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Simultaneous Determination of Four Active Flavonoids in Citrus Changshan Huyou Fruit Collected in Various Seasons by HPLC Method

JIAN-FENG SONG[1,†], JING-QIAN FENG[2,†,*], JIAN-HUA HU[3], LI-PING XU[1] and HOU-DAO FU[4]

[1]Quzhou Institute for Food and Drug Control, No. 109, Heer Road, Kecheng Dist., Quzhou 324000, Zhejiang Province, P.R. China
[2]Quzhou College of Technology, No. 18, Jiangyuan Road, Kecheng Dist., Quzhou 324000, Zhejiang Province, P.R. China
[3]Quzhou NanKong Chinese medicine Co., Ltd, No. 11, Chunyuan Zhong Road, Qujiang Dist., Quzhou 324000, Zhejiang Province, P.R. China
[4]Second People's Hospital of Ningbo, No. 41, Xibei Road, Haishu Dist., Ningbo 315010, Zhejiang Province, P.R. China

*Corresponding author: Tel: +86 570 8068339; E-mail: fengjingqian@126.com
†Contribute equally to this work and should be considered as co-first author

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An HPLC method was developed for the simultaneous determination of narirutin, naringin, hesperidin and neohesperidin to investigate active ingredients in citrus Changshan Huyou fruit collected in different harvest time. The solid phase was Agilent ZORBAX SB-C18 (250 mm × 4.6 mm, 5 μm), with isocratic elution using 0.2 % phosphoric acid solution-acetonitrile (85:15) as the mobile phase. The flow rate was 1 mL/min and the wavelength was 284 nm. The methodology all fit the analytical requests and the recoveries were 98.83-99.05 %. Quantitative analysis showed that the contents of four main flavonoids would change within the growth period. The contents of naringin and narirutin presented a decreasing trend after the two months' increasing, reached the highest in June. Meanwhile, the contents of neohesperidin and hesperidin appeared a downgrade trend during the growing period. The contents of naringin and neohesperidin met the requirements of Chinese pharmacopoeia during May and July. Based on the method, quantitative analysis is simple and rapid, accurate and reliable, which was suitable for the contents determination and was able to provide reference for determining the best harvest period of ChangShan-HuYou as the medicinal resource.

Keywords: ChangShan-HuYou, Naringin, Hesperidin, Neohesperidin, Dynamic change.

INTRODUCTION

The ChangShan-HuYou (CSHY) is the fruit of *Citrus changshan-huyou* Y.B. Chang, belonging to the family of Citrus. ChangShan-HuYou is original from HuJia village, Qingshi town, Changshan County in Quzhou of Zhejiang province, with rich resources and high yield there. ChangShan-HuYou contains lots of vitamins, trace elements and nutrients, which is also rich in enormous amount of volatile oil, flavonoids, glycosides and other components in its pericarp. It's not only a kind of delicious fruit, but also a valuable medicinal plant. The main active components of ChangShan-HuYou are flavones, possessing pharmacological actions of preventing cough and eliminating phlegm, clearing heat and detoxicating, promoting gastrointestinal function, together with reducing blood pressure, blood fat and antioxidation[1,2].

According to the literature[3], it is of much similarities with "Fructus Aurantii" recorded in the Chinese Pharmacopoeia, both in character and chemical composition. Our research also confirmed this result in the related work. While the ChangShan-HuYou were harvested in the right time, several recorded index components all met the requirements of pharmacopoeia[4].

The research of four active flavonoids in ChangShan-HuYou changing along with different growing seasons was almost blank. In addition, there was no report about the simultaneous determination of flavonoids in ChangShan-HuYou of different growing seasons yet. Considering that the flavonoids in ChangShan-HuYou have plenty of medicinal values and could be made best use for clinical treatment[5,6], an HPLC method was established for the simultaneous determination of four flavonoids, including narirutin, naringin, hesperidin and neohesperidin, in ChangShan-HuYou fruits of different growing periods[7-10], which could provide reasonable evidence for its harvest and collection.

EXPERIMENTAL

Standards of narirutin, naringin, hesperidin and neohesperidin were supplied by National Institutes for Food and Drug Control (Beijing, China). ChangShan-HuYou were collected from Changshan County in Quzhou, Zhejiang province of China. The different growing periods of Changshan Huyou fruit were indentified by Jianfeng Song, the deputy director pharmacist of Quzhou Institute for Food and Drug Control. Methanol and

acetonitrile were of HPLC grade and other used reagents were analytical grade. Deionized water was prepared using a Millipore water purification system.

HPLC conditions: An Agilent 1260 series LC system was employed in this research, consisting of a G1379B Quaternary Pumps, a G1376B Degasser, a G1316A Diode-Array Detector and a G1376B autosampler.

The analysis of the four flavonoids was carried out on a ZORBAX SB-C18 (250 mm × 4.6 mm, 5 μm, Agilent, USA). The solvents used for HPLC separation of the four flavonoids in samples were acetonitrile (A) and buffer solution (B, 0.2 % phosphoric acid solution) at the flow rate of 1 mL/min. The mobile phase was A-B (15:85, v/v) with isocratic elution and the analysis was monitored at 284 nm. The column temperature was 40 °C and the sample injection volume was 5 μL.

Preparation of sample solutions: ChangShan-HuYou samples harvested in different growing periods were pulverized into powder and then passed through a 0.45 mm sieve, about 0.2 g sample was accurately weighted and then added into a 100 mL conical flask. 50 mL methanol was added for refluxing extraction for 1.5 h. After refrigeration, weighted accurately, making complement for the lose weight by methanol and shaking for uniformity. The solution was ready for the chromatographic analysis after passing through a 0.45 μm membrane filter.

Preparation of standard solutions: Four standard solutions, reference compounds narirutin (20.16 mg), naringin (29.40 mg), hesperidin (20.40 mg) and neohesperidin (76.08 mg) were dissolved with methanol and diluted into four different concentrations.

RESULTS AND DISCUSSION

Regression equations: Linear regression analysis for each of the four flavonoids was performed by the external standard method. Calibration curves were established based on six points of narirutin with concentrations of 39.96, 79.92, 159.84, 199.80, 319.68 and 399.60 μg/mL, six points of naringin with concentrations of 28.45, 56.90, 113.80, 142.25, 227.60 and 284.50 μg/mL, six points of hesperidin with concentrations of 18.09, 36.18, 72.36, 90.45, 144.72 and 180.90 μg/mL and six points of neohesperid in with concentrations of 85.86, 171.72, 343.44, 429.30, 686.88 and 858.60 μg/mL. The calculated results were given in Table-1. All the flavonoids showed good linearity in a relatively wide concentration range.

Precision: The standard mixture solution of narirutin, naringin, hesperidin and neohesperidin was injected into HPLC for six times continuously and the area of each peak was used for the calculation of precision. The results showed that relative stand deviation (RSD) of peak area of each standard was 0.32, 0.36, 0.23 and 0.21 %, respectively (n = 6).

Repeatability: Repeatability was carried out through preparing six samples solution with the same treatment procedure. The results showed that relative stand deviation of each peak area was 2.31, 1.24, 2.50 and 1.26 %, respectively (n = 6).

Stability: For stability test, the same sample solution was analyzed at the designated time points for 48 h. The results showed that relative stand deviation of each peak area was 1.26, 0.90, 1.54 and 0.98 %, stable for the experiment.

Recovery test: The sample with determined targeted contents was spiked with certain amounts of the four standards. Then the spiked sample was processed in accordance with the established method for HPLC detection. The average recoveries for narirutin, naringin, hesperidin and neohesperidin determined were 97.56-98.99 % (Table-2). Determinations of flavonoids in different seasons (the right time for first fruit harvest is in ten days after blossom, after then, every 30 days one time for collecting fruits samples) were given in Table-3.

Application of the HPLC method for quantitative studies: 10 μL sample solution was injected into the HPLC instrument. The representative HPLC chromatograms were shown in Fig. 1, respectively. Peaks in the obtained chromatograms were identified by comparing the retention time and on-line UV spectra with those of the standards.

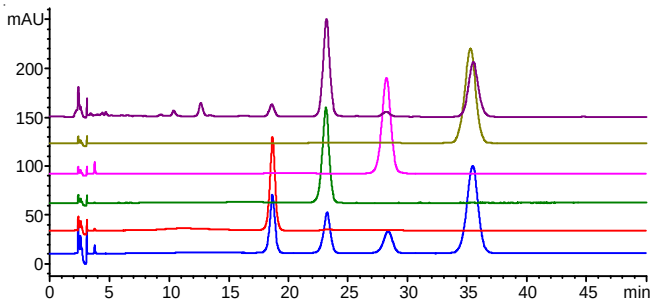

Fig. 1. Typical HPLC chromatograms of sample solution (A), neohesperidin (B,1), hesperidin (C,2), naringin (D,3), narirutin (E,4) and mixed standard solution (F)

Optimization of HPLC separation conditions: In order to get a separation with better resolution of targeted components in a shorter analytical time, we compared three different column temperature: 20, 30 and 40 °C and then we found that the higher the temperature, the shorter the retention time with almost the same resolution. Finally, we chose the column temperature of 40 °C. Besides, refluxing extraction and ultrasonic extraction were investigated in the experiments. Considering the advantages of high efficiency and complete extraction, we chose refluxing extraction as the way to prepare sample solution. In the meantime, we compared the different refluxing time, *i.e.*, 60, 90 and 120 min. Finally, taking the determination of targeted components into consideration, refluxing extraction 1.5 h with methanol was better. There were three chromatographic columns being compared in the experiment: Agilent Zorbax SB-C18 (250 mm × 4.6 mm, 5 μm), Elite Hypersil

TABLE-1
LINEAR REGRESSION EQUATION AND LINEAR RANGES

Flavonoids	Regression equation	Correlation coefficient (R)	Linear range (mg/mL)
Narirutin	A = 1236.0C + 46.5982	0.9996	39.96-399.60
Naringin	A = 1725.2C + 6.3833	0.9998	28.45-284.50
Hesperidin	A = 1764.6C + 0.4167	0.9998	18.09-180.93
Neohesperidin	A = 1914.7C + 15.892	0.9999	85.86-858.55

TABLE-2
RECOVERIES OF THE FOUR FLAVONOIDS (N = 6)

Component	Contents (mg)	Added (mg)	Determined (mg)	Recovery (%)	Mean (%)	RSD (%)
Narirutin	0.1738	0.1908	0.3609	98.07	98.59	1.30
	0.1809	0.1908	0.3652	96.57		
	0.1900	0.1908	0.3778	98.42		
	0.2041	0.1908	0.3957	100.44		
	0.1782	0.1908	0.3669	98.88		
	0.1706	0.1908	0.3598	99.15		
Naringin	2.0429	2.2701	4.2692	98.07	98.50	1.22
	2.1995	2.2701	4.3910	96.53		
	2.2816	2.2701	4.5097	98.15		
	2.3405	2.2701	4.6044	99.73		
	2.1402	2.2701	4.3836	98.82		
	2.0532	2.2701	4.3160	99.68		
Hesperidin	0.1161	0.1292	0.2445	99.39	98.99	1.54
	0.1218	0.1292	0.2469	96.80		
	0.1275	0.1292	0.2544	98.28		
	0.1360	0.1292	0.2657	100.41		
	0.1183	0.1292	0.2486	100.86		
	0.1167	0.1292	0.2436	98.20		
Neohesperidin	1.6183	1.7968	3.3755	97.80	97.56	1.17
	1.7331	1.7968	3.4607	96.15		
	1.7975	1.7968	3.5579	97.97		
	1.8187	1.7968	3.5491	96.30		
	1.6933	1.7968	3.4533	97.95		
	1.6214	1.7968	3.4033	99.17		

TABLE-3
DETERMINATION OF FOUR FLAVONOIDS OF CSHY IN DIFFERENT SEASONS (N = 6)

Sample No.	Harvest time	Days after blossom	Narirutin (mg/g)	RSD (%)	Naringin (mg/g)	RSD (%)	Hesperidin (mg/g)	RSD (%)	Neohesperidin (mg/g)	RSD (%)
1	2013-05-26	10	3.77	1.4	52.01	0.7	17.51	0.1	218.90	0.3
2	2013-06-25	40	7.06	1.2	109.66	0.2	5.48	0.2	83.11	0.2
3	2013-07-25	70	2.82	0.1	52.08	0.5	2.91	0.7	42.59	1.1
4	2013-08-24	100	2.29	1.8	25.81	1.6	1.35	1.2	19.82	1.9
5	2013-09-23	130	1.67	1.5	12.85	1.6	0.53	1.9	8.16	1.3
6	2013-10-23	160	1.41	1.7	12.62	1.2	0.42	1.8	7.97	1.6

C18 (250 mm × 4.6 mm, 5 μm) and Shim-pack VP-ODS C18 (4.6 mm × 250 mm, 5 μm). According to the effect of separation, Agilent Zorbax SB-C18 (250 mm × 4.6 mm, 5 μm) was used for further research of the methodology.

Conclusion

In this study, four flavonoids were investigated in different seasons. To the best of our knowledge, it is the first report which simultaneously determined the four main flavonoids in ChangShan-HuYou collected in different seasons, not only quantitatively but also qualitatively. Quantitative analysis showed that the contents of four main flavonoids would change within the growth period.

The contents of naringin and narirutin presented a decreasing trend after the two months' increasing, reached the highest in June. Meanwhile, the contents of neohesperidin and hesperidin appeared a downgrade trend during the growing period.

From the content point of view, we maintain that we could harvest the fruit in June for medicinal resources, which coincides with the harvest of immature fruit in June in the Chinese Pharmacopoeia. The study on dynamic changes of the four main flavonoids in ChangShan-HuYou in different growing periods could provide guidance for the plantation, harvest and scientific evaluation of ChangShan-HuYou. According to the Chinese Pharmacopoeia, to meet the requirements of the contents of naringin and neohesperidin, we recommend that harvesting immature fruit from May to July is probably a more appropriate choice and what is more, it will definitely have great significance to the harvest and further development of ChangShan-HuYou.

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