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ARTICLE

Simultaneous Determination of Five Bioactive Flavonoids in Pericarpium Citri Reticulatae from China by High-Performance Liquid Chromatography with Dual Wavelength Detection

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To evaluate the quality of Pericarpium Citri Reticulatae from China, a new, simple, and accurate method involving high-performance liquid chromatography with dual wavelength detection was developed for the simultaneous determination of five bioactive flavonoids, hesperidin, nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, tangeretin, and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone for the first time. Under the optimal condition, analysis was performed on a Dikma Diamonsil C_{18} column and gradient elution with a solvent system of acetonitrile and water at a flow rate of 1.0 mL/min and detected at 283 and 330 nm, respectively. All five calibration curves exhibited good linearity ($R^2 > 0.9997$). The relative standard deviation values for intra- and interday precision were less than 5% with accuracies between 95.16% and 104.71%. The recoveries were in the range of 96.91−103.20%. The established method was successfully applied to determine above five flavonoids in 32 samples collected from different districts of China, and the results demonstrated that the method may be used as strong research tools for quality control of Pericarpium Citri Reticulatae and chemotaxonomic investigation in botanical sciences of *Citrus* species.

KEYWORDS: Pericarpium Citri Reticulatae; flavonoids; HPLC; dual wavelength detection; quantification

INTRODUCTION

Pericarpium Citri Reticulatae (PCR, Chenpi in Chinese), the dried ripe pericarp of *Citrus reticulata* Blanco or its cultivars, is one of the most popular traditional medicinal herb with great medicinal values which has been widely used in traditional Chinese medicine and is officially listed in the Chinese Pharmacopoeia (*1*). It has been exploited for some applications in foods and drugs with the effectivenesses of regulating qi, normalizing the function of spleen and stomach, resolving phlegm, etc. (*2*). Generally, the main cultivars of *Citrus reticulata* Blanco in China are *Citrus reticulata* 'Chachi' (Chachigan), *Citrus reticulata* 'Unshiu' (Wenzhoumigan), *Citrus reticulata* 'Subcompressa' (Zaoju), *Citrus reticulata* 'Tangerina' (Fuju or Chuanju), *Citrus reticulata* 'Dahongpao' (Dahongpao), *Citrus reticulata* 'Kinokuni' (Nanfengmiju), *Citrus reticulata* 'Erythrosa' (Zhuju), *Citrus reticulata* 'Ponkan' (Ponkan), and so on. Among them, the dried ripe pericarp of *Citrus reticulata* 'Chachi', mainly produced in Xinhui district of Guangdong Province in China, named Guang Chenpi in Chinese, is regarded as a genuine medicinal herb on account of its excellent quality.

Apart from essential oils, the primary active biological constituents of PCR are flavonoids, which are generally categorized into two groups, flavanone glycosides (mainly include hesperidin, etc.) and polymethoxylated flavones (mainly include nobiletin, tangeretin, etc.). At present, owing to its extremely high concentration (no less than 3%) and wealth of various pharmacological activities such as anticarcinogen (*3*), antioxidation (*4*,*5*), and anticonvulsion (*6*), hesperidin is chosen as a chemical reference substance singly for quality control of PCR in the Chinese pharmacopoeia. However, with the progress of scientific research, polymethoxylated flavones (PMFs) are also found to play critical roles in influencing the quality of PCR. A lot of studies have shown that PMFs possess various biological activities, including anticarcinogenic (*7*, *8*), antimutagenic (*6*, *9*), anti-inflammatory (*10*, *11*), antioxidative (*12*), and cardioprotective properties (*13*). Moreover, the types and contents of PMFs vary among different varieties of *Citrus* species (*14*), which may serve chemotaxonomic purposes in botanical sciences. Therefore, it is necessary to evaluate the quality of PCR more comprehensively and effectively.

Several studies have reported the determination of flavonoids in *Citrus* herbs by using TLC (*15*), HPLC-UV (*16*, *17*), HPLC-MS (*18*), HPLC-ECD (*19*), and CE-ECD (*20*). However, most of them are focused on the determination of flavonoids in citrus fruits or juices of only one or two species of *Citrus* herbs. In addition, there are very few studies about citrus flavonoids in PCR among locally grown cultivars, especially in China.

Thus, in this paper, a new, simple, accurate, and reliable HPLC method for the simultaneous determination of five bioactive flavonoids (**Figure 1**) including hesperidin (1), nobiletin (2), 3,5,6,7,8,3′,4′-heptamethoxyflavone (3), tangeretin (4), and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (5) was established to comprehensively analyze 32 samples collected from different main citrus producing areas in China, such as Guangdong Province, Guangxi Province,

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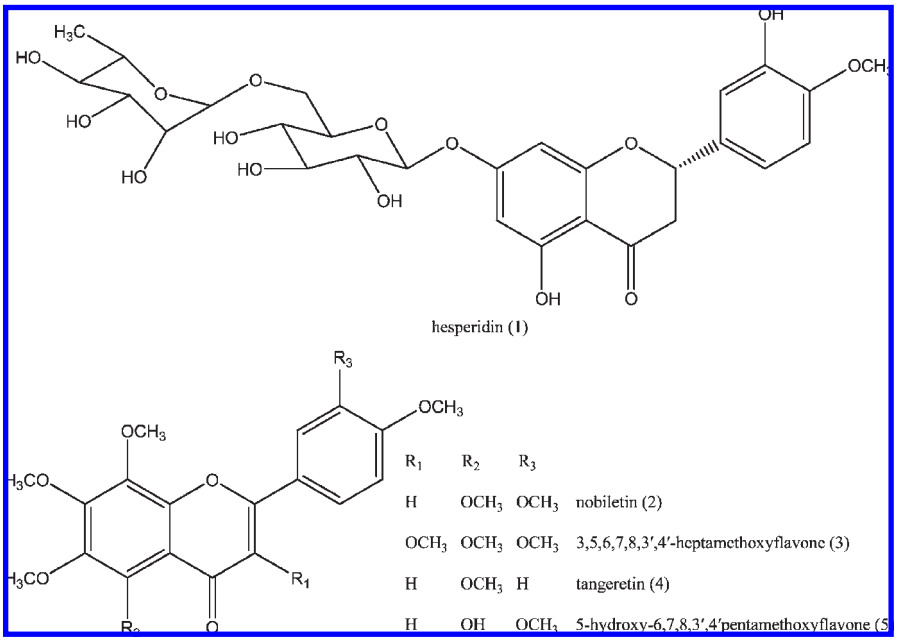

Figure 1. Chemical structures of bioactive flavonoids **1**−**5**.

Table 1. Calibration Curve Data for Reference Compounds **1**−**5** (n = 3)

compd	regression equation ($y = ax + b$) [a]	R^2	linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
1	y = 19, 930.33x − 152, 459.75	0.9998	19.50−1, 248.00	0.54	1.87
2	y = 83, 771.88x − 42, 750.37	0.9999	1.50−192.00	0.32	1.01
3	y = 55, 858.03x − 26, 196.57	0.9997	2.69−86.00	0.27	0.87
4	y = 93, 661.10x − 51, 701.45	0.9999	1.59−204.00	0.34	1.08
5	y = 53, 827.97x − 10, 185.66	0.9999	1.39−89.00	0.22	0.72

[a] Where y and x denote the peak area and corresponding injection concentration (μg/mL), respectively, and where a and b denote the slope and intercept of the regression line, respectively.

Table 2. Intra- and Interday Precision of the Developed Method (n = 6)

		intraday			interday		
compd	concn (μg/mL)	detected[a] (μg/mL)	accuracy (%)	RSD (%)	detected[a] (μg/mL)	accuracy (%)	RSD (%)
1	19.50	20.06 ± 0.38	102.86	1.89	20.42 ± 0.74	104.71	3.63
	78.00	76.65 ± 2.73	98.26	3.56	76.85 ± 2.00	98.52	2.61
	624.00	601.02 ± 13.20	96.32	2.20	604.03 ± 14.23	96.80	2.36
2	6.00	5.86 ± 0.11	97.59	1.93	5.93 ± 0.28	98.82	4.78
	24.00	24.57 ± 0.47	102.37	1.91	23.81 ± 0.65	99.22	2.72
	192.00	191.21 ± 1.40	99.59	0.73	192.57 ± 4.03	100.30	2.09
3	5.375	5.20 ± 0.12	96.77	2.26	5.11 ± 0.10	95.16	1.93
	21.50	21.44 ± 0.77	99.74	3.61	21.08 ± 0.37	98.04	1.76
	172.00	178.35 ± 3.02	103.69	1.69	173.74 ± 5.83	101.01	3.36
4	6.38	6.35 ± 0.12	99.53	1.85	6.29 ± 0.10	98.62	1.62
	25.50	26.12 ± 0.91	102.42	3.48	26.59 ± 0.77	104.29	2.90
	204.00	205.42 ± 3.62	100.70	1.76	199.73 ± 4.79	97.91	2.40
5	5.56	5.42 ± 0.14	97.36	2.52	5.32 ± 0.14	95.55	2.56
	22.25	22.56 ± 0.37	101.39	1.62	21.86 ± 0.70	98.24	3.22
	178.00	183.78 ± 1.49	103.25	0.81	181.95 ± 2.69	102.22	1.48

[a] Data are represented as the mean ± SD.

Sichuan Province, Fujian Province, Zhejiang Province, Jiangxi Province, Hubei Province, and Hunan Province. And the results provided detailed information for the identification of botanical origin and chemotaxonomic investigation of *Citrus* species.

MATERIALS AND METHODS

Materials and Reagents. Thirty-two samples including nine different cultivars were collected from different main citrus producing regions in China (**Table 5**). About 8−10 kg of fresh fruits was collected from each sampling area. Then, the citrus peels were removed and dried in the sun for about 5−7 days, which were used for the tested sample. The voucher specimens, identified by Prof. DePo Yang, have been deposited at the Laboratory of Pharmacognosy and Natural Medicinal Chemistry, School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou University City, Guangdong Province, China.

HPLC-grade acetonitrile (Tedia, Fairfield, OH) was used for HPLC analysis. Water for HPLC analysis was purified using a Millipore Milli-Q plus system (Milford, MA). All other reagents were of analytical

grade (Tianjin Chemical Reagent Factory, Tianjin, China). The reference standards of flavonoids 1−5 were isolated and purified from PCR by conventional column chromatography coupled with high-speed counter-current chromatography (HSCCC). Their structures were identified by EI-MS, ^{1}H NMR, and ^{13}C NMR in comparison with the literature data (*21*, *22*), and purities were determined to be >98% by HPLC-PDA analysis based on a peak area normalization method.

HPLC Conditions. The HPLC analysis was performed on a Shimadzu LC-20AT HPLC system (Shimadzu, Suzhou, China) equipped with a dual pump, a 7725i injection valve (20 μL, Rheodyne) and a SPD-20A UV/vis detection coupled with LC solution software. The separation was carried out on a Dikma Diamonsil C_{18} column (250 mm × 4.6 mm i.d., 5 μm) and at a column temperature of 25 °C. The mobile phase was acetonitrile and water with a gradient elution programmed as follows: 0−15 min, 25−50% acetonitrile; 15−35 min, 50−60% acetonitrile; 35−40 min, 60−85% acetonitrile. The flow rate was 1.0 mL/min, and sample injection volume was 20 μL. The effluent was monitored by UV detection at 283 nm for compound **1** and 330 nm for compounds **2**−**5**.

Table 3. Recovery Data of the Developed Method (n = 6)

	concentration of analyte				
compd	original (μg/mL)[a]	spiked (μg/mL)	found (μg/mL)[a]	recovery (%)[a]	RSD (%)
1	444.03 ± 0.41	124.80	566.67 ± 5.01	98.27 ± 3.88	3.94
2	56.85 ± 0.05	62.40	119.92 ± 2.08	101.08 ± 3.37	3.34
3	6.29 ± 0.01	5.60	11.75 ± 0.23	97.64 ± 4.11	4.20
4	34.99 ± 0.03	57.20	94.02 ± 1.64	103.20 ± 2.85	2.76
5	4.26 ± 0.01	5.20	9.30 ± 0.23	96.91 ± 4.45	4.60

[a] Data are represented as the mean ± SD.

Preparation of Sample Solutions. An amount of 100 g of each tested sample was ground into thin powder (140 mesh), and an aliquot (0.4 g) of the sample was added to 40 mL of methanol with ultrasonication at room temperature for 30 min. After centrifugation at a speed of 4000 rpm for 5 min, the supernatant was collected and evaporated to small volume under vacuum. Subsequently, the enriched liquid was transferred to a 25 mL volumetric flask and diluted to volume with methanol. The obtained solution was filtered through a 0.45 μm of filter membrane, and 20 μL of the filtrate was subjected to HPLC analysis.

Preparation of Standard Solutions. Standard stock solution was prepared by dissolving the reference compounds **1**−**5** in methanol to final concentrations of 1.248, 0.384, 0.344, 0.408, and 0.356 mg/mL, respectively. Working standard solutions were prepared from the standard stock solution by serial dilutions with the appropriate volume of methanol. These solutions were stored in a refrigerator at 4 °C for further HPLC analysis.

Calibration Curves: Limits of Detection and Limits of Quantification. Linear correlation analysis for each of the five flavonoids was determined in triplicate using six different concentrations of the above working standard solutions. Calibration curves were constructed by plotting the peak area (Y) vs the corresponding concentration of the

Table 4. Analysis Repeatability and Stability of the Developed Method

	repeatability (n = 6)			stability (n = 8)		
compd	RT (min)[a]	content (mg/g)[a]	RSD (%)	RT (min)[a]	content (mg/g)[a]	RSD (%)
1	8.77 ± 0.07	42.736 ± 0.647	1.52	8.75 ± 0.06	42.295 ± 0.957	2.26
2	24.42 ± 0.04	6.424 ± 0.141	2.20	24.45 ± 0.05	6.735 ± 0.179	2.66
3	26.80 ± 0.05	0.697 ± 0.008	1.20	26.82 ± 0.06	0.716 ± 0.018	2.54
4	28.77 ± 0.08	3.676 ± 0.069	1.87	28.78 ± 0.08	3.851 ± 0.089	2.32
5	33.75 ± 0.25	0.466 ± 0.014	2.90	33.74 ± 0.24	0.479 ± 0.010	2.05

[a] Data are represented as the mean ± SD.

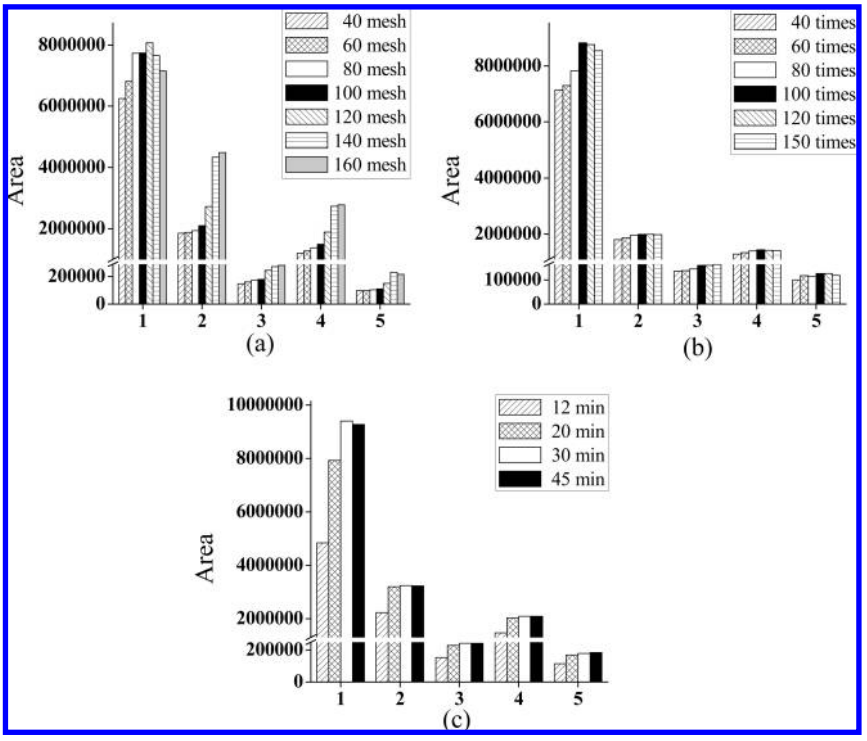

Figure 2. Extraction efficiencies of the compounds **1**−**5** by different sizes of sample powders (**a**), different times the volume of methanol (**b**), and different extraction time (**c**).

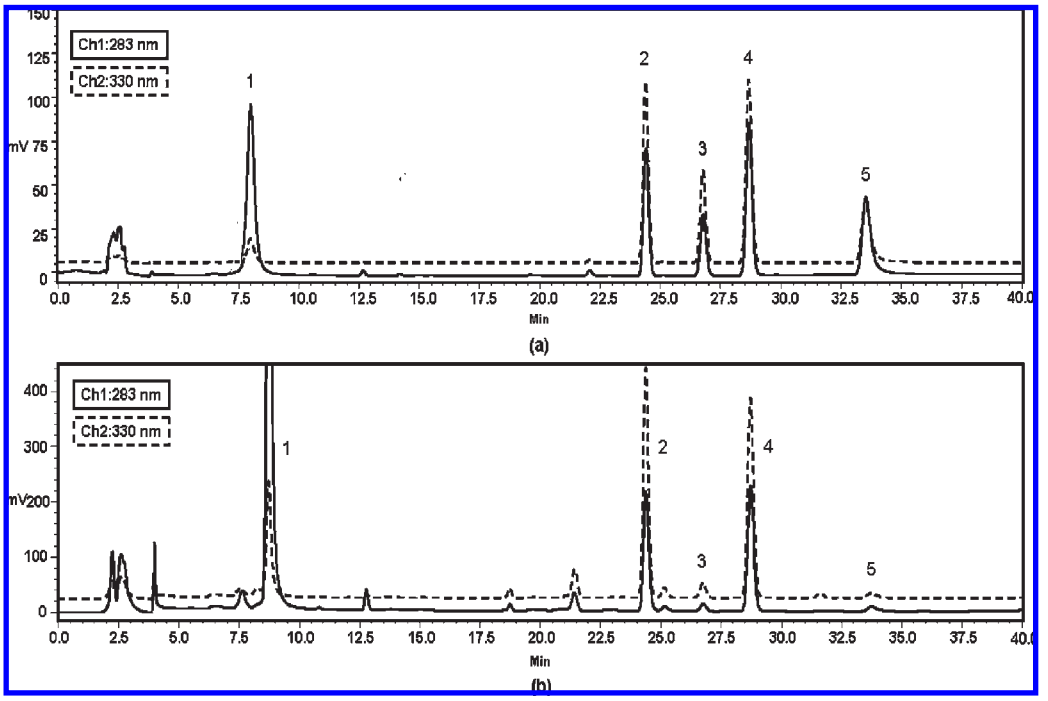

Figure 3. Representative HPLC chromatograms of standards mixture (**a**) and sample (**b**). The numbers indicate bioactive flavonoids **1**−**5**.

standard solutions (X, μg/mL). The limits of detection (LOD) and quantification (LOQ) were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively. The calculated results are shown in **Table 1**.

Precision, Repeatability, Recovery, and Stability. The intra- and interday precisions of the method were determined using multiple analyses (n = 6) of the standard solutions prepared at three different concentration levels (low, medium, and high) for each compound within 1 day and on 6 consecutive days. As shown in **Table 2**, the relative standard deviation (RSD) values were less than 5% and accuracies were in range of 95.16−104.71%.

To determine the repeatability of the analysis method, six samples from the same source were prepared and analyzed as the method described above. As shown in **Table 3**, the RSD values of the analytes were less than 3%.

Recovery tests were used to evaluate the accuracy of the developed method. A known amount of the mixed standard solution was added to a 0.2 g of the sample quantified previously, then extracted and analyzed using the described method. These experiments produced recovery rates in the range of 96.91−103.20% with their RSD values less than 5%.

The stability of the sample solutions was tested by injecting the same sample solution at 0, 2, 4, 6, 8, 10, 24, 48 h after preparation using the above established method. The results showed that the sample solutions would be stable within 48 h (**Table 4**).

RESULTS AND DISCUSSION

Optimization of Extraction Method. As a simple and efficient extraction method, ultrasonic extraction was chosen for the present experiments. Then, the efficiencies of extraction with different solvents such as petroleum ether (bp 60−90 °C), ethyl acetate, ethanol, and methanol were investigated preliminarily, and the results showed that methanol was an ideal solvent, which resulted in the highest extraction yield on both flavanone glycoside and PMFs. Moreover, to obtain the optimal extraction efficiency, effects including the particle size of crude drug, solvent volume, and extraction time on the extraction performance were evaluated. An almost equal amount of sample (0.2 g) was extracted and analyzed using the described procedure. As a result, compared with the extraction yields of different sizes of sample powders (40, 60, 80, 100, 120, 140, and 160 mesh), different factors of the volume of methanol (40, 60, 80, 100, 120, and 150 times), and extraction time (12, 20, 30, and 45 min) for five tested compounds, it was found that 140 mesh powders, 100 times the volume of methanol, and ultrasonication for 30 min would be optimum (**Figure 2**).

Optimization of HPLC Conditions. In order to establish an optimal and reliable chromatographic condition, the effects of different mobile phase compositions (methanol−water and acetonitrile−water), different concentrations of acid (formic acid and acetic acid), and detection wavelength were examined. The results showed that compounds **2**−**5** were difficult to reach baseline separations if the solvent system of methanol−water was used, and there were no significant improvements when different concentrations of acid were used. However, it was found that the peak shape and separation efficiency of five flavonoids could be improved significantly when the mobile phase of acetonitrile−water was applied with gradient elution. As a result, the solvent system of acetonitrile−water was chosen as the mobile phase with procedure of gradient elution programmed as described above. Moreover, according to the result of HPLC-PDA analysis, the maximum absorption wavelength of compounds **1**−**5** were 283.7, 334.4, 341.5, 323.7, 345.1 nm, respectively. So compound **1** and compounds **2**−**5** were monitored with a UV detector at 283 and 330 nm, respectively. Representative HPLC chromatograms of standards mixture and sample under the optimized conditions are shown in **Figure 3**.

Sample Analysis. The developed analytical method was then applied to the simultaneous determination of above five flavonoids in 32 batches of PCR collected from different main citrus producing regions in China. The contents of the analyzed compounds are summarized in **Table 5** (owing to the particularly high content of tested compounds in some parts of sample, the sample solution would be further diluted twice with methanol if the concentration of tested compounds was beyond the linear range). The data indicate that the content of each flavonoid varied significantly among the different cultivars and regions, especially different cultivars. Except for 5 of the 32 examined, samples were free of 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (compound **5**) and eight samples were not be quantified owing to its extremely low content; all five bioactive flavonoids could be determined accurately in the remaining samples.

Among the cultivars examined, hesperidin (compound **1**) is the richest in 32 samples of content varied from 38.021 to 121.428 mg/g. As a genuine medicinal herb, Guang Chenpi (*Citrus reticulata* 'Chachi') produced in Xinhui district exhibits relatively lower

Table 5. Contents of Compounds **1**−**5** in Pericarpium Citri Reticulatae Collected from Different Regions in China

sample no.	cultivars	place of collection	time of collection	content of compounds (mg/g, $n = 3$)[a] **1**	**2**	**3**	**4**	**5**
1	*Citrus reticulata* 'Chachi'	Siqian Town, Xinhui District, Jiangmen City, Guangdong Province	2007/11	51.800 ± 1.262	4.800 ± 0.081	0.523 ± 0.014	4.439 ± 0.084	0.366 ± 0.010
2	*Citrus reticulata* 'Chachi'	Yanan Town, Xinhui District, Jiangmen City, Guangdong Province	2007/11	53.914 ± 0.730	5.851 ± 0.121	0.644 ± 0.016	3.008 ± 0.045	0.373 ± 0.008
3	*Citrus reticulata* 'Chachi'	Daze Town, Xinhui District, Jiangmen City, Guangdong Province	2008/11	50.123 ± 0.200	3.694 ± 0.075	0.406 ± 0.007	2.286 ± 0.046	0.308 ± 0.007
4	*Citrus reticulata* 'Chachi'	Huicheng Town, Xinhui District, Jiangmen City, Guangdong Province	2007/12	42.817 ± 0.496	3.281 ± 0.088	0.381 ± 0.008	1.976 ± 0.037	0.277 ± 0.008
5	*Citrus reticulata* 'Chachi'	Shuangshui Town, Xinhui District, iangmen City, Guangdong Province	2007/11	55.379 ± 0.476	7.090 ± 0.093	0.784 ± 0.009	4.364 ± 0.101	0.531 ± 0.008
6	*Citrus reticulata* 'Chachi'	Sanjiang Town, Xinhui District, Jiangmen City, Guangdong Province	2008/12	38.021 ± 0.544	2.830 ± 0.070	0.367 ± 0.010	1.749 ± 0.049	0.210 ± 0.006
7	*Citrus reticulata* 'Chachi'	Shadui Town, Xinhui District, Jiangmen City, Guangdong Province	2008/11	59.785 ± 0.365	4.010 ± 0.064	0.524 ± 0.009	2.905 ± 0.048	0.328 ± 0.007
8	*Citrus reticulata* 'Chachi'	Gujing Town, Xinhui District, Jiangmen City, Guangdong Province	2007/10	51.854 ± 0.950	9.473 ± 0.273	1.046 ± 0.024	5.166 ± 0.101	0.571 ± 0.015
9	*Citrus reticulata* 'Chachi'	Luokeng Town, Xinhui District, Jiangmen City, Guangdong Province	2008/11	52.394 ± 0.480	3.682 ± 0.052	0.461 ± 0.007	2.619 ± 0.042	0.302 ± 0.008
10	*Citrus reticulata* 'Chachi'	Xiaogang Town, Xinhui District, Jiangmen City, Guangdong Province	2007/10	45.957 ± 0.247	9.467 ± 0.243	1.129 ± 0.007	5.596 ± 0.041	0.728 ± 0.009
11	*Citrus reticulate* 'Chachi'	Shuinan Town, Gaoyao City, Guangdong Province	2008/09	70.735 ± 0.236	5.375 ± 0.091	0.502 ± 0.010	3.666 ± 0.045	0.465 ± 0.007
12	*Citrus reticulata* 'Chachi'	Longmen County, Huizhou City, Guangdong Province	2008/10	65.470 ± 0.958	6.786 ± 0.063	0.520 ± 0.005	5.519 ± 0.040	0.541 ± 0.008
13	*Citrus reticulata* 'Unshiu'	Longsheng County, Guilin City, Guangxi Zhuang Autonomous Region	2008/09	96.781 ± 1.865 (2)[b]	0.414 ± 0.009	0.493 ± 0.013	0.224 ± 0.005	UD[c]
14	*Citrus reticulata* 'Unshiu'	Yangshuo County, Guilin City, Guangxi Zhuang Autonomous Region	2008/10	90.871 ± 1.452 (2)[b]	0.699 ± 0.008	1.142 ± 0.004	0.334 ± 0.005	UD[c]
15	*Citrus reticulata* 'Unshiu'	ChaheTown, Honghu City, Hubei Province	2008/10	101.454 ± 0.881 (2)[b]	0.829 ± 0.019	1.506 ± 0.041	0.360 ± 0.007	UD[c]
16	*Citrus reticulata* 'Unshiu'	Yidu City, Yichang City, Hubei Province	2008/11	121.428 ± 0.745 (2)[b]	0.415 ± 0.008	1.089 ± 0.010	0.234 ± 0.005	ND[d]
17	*Citrus reticulata* 'Unshiu'	Shimen County, Changde City, Hunan Province	2008/10	76.552 ± 1.343	0.387 ± 0.004	0.847 ± 0.007	0.203 ± 0.003	UD[c]
18	*Citrus reticulata* 'Unshiu'	Huaning County, Yuxi City, Yunnan Province	2008/10	92.375 ± 2.335 (2)[b]	0.739 ± 0.005	0.919 ± 0.010	0.305 ± 0.005	UD[c]
19	*Citrus reticulata* 'Subcompressa'	Huangyan District, Taizhou City, Zhejiang Province	2008/11	76.392 ± 0.884	0.300 ± 0.006	0.704 ± 0.006	0.117 ± 0.003	ND[d]
20	*Citrus reticulata* 'Subcompressa'	Yongquan Town, Linhai City, Zhejiang Province	2008/11	91.615 ± 0.680 (2)[b]	0.422 ± 0.009	1.134 ± 0.014	0.178 ± 0.004	ND[d]
21	*Citrus reticulata* 'Subcompressa'	Pujiang County, Chengdu City, Sichuan Province	2008/11	99.738 ± 0.783 (2)[b]	0.198 ± 0.003	0.396 ± 0.007	0.127 ± 0.001	ND[d]
22	*Citrus reticulata* 'Subcompressa'	Nanchang County, Nanchang City, Jiangxi Province	2008/10	69.312 ± 0.833	1.750 ± 0.046	1.868 ± 0.023	1.071 ± 0.029	0.146 ± 0.004
23	*Citrus reticulata* 'Tangerina'	Yongtai county, Fuzhou City, Fujian Province	2008/11	89.613 ± 0.448 (2)[b]	0.717 ± 0.005	1.164 ± 0.017	0.310 ± 0.002	UD[c]
24	*Citrus reticulata* 'Tangerina'	Shaxian County, Sanming City, Fujian Province	2008/11	91.974 ± 1.064 (2)[b]	0.567 ± 0.008	0.869 ± 0.011	0.278 ± 0.007	UD[c]
25	*Citrus reticulata* 'Tangerina'	Nanxi County, Yinbin City, Sichuan Province	2008/11	71.849 ± 0.426 (2)[b]	0.305 ± 0.004	0.437 ± 0.011	0.137 ± 0.003	ND[d]
26	*Citrus reticulata* 'Dahongpao'	Wanzhou District, Chongqing Municipality	2008/12	80.441 ± 0.385 (2)[b]	3.251 ± 0.056	0.551 ± 0.009	1.968 ± 0.012	0.573 ± 0.007
27	*Citrus reticulata* 'Dahongpao'	Fruit Tree Research Institute, Sichuan Academy of Agricultural Science	2008/12	49.269 ± 0.444	5.297 ± 0.076	0.293 ± 0.008	2.457 ± 0.036	0.372 ± 0.010
28	*Citrus reticulata* 'Kinokuni'	Nanfeng County, Fuzhou City, Jiangxi Province	2008/11	81.726 ± 0.959 (2)[b]	15.686 ± 0.401 (2)[b]	0.528 ± 0.011	8.020 ± 0.132	1.625 ± 0.040
29	*Citrus reticulata* 'Erythrosa'	Shimen County, Changde City, Hunan Province	2008/10	104.582 ± 1.735 (2)[b]	0.820 ± 0.024	1.099 ± 0.029	0.313 ± 0.007	UD[c]
30	*Citrus reticulata* 'Ponkan'	Yongchun County, Quanzhou City, Fujian Province	2008/11	105.776 ± 0.688 (2)[b]	6.363 ± 0.089	0.231 ± 0.004	4.450 ± 0.034	1.272 ± 0.017
31	*Citrus reticulata* 'Ponkan'	Jiuhu Town, Longhai City, Fujian Province	2008/11	86.524 ± 1.060 (2)[b]	12.145 ± 0.113 (2)[b]	0.783 ± 0.016	8.340 ± 0.087	2.552 ± 0.038
32	*Citrus reticulata* 'Shiyueju'	Huangtian Town, Sihui City, Guangdong Province	2008/11	57.946 ± 1.300 (2)[b]	12.013 ± 0.085 (2)[b]	0.414 ± 0.004	5.638 ± 0.029	1.087 ± 0.009

[a] Data are represented as the mean ± SD. [b] '(2)' denotes that the sample was further diluted twice with methanol. [c] UD denotes under LOQ. [d] ND denotes not detected.

content of hesperidin (38.021−59.785 mg/g), compared to the other two regions of Guangdong Province (Shuinan Town and Longmen County) and the other cultivars (except for *Citrus reticulata* 'Dahongpao' and *Citrus reticulata* 'Shiyueju').

Apart from hesperidin, the differences of the four PMFs (compounds **2**−**5**) in various cultivars may be more significant. Nobiletin (compound **2**) and tangeretin (compound **4**) were found to be rich in the peel of *Citrus reticulata* 'Chachi' (2.830−9.473 and 1.749−5.596 mg/g, respectively), *Citrus reticulata* 'Dahongpao' (3.251−5.297 and 1.968−2.457 mg/g, respectively), *Citrus reticulata* 'Kinokuni' (15.686 and 8.020 mg/g, respectively), *Citrus reticulata* 'Ponkan' (6.363−12.145 and 4.450−8.340 mg/g, respectively), and *Citrus reticulata* 'Shiyueju' (12.013 and 5.638 mg/g, respectively), and their contents are much higher than 3,5,6,7,8,3′,4′-heptamethoxyflavone (compound **3**) and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (compound **5**) in those cultivars. On the contrary, the content of 3,5,6,7,8,3′,4′-heptamethoxyflavone was found to be higher than nobiletin and tangeretin in the other cultivars with content ranging from 0.396 to 1.868 mg/g, such as the peel of *Citrus reticulata* 'Unshiu', *Citrus reticulata* 'Subcompressa', *Citrus reticulata* 'Tangerina', and *Citrus reticulata* 'Erythrosa'. As shown in **Table 5**, generally, 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone presented the lowest content of all five bioactive flavonoids in most of samples. However, the peel of *Citrus reticulata* 'Kinokuni', *Citrus reticulata* 'Ponkan', and *Citrus reticulata* 'Shiyueju' were found to have relatively high content of 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (1.087−2.552 mg/g), with its content even more than 3,5,6,7,8,3′,4′-heptamethoxyflavone.

In summary, the results showed that there was a great deal of regional variability across China in the flavonoid composition in PCR. The variation may be due to a lot of factors, such as their genetic origin, growing environment, time of collection, and storage conditions, especially the genetic origin. As a traditional Chinese medicine for PCR, it is particularly important for the selection of medicinal plants and its quality control.

In the present study, after various factors of extraction method and HPLC conditions were optimized preliminarily, a new, simple, accurate, and reliable HPLC method coupled with dual wavelength detection was successfully developed and applied to simultaneous determination of five bioactive flavonoids in 32 PCR samples collected from different districts in China with acceptable levels of linearity, precision, repeatability, accuracy, and stability. Thus, instead of monocomponent analysis such as hesperidin, multicomponent of flavonoids including flavanone glycosides and polymethoxylated flavones become more conducive to indicate the characteristics of each *Citrus* cultivar, which can be employed to standardize the quality of PCR and chemotaxonomic investigation in botanical sciences of *Citrus* species.

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