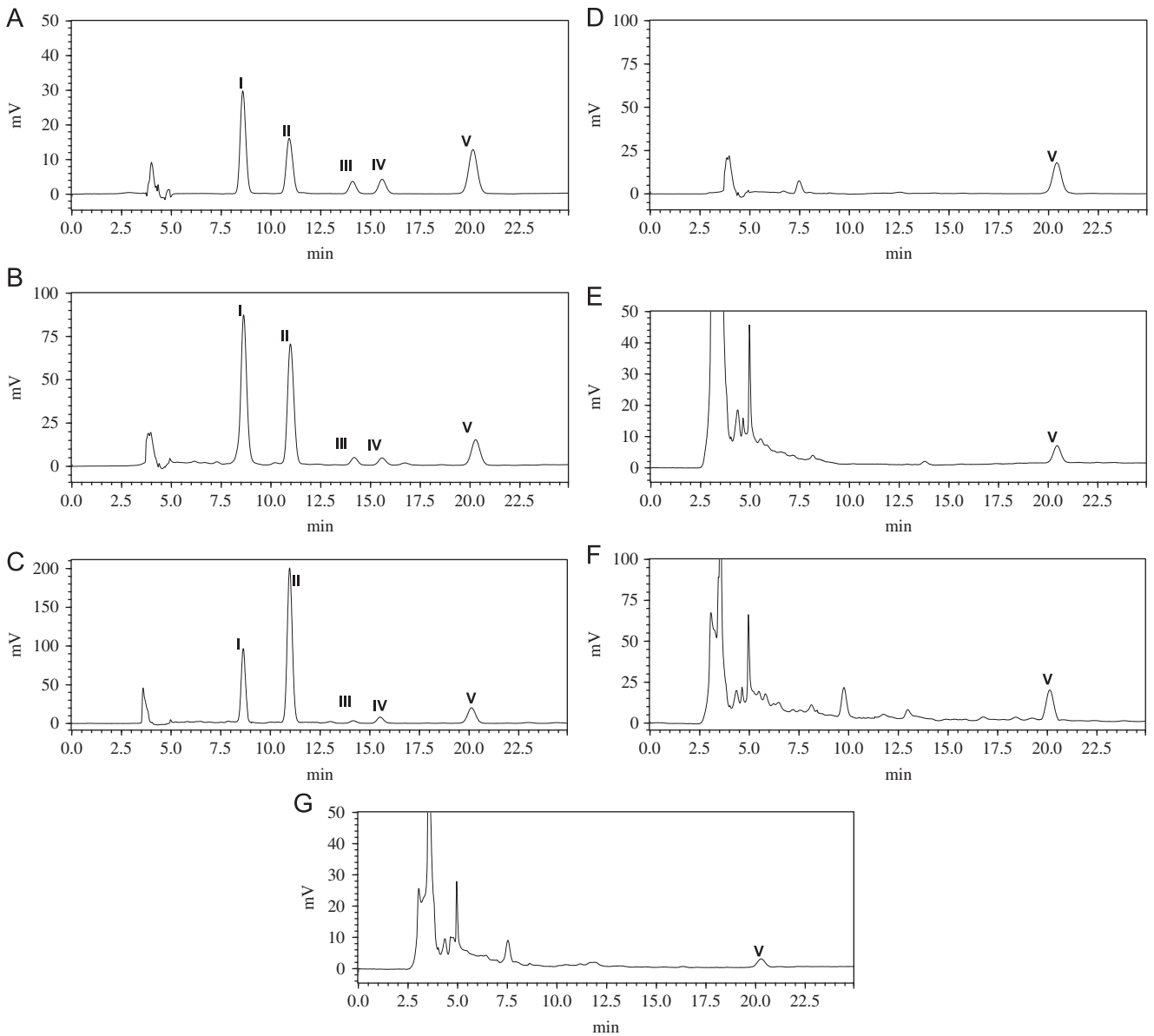

Figure 2 Typical chromatograms of five flavonoids in hawthorn leaves, hawthorn fruits and the preparations containing hawthorn leaves or fruits determined by HPLC-UV. (A) mixed standard solution of vitexin-2″-O-glucoside(I), vitexin-2″-O-rhamnoside(II), rutin(III), vitexin(IV), and hyperoside(V); (B) an unknown sample of hawthorn leaves; (C) an unknown sample of Yixintong tablets; (D) an unknown sample of hawthorn fruits; (E) an unknown sample of Jiangzhining tablets; (F) an unknown sample of Baohe pills; and (G) an unknown sample of Dashanzha pills.

Table 1 Calibration curve, LOD, and LOQ data of five flavonoid standards and recovery of five flavonoids in hawthorn leaves determined by HPLC-UV.

Compound	Linearity range (μg/mL)	Calibration equation	Correlation coefficient (r)	LOD (ng)	LOQ (ng)	Recovery (%)
Vitexin-2″-O-glucoside	4.12–206.00	$y=1.329\times10^{6}x+3818.831$	0.9982	0.6	2	96.7
Vitexin-2″-O-rhamnoside	4.05–202.50	$y=1.343\times10^{6}x+1540.220$	0.9998	0.6	2	101.4
Rutin	1.64–82.00	$y=8.532\times10^{5}x+936.965$	0.9989	0.3	1	97.9
Vitexin	1.74–87.00	$y=3.457\times10^{5}x+1074.241$	0.9990	1	3	98.5
Hyperoside	1.41–70.60	$y=1.975\times10^{6}x+369.925$	0.9999	0.5	2	101.2

Table 2 The contents of vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin, and hyperoside in hawthorn leaves, hawthorn fruits, and the preparations containing hawthorn leaves or fruits. (Mean ± SD).

Sample	Content (mg/g)				
	Vitexin-2″-O-glucoside	Vitexin-2″-O-rhamnoside	Rutin	Vitexin	Hyperoside
Hawthorn leaves	5.04 ± 0.11	3.51 ± 0.09	0.02 ± 0.00	0.35 ± 0.01	0.80 ± 0.02
Yixintong tablets	61.80 ± 0.52	108.10 ± 1.26	1.80 ± 0.06	4.70 ± 0.11	16.90 ± 0.26
Hawthorn fruits	ND	ND	ND	ND	1.61 ± 0.04
Jiangzhining tablets	ND	ND	ND	ND	0.40 ± 0.01
Dashanzha pills	ND	ND	ND	ND	0.03 ± 0.00
Baohe pills	ND	ND	ND	ND	0.52 ± 0.01

ND, not detected.

3.2. *Calibration and method validation*

The developed HPLC method was validated for different parameters like linearity range, LOD, LOQ, accuracy, precision, repeatability, and recovery. The series of calibration solutions were prepared and separated under the optimal conditions as described above. The calibration curves were found to be linear in the range of 4.12–206.00 μg/mL for vitexin-2″-O-glucoside, 4.05–202.50 μg/mL for vitexin-2″-O-rhamnoside, 1.64–82.0 μg/mL for rutin, 1.74–87 μg/mL for vitexin, and 1.41–70.60 μg/mL for hyperoside. Regression equation and correlation coefficient ranging from 0.9982 to 0.9999 revealed a good linearity response for developed method, which are presented in Table 1.

The LODs obtained for vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin and hyperoside were 0.6, 0.6, 0.3, 1, and 0.5 ng, while the LOQs obtained were 2, 2, 1, 3, and 2 ng, respectively (Table 1). This indicated that the proposed method exhibits a good sensitivity for the quantification of the five flavonoids in the hawthorn leaves and fruits.

The intra- and inter-day precisions (expressed as RSD) and accuracy (expressed as recovery) for the five analytes were determined by the spiked samples with the standard solutions of the five flavonoids ($n=6$), consecutively, using the analytical method above. The intra- and inter-day precisions were 1.82% and 2.11% for vitexin-2″-O-glucoside, 1.64% and 1.88% for vitexin-2″-O-rhamnoside, 1.77% and 2.05% for rutin, 1.94% and 2.39% for vitexin, 1.84% and 2.53% for hyperoside.

The repeatability was calculated by measurement of peak area for the five analytes of interest in the same sample and was observed in the range of 1.05–2.78%, which demonstrated the good repeatability of the proposed method.

Good recoveries in the range were obtained by the fortification of the samples at three concentration levels for vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin and hyperoside. It was evident from the results that the percent recoveries for all the five analytes of interest were in the range of 97.7–101.5%, and the RSD was less than 3%.

3.3. *Sample analysis*

Hawthorn leaves, hawthorn fruits, and the preparations containing hawthorn leaves or fruits were extracted following the procedure above, and analyzed using the developed HPLC method. The content of the five analytes of interest was calculated by the corresponding regression equation and is summarized in Table 2. The samples of hawthorn leaves and its preparation (Yixintong tablets) were found to contain the five analytes of interest, but the samples of hawthorn fruits and its preparation (Jiangzhining tablets, Baohe pills, and Dashanzha pills) only to the one analyte of interest (hyperoside).

4. Conclusion

A simple, precise, and rapid HPLC-UV assay method has been developed for simultaneous determination of vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, rutin, vitexin, and hyperoside, which could improve the quality control of hawthorn leaves and fruits. Besides, the developed HPLC method can be applied to the analysis of five important flavonoids in the preparations containing hawthorn leaves or fruits.

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