







## Research Article

# The Content Variation of Four Active Components in *Amygdalus persica* L. during Different Harvesting Periods

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In this study, a quantitative method for the content determination of rutin, 5-O-coumaroylquinic acid methyl ester, chlorogenic acid butyl ester, and kaempferol in *Amygdalus persica* L. flowers during different harvest periods was established to investigate its various rules and determine the optimal harvesting period. The determination was performed on the XTERRA MS C18 column with a mobile phase consisting of 0.1% formic acid aqueous solution and acetonitrile (gradient elution) at a flow rate of 1.0 mL/min. In combination with other validation data, including precision, stability, and recovery tests, this method demonstrated good reliability and sensitivity. The results showed that the contents of rutin, 5-O-coumaroylquinic acid methyl ester, chlorogenic acid butyl ester, and kaempferol in *A. persica* flowers during different harvest periods were quite different, and the content in samples at the early blooming stage was the highest. The method is simple, accurate, and rapid for determining the contents of four active ingredients in *A. persica* flowers.

## 1. Introduction

*Amygdalus persica* L., belonging to Rosaceae, was widely distributed in most regions of China and had the effect of reducing diarrhea and defecation, promoting water, and reducing swelling [1]. *A. persica* has an effect on beauty and health care and is widely used in the fields of food and medicine [2, 3]. *A. persica* flowers mainly contain flavonoids, polyphenols, polysaccharides, and other chemical components, which have antioxidant and antibacterial activities [4, 5]. In addition, Zhang et al. found the major volatile constituents of *A. persica* flowers were linolenic alcohol, *n*-hexadecanoic acid, cyclohexane, and octadecanoic acid [6]. Li et al. found *A. persica* flowers polyphenol can significantly

increase the brain 5-hydroxytryptamine and norepinephrine levels in the hippocampus of mice with chronic depression [7]. Liu et al. studied the inhibitory effect and kinetics of a methanol extract of *A. persica* flowers on tyrosinase. Results showed that *A. persica* flowers extract could inhibit the monophenolase activity of tyrosinase effectively as well as diphenolase activity [8].

At present, although *A. persica* can be used as medicine and the curative effect is accurate, it is only used in traditional Chinese medicine prescription [9–11]. *A. persica* was not included in the Chinese Pharmacopoeia and local standards, and there was no unified standard for its quality control. In our previous study, we investigated the chemical constituents and coagulation activity of *A. persica* flowers,

and we found that rutin and kaempferol possessed significant procoagulant activity, while chlorogenic acid butyl ester had anticoagulant activity in vitro [12, 13]. Therefore, the content variation of rutin, 5-O-coumaroylquinic acid methyl ester, chlorogenic acid butyl ester, and kaempferol in *A. persica* flowers in different harvest periods was simultaneously determined for the first time in this study. The content differences and dynamic changes of four effective components were compared and analyzed in order to understand the dynamic accumulation law in different growing periods and to provide a theoretical basis for strictly controlling the quality, timely harvesting, and rational development and utilization of *A. persica* flowers.

## 2. Materials and Reagents

**2.1. Instruments.** All the analyses were performed on a Waters 2695 liquid chromatography system (Waters, Milford, USA) equipped with a vacuum degasser, a quaternary solvent delivery system, an autosampler, a column compartment, and a w2489 UV visible detector. The Kq-250db CNC ultrasonic cleaner was purchased from Kunshan Ultrasonic Instrument Co., Ltd. (Kunshan, China). The AG285 electronic analytical balance was purchased from Mettler Toledo (Switzerland).

**2.2. Chemicals and Reagents.** Rutin, 5-O-coumaroylquinic acid methyl ester, chlorogenic acid butyl ester, and kaempferol were provided by Henan Engineering Research Center for comprehensive utilization of edible and medicinal plant resources and Huanghe Science and Technology College, and their purities were up to 98%. Deionized water was prepared using a Milli-Q ultrapure water purifier (ELGA, Labwater, Marlow, UK). Acetonitrile and methyl alcohol were purchased from Thermo Fisher Technologies Ltd. All other reagents were in analytical grade.

**2.3. Plant Material.** *A. persica* flower samples (No: S1–S14) were collected in the medicinal botanical garden of Henan University and identified by Professor Changqin Li of Henan University. The voucher specimens were deposited in the Institute of Natural Medicine of Huanghe Science and Technology College. And the information on samples of *A. persica* flowers are given in Table 1.

## 3. Methods and Results

**3.1. Chromatographic Conditions.** All analyses were performed on a Waters e2695 HPLC system (Waters, Milford, USA). The chromatographic separation was achieved using an XTERRA MS C18 column (4.6 mm × 250 mm, 5 μm) (Waters, Milford, USA), with the column oven temperature maintained at 25°C. The mobile phase consisted of 0.1% formic acid solution (Solvent A) and acetonitrile (Solvent B) and employed gradient elution at a flow rate of 1.0 mL/min. The elution program was designed as follows: from 0 to 15 min, 5–23% B; from 15 to 35 min, 23–30% B; from 35 to 40 min, 30–40% B. After a 5 min equilibration period, the

TABLE 1: Information of samples of *A. persica* flowers.

Lot no.	Collecting time
S1	2020-03-20
S2	2020-03-21
S3	2020-03-22
S4	2020-03-23
S5	2020-03-24
S6	2020-03-25
S7	2020-03-26
S8	2020-03-27
S9	2020-03-28
S10	2020-03-29
S11	2020-03-30
S12	2020-03-31
S13	2020-04-01
S14	2020-04-02

samples were used for injection. The sample injection volume was 10 μL. The column effluent was monitored at 360 nm.

### 3.2. Preparation of Solutions

**3.2.1. Standard Solutions.** Standard stock solutions of rutin, 5-O-coumaroylquinic acid methyl ester, chlorogenic acid butyl ester, and kaempferol were prepared at the concentrations of 170.4, 118.0, 155.0, and 185.6 μg/mL in methanol, respectively. Precisely measure the right amount of standard stock solutions placed in a 10 mL volumetric flask and add methanol to the constant volume. The concentrations of rutin, 5-O-coumaroylquinic acid methyl ester, chlorogenic acid butyl ester, and kaempferol of the mixed standard solution were 34.1, 35.4, 46.5, and 37.1 μg/mL, respectively.

**3.2.2. Sample Solutions.** *A. persica* flowers were dried in the shade, triturated with a pulverizer, and passed through a 40-mesh sieve. Accurately 1.0 g of *A. persica* flowers was put into a conical flask with plug and added with 25 mL of methanol. After weighing, the solution was treated by ultrasound for 30 min, cooled, and weighed again. The lost weight was complemented with methanol. The solution was shaken well, centrifuged, filtrated, and the filtrate was obtained.

All solutions were stored at 4°C and filtered through 0.22 μm membrane filters before being injected into the HPLC system for analysis. Methanol was used as a blank control solution.

**3.3. System Suitability.** Standard solutions, sample solutions (No: S1), and methanol blank control solutions were taken for sample injection and determination to analyze system suitability according to chromatographic conditions in Section 3.1. The result showed that methanol as a solvent had no interference with the detection. The theoretical plate numbers were all more than 3000, the chromatographic peaks of each component reached the baseline separation, and the separation degree from the adjacent

chromatographic peaks was greater than 1.5. All results were obtained within acceptable ranges (Figure 1).

**3.4. Method Validation.** In the validation of the analytical method used for the quantification of rutin, 5-O-coumaroylquinic acid methyl ester, chlorogenic acid butyl ester, and kaempferol of *A. persica* flowers, the following parameters were determined: linearity, stability, precision, repeatability, and recovery.

**3.4.1. Investigation of Linear Relations.** 0.2 mL, 0.6 mL, 0.8 mL, 1.2 mL, 1.5 mL, and 2.0 mL of mixed standard stock solutions were, respectively, accurately absorbed and placed in a 20 mL volumetric flask. Methanol was added at a constant volume to obtain standard solutions of various concentrations. The standard solutions were detected according to the chromatographic conditions and mobile phase conditions described in Section 3.1, and the peak area was recorded. Standard curves of the investigated components were established by plotting the peak areas ( $Y$ ) versus the concentration of each standard compound ( $X$ ). The limits of detection (LOD) under the chromatographic conditions were determined at the lowest detectable concentration with a signal-to-noise ratio (S/N) greater than three, and the limits of quantification (LOQ) were determined at the lowest concentration with an S/N greater than ten. All the calibration curves of the four analytes were gained with a good linear relationship, and the correlation coefficients of all the calibration curves were found to be higher than 0.9990. The results are shown in Table 2.

**3.4.2. Precision Test.** Intraday and interday variations were utilized to evaluate the precision of the developed method. The mixed standard solution was repeatedly sampled six times and detected according to the chromatographic conditions and mobile phase conditions described in Section 3.1, and the peak area was recorded. The results showed that the RSD of rutin, 5-O-coumaroylquinic acid methyl ester, chlorogenic acid butyl ester, and kaempferol peak area were 0.78%, 1.15%, 0.94%, and 1.13%, which indicated that the liquid chromatograph had good precision.

**3.4.3. Repeatability Test.** 1.0 g of *A. persica* flowers (No: S1) was accurately weighed in six replicates, prepared into solutions according to the methods described in Section 3.2.2, and detected according to the chromatographic conditions and mobile phase conditions described in Section 3.1 to determine the peak area of each sample. The results showed that the peak area RSD values of rutin, 5-O-coumaroylquinic acid methyl ester, chlorogenic acid butyl ester, and kaempferol were 0.83%, 1.15%, 1.06, and 0.94%, which indicated good repeatability.

**3.4.4. Stability Test.** 1.0 g of *A. persica* flowers (No: S1) was accurately weighed and prepared into solutions according to the methods described in Section 3.2.2. Samples were

detected according to the chromatographic conditions and mobile phase conditions described in Section 3.1 at 0 h, 2 h, 4 h, 8 h, 12 h, and 24 h to determine the peak areas of each sample. The RSD values of the peak areas were 1.22%, 1.05%, 0.95%, and 1.14%, respectively. This result suggested that the sample was stable within 24 h.

**3.4.5. Recovery Test.** The recovery experiment was performed by adding 50%, 100%, and 150% of individual standards to a known concentration of *A. persica* flowers. 1.0 g of *A. persica* flowers (No: S1) was accurately weighed in nine replicates, prepared into solutions according to the methods described in Section 3.2.2, and detected according to the chromatographic conditions and mobile phase conditions described in Section 3.1, to determine the peak area of each sample. The results are shown in Table 3, and the method had good accuracy.

**3.5. Determination of Sample Content.** The established analytical method was successfully applied to the simultaneous analysis of the four active ingredients (rutin, 5-O-coumaroylquinic acid methyl ester, chlorogenic acid butyl ester, and kaempferol) of *A. persica* flowers samples (No.: S1–S14). The contents of the four analytes in the samples were quantified, and the results are listed in Table 4 with the mean content of three replicated analyses ( $n = 3$ ).

As shown in Figure 2, the contents of rutin, 5-O-coumaroylquinic acid methyl ester, and kaempferol in the *A. persica* flowers during different harvest periods fluctuated slightly, and the three components showed a downward trend. The content of chlorogenic acid butyl ester fluctuates relatively large, and the overall trend was downward. The contents of rutin and 5-O-coumaroylquinic acid methyl ester in the *A. persica* flowers harvested on March 21 were the highest (26.265  $\mu\text{g/g}$  and 31.028  $\mu\text{g/g}$ , respectively) and reached the lowest on April 1 (16.203  $\mu\text{g/g}$  and 13.243  $\mu\text{g/g}$ , respectively). The content of chlorogenic acid butyl ester in *A. persica* flowers harvested on March 20 was the highest (81.094  $\mu\text{g/g}$ ) and showed a downward trend, reaching the lowest on April 1 (19.352  $\mu\text{g/g}$ ). The content of kaempferol in *A. persica* flowers harvested on March 22 was the highest (21.551  $\mu\text{g/g}$ ) and showed a significant downward trend, reaching the lowest on March 28 (6.956  $\mu\text{g/g}$ ). The total content of the four active ingredients in *A. persica* flowers during different harvest periods was the highest at the beginning of flowering, showing a downward trend, reaching the lowest on April 1.

## 4. Discussion

The ingredients of traditional Chinese medicine are complex, and the efficacy of traditional Chinese medicine is often the result of the synergistic effect of many ingredients [14, 15]. If only one or two ingredients are used as quality evaluation indexes, it is difficult to reflect the true quality of traditional Chinese medicine, while the multi-index evaluation method can more comprehensively characterize the quality of traditional Chinese medicine [16, 17]. Therefore,

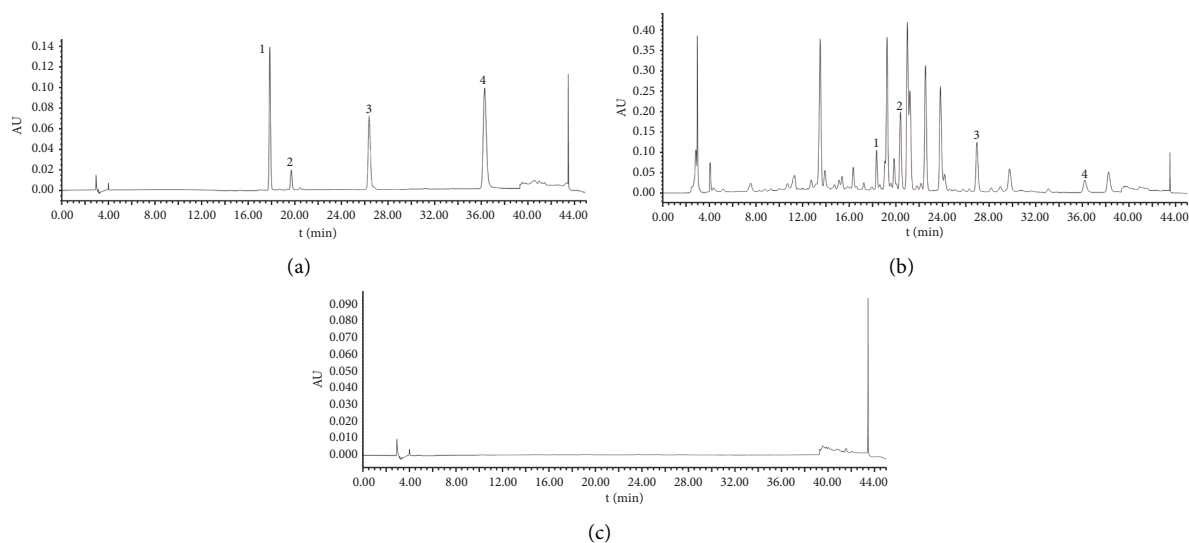


FIGURE 1: HPLC chromatograms of reference substances (a), samples S8 (b), and methanol blank control (c). (1) rutin; (2) 5-O-coumaroylquinic acid methyl ester; (3) chlorogenic acid butyl ester; (4) kaempferol.

TABLE 2: Regression equation and linear range of four active ingredients.

Components	Regression equation	$R^2$	Linear range/ $(\mu\text{g})$
Rutin	$y = 33772x - 13189$	0.9998	0.0682~0.5456
5-O-Coumaroylquinic acid methyl ester	$y = 79565x - 1669$	0.9996	0.0708~0.5664
Chlorogenic acid butyl ester	$y = 32987x - 31325$	0.9999	0.0930~0.7440
Kaempferol	$y = 52143x - 65703$	0.9995	0.0742~0.5936

the content determination of multi-ingredients has become the development trend of quality evaluation of traditional Chinese medicine [18, 19]. *A. persica* flowers contain a variety of effective ingredients, of which kaempferol has antioxidant, anti-inflammatory, antitumor, and other activities [20–23]. Sun et al. found that, in a certain concentration range, rutin had an obvious protective effect on HUVECs injured by  $\text{H}_2\text{O}_2$  and glucose. And the mechanism was related to the inhibition of the intercellular adhesion molecule expression and the regulation of NO and TNF- $\alpha$  production [24]. The anti-inflammatory and cytotoxic activities of rutin were determined by the Griess and CCK-8 methods; then, the results of screening demonstrated that rutin showed moderate NO inhibitory effects [25]. Combined with the previous study on the coagulation activity of *A. persica* flowers in vitro, rutin, 5-O-coumaroylquinic acid methyl ester, chlorogenic acid butyl ester, and kaempferol were selected as the quality control indexes of *A. persica* flowers, which can reflect the quality of *A. persica* flowers more comprehensively.

**4.1. Optimization of Extraction Methods.** The main chemical components of *A. persica* flowers are flavonoids and phenolic acids, which are easily soluble in polar solvents. The components and contents obtained by extraction with different polar solvents are different. Therefore, in the early stages of this study, three different polar solvents, namely, ultrapure water, methanol, and ethanol, were used for extraction. The results show that, compared with water and

ethanol, methanol extract has a better peak shape and the largest absorption peak intensity, so methanol was the best pure solvent for *A. persica* flowers extraction. Furthermore, the effects of different volume fractions of methanol (10%, 20%, 40%, 60%, 80%, and 100%) and different extraction methods (ultrasonic extraction [26] and reflux extraction [27]) on the extraction of active components from *A. persica* flowers were compared by the single factor method. The results showed that when 100% methanol was used as the solvent, the peak areas of rutin and other four components were higher and the method stability was better. At the same time, the author found that the effects of ultrasonic extraction and reflux extraction were similar. Considering the simplicity of operation and the repeatability of the method, ultrasonic extraction was selected in this study. On this basis, this research also investigated the effects of different extraction times (30, 45, and 60 min), different solid-liquid ratios (1.0 g: 10 mL, 1.0 g: 25 mL, and 1.0 g: 50 mL), and other factors on the extraction of rutin from *A. persica* flowers. The results showed that the results of different extraction times were similar, so the shortest extraction time was 30 min. According to different solid-liquid ratios, the extraction rate of 1.0 g: 25 mL is high, which can better reflect the chemical information of *A. persica* flowers. Therefore, it was determined that the extraction method was ultrasonic extraction, the extraction solvent was 100% methanol, the extraction time was 30 min, and the extraction solid-liquid ratio was 1.0 g: 25 mL.

TABLE 3: Test results of sample recovery ( $n = 9$ ).

Compounds	Mass (sample)/g	Mass (original)/ $\mu\text{g}$	Mass (added)/ $\mu\text{g}$	Mass (found)/ $\mu\text{g}$	Recovery/%	Average recovery/%	RSD/%
Rutin	1.0006	25.98	12.99	38.94	99.77	99.64	0.43
	1.0025	26.00	12.99	38.93	99.54		
	1.0017	25.98	12.99	38.98	100.08		
	1.0015	25.97	25.98	51.94	99.96		
	1.0014	25.97	25.98	51.67	98.92		
	1.0024	26.00	25.98	51.94	99.85		
	1.0019	25.98	38.97	64.79	99.59		
	1.0035	26.02	38.97	65.01	100.05		
	1.0027	26.00	38.97	64.58	99.00		
5-O-Coumaroylquinic acid methyl ester	1.0016	30.50	15.25	45.63	99.21	99.20	0.83
	1.0020	30.51	15.25	45.76	100.00		
	1.0016	30.50	15.25	45.38	97.57		
	1.0025	30.53	30.5	60.58	98.52		
	1.0024	30.53	30.5	60.80	99.25		
	1.0037	30.57	30.5	61.03	99.87		
	1.0018	30.51	45.75	76.35	100.20		
	1.0014	30.50	45.75	76.00	99.45		
1.0023	30.52	45.75	75.70	98.75			
Chlorogenic acid butyl ester	1.0028	81.32	40.66	122.40	101.03	98.96	1.33
	1.0016	81.22	40.66	121.68	99.51		
	1.0026	81.30	40.66	121.70	99.36		
	1.0017	81.23	81.32	160.30	97.23		
	1.0033	81.36	81.32	161.67	98.76		
	1.0025	81.30	81.32	161.86	99.07		
	1.0019	81.25	121.98	203.78	100.45		
	1.0020	81.26	121.98	199.83	97.20		
1.0027	81.31	121.98	200.93	98.07			
Kaempferol	1.0012	19.09	9.55	28.57	99.27	99.35	1.03
	1.0007	19.08	9.55	28.46	98.22		
	1.0014	19.10	9.55	28.58	99.27		
	1.0026	19.12	19.1	38.48	101.36		
	1.0009	19.09	19.1	38.03	99.16		
	1.0026	19.12	19.1	37.87	98.17		
	1.0013	19.10	28.65	47.57	99.37		
	1.0023	19.11	28.65	47.43	98.85		
	1.0030	19.13	28.65	47.92	100.49		

TABLE 4: The content of four active ingredients of samples ( $n = 3$ ,  $\mu\text{g/g}$ ).

Lot no.	Rutin	5-O-Coumaroylquinic acid methyl ester	Chlorogenic acid butyl ester	Kaempferol
S1	25.934	30.454	81.094	19.071
S2	26.265	31.028	78.144	6.992
S3	24.155	24.415	59.358	21.551
S4	21.859	20.329	54.414	6.953
S5	20.818	24.675	53.332	9.632
S6	19.071	21.741	41.730	9.073
S7	19.570	25.772	37.930	11.504
S8	22.226	24.801	33.860	12.051
S9	21.782	20.802	33.860	6.956
S10	17.720	21.640	43.103	12.625
S11	21.595	14.709	31.262	10.694
S12	21.978	14.078	26.347	13.187
S13	16.203	13.243	19.352	7.980
S14	20.725	20.065	26.463	12.066

4.2. Investigation of Chromatographic Conditions. According to references [28, 29], the four components were scanned in the wavelength range of 200~400 nm, and the four components had a better linear relationship at 360 nm.

At this wavelength, the interference of other components in the sample to the four components was less, and the baseline was more stable. Meanwhile, this research investigated the separation effects of two mobile phase systems, acetonitrile-

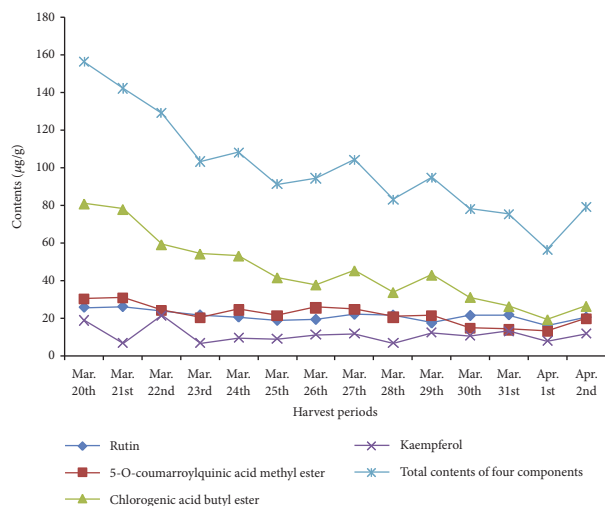


FIGURE 2: Dynamic change of the contents of four active ingredients in *A. persica* flowers during different harvesting periods.

water and acetonitrile-0.1% formic acid solution. The results showed that when the mobile phase was acetonitrile-water, the chromatographic peaks and separation degrees of chlorogenic acid butyl ester and kaempferol were poor. When an acetonitrile-0.1% formic acid solution was used as the mobile phase, the four components, such as kaempferol, could be well separated and the peak time was stable. Therefore, an acetonitrile-0.1% formic acid solution was selected as the mobile phase.

**4.3. Effect of Harvesting Time on the Content of Active Ingredients.** It can be seen that it is difficult to evaluate the overall quality of *A. persica* flowers medicinal materials with a single component, so this study simultaneously measured the contents of four active ingredients in *A. persica* flowers for the first time. The contents of rutin, 5-O-coumaroylquinic acid methyl ester, chlorogenic acid butyl ester, and kaempferol in *A. persica* flowers reached the highest value at the beginning of flowering and then began to decline and reached the lowest on April 1. The results showed the optimum harvesting period for *A. persica* flowers in the early flowering stage. The variation in the four components was thought to be due to differences in origin, harvesting time, and environmental conditions of raw plant materials, as well as differences in temperature. So, there is important academic significance and application value to carry out research on the chemical composition and pharmacological action of *A. persica* flowers during the early stage of anthesis.

## 5. Conclusions

A simple, rapid, and sensitive HPLC method for the determination of four active ingredients in *A. persica* flowers during different harvest periods was developed and validated. The results of the present study indicate that the early stage of anthesis is the optimum harvesting period for *A. persica* flowers. The method will provide a scientific basis for the quality control of *A. persica*.

## Data Availability

The data used to support the findings of this study are included within the article.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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