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Quality Analysis and High-Performance Liquid Chromatographic Fingerprint Analysis of New Cultivated Kind of *Lonicerae japonicae* Flos "Hua Jin 6" from Different Harvest Times

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Abstract

Lonicerae japonicae Flos (LJF) is widely used in traditional Chinese medicines for the treatment of various diseases, which is now in great demand every year and has a broad development prospect. However, the flowering phase of common LJF varieties is so short, which seriously restricts the development of LJF industry. As a new cultivated kind of *Lonicerae japonicae* Flos, "Hua Jin 6" has characteristics in long flowering phase and conveniently picking, which makes it have a broad development prospect. The aim of this study is to provide scientific guidance for its suitable harvest period by measuring yield and quality of "Hua Jin 6" from different harvest time. Studies show that flower size had a slowly rising trend from the first day to the seventh day, and then slowly declined or kept stable. There were no significant differences of total phenolic acid contents in different samples from different days, but contents of total flavonoids were on the rise and up to maximum in the ninth day. The contents of total iridoids had an increasing tendency from the first day to the fifth day and then kept relatively stable in other days. We demonstrated that the quality of "Hua Jin 6" is relatively stable and suitable for harvesting in all flower buds white stage in term of HPLC fingerprints. Our findings can make it possible to select the suitable time for different harvest purpose.

Keywords

Lonicerae japonicae Flos, New Cultivate Kind, "Hua Jin 6", Quality Analysis, HPLC Fingerprint, Harvest Time

1. Introduction

Lonicerae japonicae Flos (LJF), the dried flower buds of *Lonicera japonica*

thunb., is well known and commonly used as herbal medicine for treatment of various diseases in traditional Chinese medicine (TCM) practice, such as anti-inflammatory, anti-bacterial, anti-viral, anti-oxidative and anti-diabetic activities [1] [2] [3] [4] [5]. Generally, the flowering stage of LJF has been divided into three stages: the green bud stage (Bud size 1.0 - 3.5 cm, green), the white bud stage (Bud size 3.0 - 4.5 cm, white), the flowering stage (Blooms) [6]. Research has shown that the white bud stage is the most appropriate harvest time of LJF according to the maximum yield and superior quality [7]. However, the lasting time from the white bud stage to the flowering stage is so short (2 - 3 days) and the opening buds are not up to official standards. Therefore, a lot of manpower are needed to pick flower buds in the period of flower phase and numerous wealth are consumed. As a result, many farmers have abandoned cultivating or collecting LJF and a great deal of economic losses [8] [9].

"Hua Jin 6" is a new *Lonicerae japonicae* Flos variety selected in ten years from natural hybrid progeny of local variety, and the most prominent characteristics of the variety is the white bud stage which can last up to 15 days then to open or directly wither and fall. Furthermore, the high output is another feature of "Hua Jin 6" because the flower buds are much denser and bigger than common varieties. As a result, white flower buds can be a one-time picking if farmers cultivate "Hua Jin 6" which could slash personnel costs and improve economic returns. Besides, previous studies showed that the output of "Hua Jin 6" was 1.64 times higher than that of "Hua Jin 2"; contents of chlorogenic acid and cynaroside were respectively 2.03 times and 2.8 times higher than those of "Hua Jin 2" [9]. Therefore, its potential applications are fantastic from above mentioned characteristics. However, whether it is suit to pick during all white flower bud stage or not depends on the flowering quality change laws, so it is the most important task to study on dynamic changes of "Hua Jin 6" quality and yield during all white flower bud stage to determine the suitable harvest time.

Generally, the suitable harvest time of herbal medicines is determined according to the maximal yield and the best quality. Similarly, the best harvest time of "Hua Jin 6" also depend on yield and quality. Usually, yield refers to flower buds weight and size, but there are not unity standard index to evaluate the quality of LJF. In the Chinese Pharmacopoeia (2015 version), only two components (chlorogenic and cynaroside) were used to evaluate the quality of LJF products. While, we all known that for the herbal medicines the safety and efficiency are not just attributed to one or several particular constituents, it usually depends on the interactions among these constitutes which can make their relationship to the safety and efficacy much more complicated than that pure components [10] [11]. Therefore, we should take into account as many as possible constitutes to evaluate the quality of the herbal medicines. Besides, chromatographic fingerprint using high-performance liquid chromatography (HPLC), which focuses on the systemic characterization of composition of the complex chemical mixture, is becoming increasingly recognized as an important separation technique. Recently, several studies on the chromatographic fingerprinting using HPLC tech-

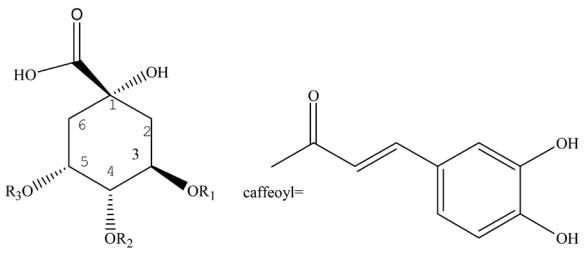
nique have been reported [12] [13] [14].

In this study, we determined morphological characteristics (bud length and bud diameter) and buds dried weight of "Hua Jin 6" from the first day to the eleventh day of the white bud stages to evaluate the yield. Meanwhile, a rapid and simple HPLC method for quantitative determination of 11 major active components from LJF was established, which included 6 phenolic acids, 2 flavonoids and 3 iridoids, the chemical structures of 11 components are shown in Figure 1. Finally, chromatographic fingerprint analysis with HPLC was developed to evaluate the stability of "Hua Jin 6" from different harvest time.

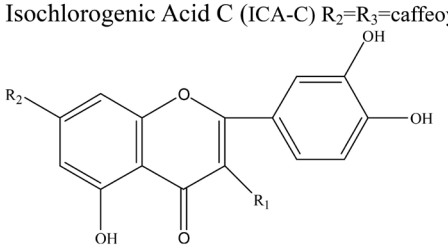
2. Experimental

2.1. Plant Material

Samples of "Hua Jin 6" from different harvest times were collected in medicinal botanical garden of Shan Dong University of TCM of China and identified by

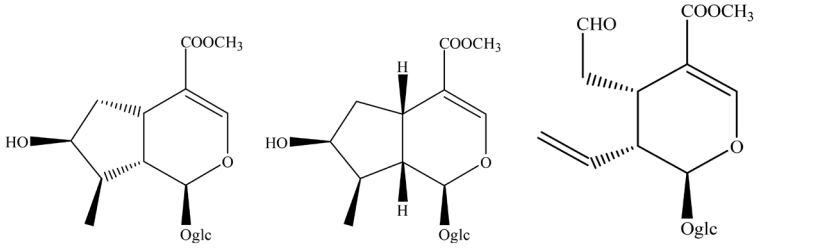


Chlorogenic Acid (CA) R_1=caffeoyl R_2= R_3=H
Cryptochlorogenic Acid (CCA) R_2=caffeoyl R_1= R_3=H
Neochlorogenic Acid (NCA) R_3=caffeoyl R_1= R_2=H
Isochlorogenic Acid B (ICA-B) R_1=R_2=caffeoyl R_3=H
Isochlorogenic Acid A (ICA-A) R_1=R_3=caffeoyl R_2=H
Isochlorogenic Acid C (ICA-C) R_2=R_3=caffeoyl R_1=H



Rutin (RT) R_1=O-glc-rha R_2=OH

Cynaroside (CN) R_1=H R_2=O-glc



loganin (LG) loganic acid (LGA) Secoxylogain (SCLG)

Figure 1. Chemical structures of 11 active components.

Prof. Zhang Yongqing from Shandong University of TCM. White flower buds of "Hua Jin 6" were picking from the first day to the eleventh day of the white bud stage and all samples were stored in Silica gel box to dry. Voucher specimens were preserved at herbarium of Shandong university of TCM (SDCM).

2.2. Chemicals and Solvents

The standard samples of RT was purchased from The National Institute For the Control of Pharmaceutical and Biological Products, NCA, CA, CCA, ICA-B, ICA-A, ICA-C, LG, CN, HP, LGA were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. SCLG was purchased from Shanghai Guyan Bio-Technology Co., Ltd. The purities of above mentioned chemical components were all ≥ 98%, and their structures are shown in Figure 1.

Acetonitrile and formic acid of HPLC grade were chromatographically pure and other reagents were analytically pure. The double distilled water was self-made. Analytical grade methanol was purchased from Tianjin Fuyu Fine Chemical Co., Ltd. and used for sample preparation.

2.3. Flower Bud Sizes and Dried Weight

Flower bud sizes: Samples from different harvest times were randomly divided into 10 groups, each groups included 10 dried buds, we determined bud length and bud diameter using ruler (0.1 mm) and vernier caliper (0.01mm) respectively, and take an average.

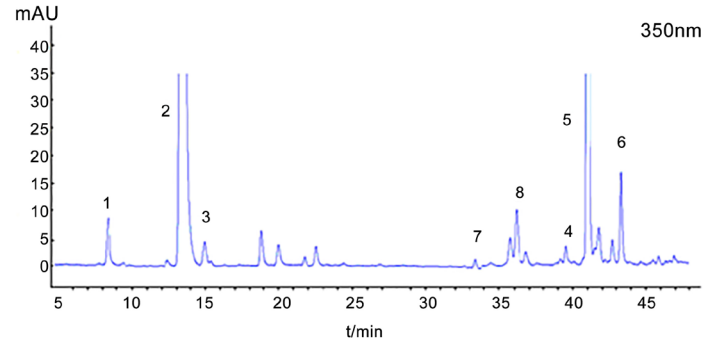
Flower dried weight: Samples from different harvest times were randomly divided into 10 groups, each groups included 10 dried buds, we determined flower dried weight of each group using electronic balance and then take an average.

2.4. HPLC Analysis [10]

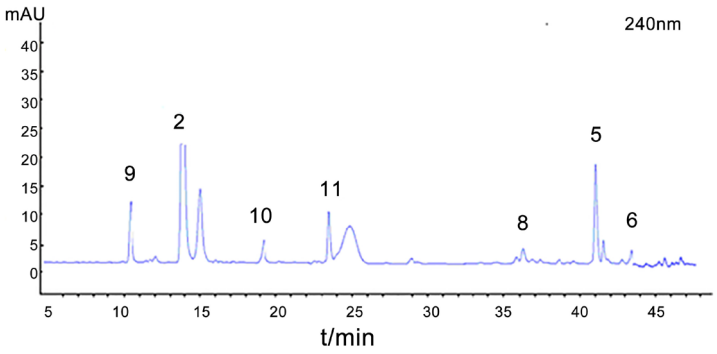
The analysis were performed on an Agilent 1260 liquid chromatography system, equipped with a quaternary gradient pump, an autosampler a diode array detector; A ZORBAX SB-C18 (4.6 mm × 250 mm, 5 um) column at temperature of 25˚C was applied for all analyses. Detection wavelengths were sat at 327 nm for phenolic acids (NCA, CA, CCA, ICA-B, ICA-A, ICA-C), 350 nm for flavonoids (RT, CN), 240 nm for iridoids (LG, LGA, SCLG). The mobile phase consisted of 0.2% aqueous formic acid (A) and acetonitrile (B) in a gradient elution mode was as follows: 0 - 10 min: 92% - 85% A, 10 - 20 min: 85% A, 20 - 30 min: 75% A, 30 - 40 min: 75% - 55% A, 40 - 60 min: 55% - 0% A. The flow rate was 1.0 ml/min and aliquots of 20 uL were injected. The HPLC peaks were identified by comparing the retention times and the UV absorption of the 11 active components in each sample with those of the standard solutions. HPLC chromatograms of samples and mixture reference substances are shown in Figure 2.

2.5. Similarity Analysis

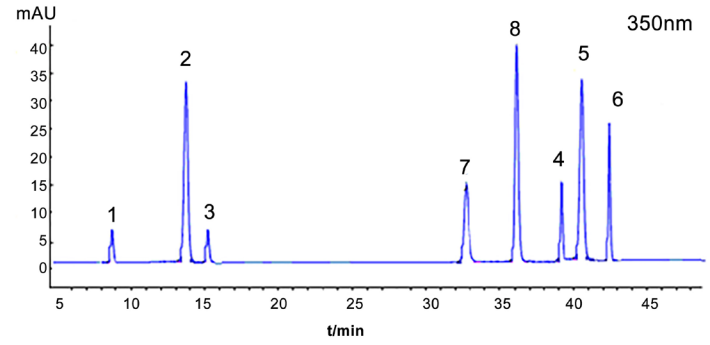
On the basis of the HPLC data, the similarities of LJF samples were performed by a professional software named Similarity Evaluation System for Chromatographic



(a)



(b)



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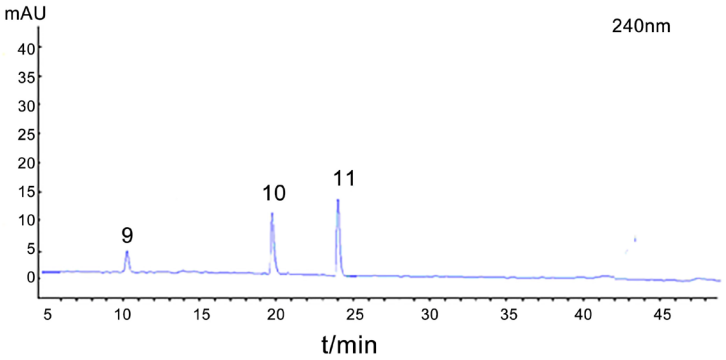


(d)

Figure 2. HPLC chromatogram of sample ((a) 350 nm; (b) 240 nm) and mixture reference substances (c) 350 nm; (d) 240 nm): (1) NCA; (2) CA; (3) CCA; (4) ICA-B; (5) ICA-A; (6) ICA-C; (7) RT; (8) CN; (9) LGA; (10) LG; (11) SCLG.

Fingerprint (SESCF) of TCM (Version 2004 A) composed by Chinese Pharmacopoeia Committee, which was recommended by CFDA of China. Simultaneously, a reference chromatogram, including all the common peaks in the chromatograms of the 11 samples, was carried out by the median method, and the similarities between the reference chromatogram and the 11 samples were calculated.

2.6. Statistical Analysis

Statistical evaluation was carried out using one-way analysis of variance (ANOVA followed by Dunnett's post hoc test). Statistical differences were considered to be significant at $P < 0.05$. The programs were analyzed using the software named GraphPad Prism 5.

3. Results

3.1. Validation of the HPLC Quantitative Method

The calibration curves were constructed using external standard method. The contents of 11 analytes were the average values of three replicate injections. The corresponding regression equation and other characteristic parameters for the determination of analytes were shown in Table 1. All calibration curves exhibited excellent linear behaviour (R2 > 0.999) in a relatively wide concentration range. The LODs (S/N = 3) and LOQs (S/N = 10) for the analytes were less than 0.28 and 0.78 μg/mL, respectively, indicating that the proposed HPLC method presented excellent sensitivity and was successfully applied for determination of 11 active components in LJF.

3.2. Changes of Flower Bud Length and Diameter of LJF "Hua Jin 6" from Different Harvest Time

Changes of flower bud length and diameter are shown in Figure 3. From the figure we can know that flower bud length and diameter had a slowly rising ten-

Table 1. Calibration curves, LODs and LOQs for 11 active components.

Analyte	Linearity range (μg/mL)	Calibration curve	R^2 (n = 3)	LOD (μg/mL)	LOQ (μg/mL)
NCA	2.69 - 134.34	y = 1822.3x + 10.364	0.9999	0.21	0.66
CA	2.73 - 1563.60	y = 1851.3x + 13.69	0.9997	0.28	0.72
CCA	4.51 - 43.31	y = 1432.7x + 3.3910	0.9995	0.18	0.62
ICA-B	3.70 - 27.73	y = 2497.9x − 9.6004	0.9999	0.19	0.66
ICA-A	52.20 - 1257.10	y = 2229.8x + 27.950	0.9999	0.26	0.72
ICA-C	13.08 - 303.30	y = 1068.4x − 4.9689	0.9998	0.21	0.70
RT	1.926 - 441.11	y = 591.67x − 0.6221	0.9998	0.23	0.71
CN	7.21 - 515.11	y = 867.31x − 4.3101	0.9998	0.20	0.78
LG	11.37 - 30.32	y = 1406.7x − 2.0102	0.9996	0.17	0.71
LGA	73.25 - 136.50	y= 0.00125x + 0.00799	0.9998	0.24	0.73
SCLG	39.50 - 158.00	y = 1374.4x + 29.571	0.9998	0.24	0.71

dency and then had a slight reduce from the first day to the eleventh day. Among of them, flower bud diameter slowly increased from 0.2466 cm to 0.3424 cm during the first day to the tenth day, and then had a slightly reduce to 0.3272 cm in the eleventh day; flower bud length also had a minor increase from 2.8030 cm to 3.4336 cm from the first day to the ninth day, and then had a slight reduce to 3.0300 cm in the eleventh day. The result showed that the flower buds were also in the stage of vigorous growth during the first day to the tenth day and in the period of decline from the eleventh day.

3.3. Changes of Flower Bud Dried Weight of LJF Hua Jin 6 from Different Harvest Time

To a certain degree, flower bud dried weight has always been the main index limiting LJF yield. Changes of flower bud dried weight are shown in Figure 4, from the figure we can see clearly that flower bud dried weight was relatively

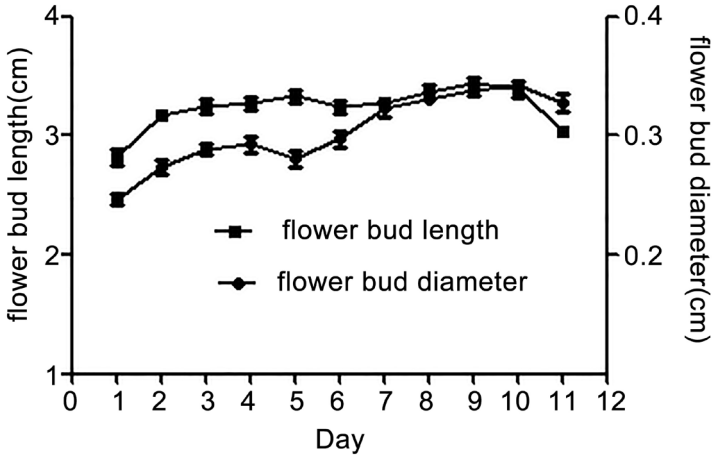


Figure 3. Changes of flower bud length and diameter (cm) of LJF "Hua Jin 6" from different harvest time.

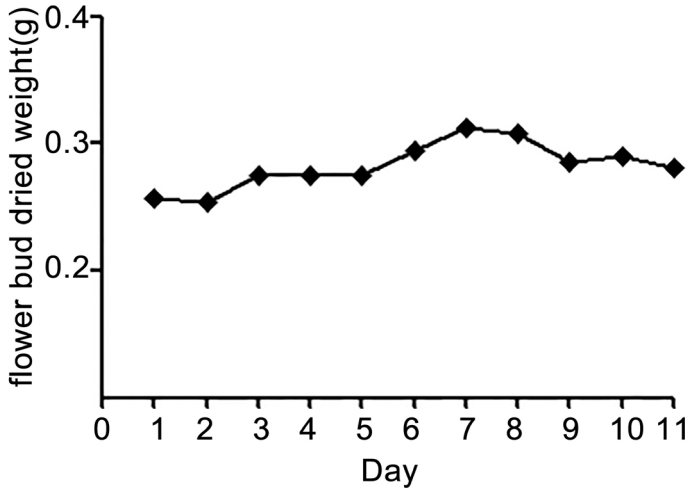


Figure 4. Changes of bud dry weight (g/10 buds) of LJF "Hua Jin 6" from different harvest time.

stable during all white bud stage. Flower bud dried weight was slightly rising from 0.2577 g to 0.3129 g during the first day to the seventh day, and then had a slight reduce to 0.2905 g in eleventh day. From the result we can know that the accumulated amount of dry matter of LJF reached maximum in the seventh day and then slightly reduced or kept stable in another days. Therefore, we assumed the yield of LJF was much higher after the seventh day harvesting.

3.4. Contents of Active Constituent of LJF "Hua Jin 6" from Different Harvest Time

3.4.1. Phenolic Acid Contents Analysis

Contents of active constituent of LJF Hua Jin 6 from different harvest time are shown in Figure 5. From the figure we can see that except chlorogenic, the contents of other compounds were increase slowly as a whole, and the content of chlorogenic acid slowly reduces during the whole white flower bud stages from the first day to the eleventh day during white flower bud stages.

We also calculated the total contents of phenolic acids during the all flower buds white stage. From the Figure 5, we can know that there were no significant different of phenolic acid contents in different sample from different days (Figure 5). We speculated that chlorogenic acid synthesized into other phenolic acids with the growth of LJF. Therefore, the total phenolic acids contents were relatively stable in all flower buds white stage. The highest phenolic acid contents were detected in sample from the second day (33.9890 mg/g), while flower buds from the eighth day had the lowest content (27.2338 mg/g).

3.4.2. Flavonoid Contents Analysis

Changes of flavonoid contents of LJF "Hua Jin 6" from different harvest time are shown in Figure 6. From the figure we can clearly see that there were significantly different in flavonoid constituents. The variation law of rutin was not obvious. The content of cynaroside slowly increased in all days from 0.9444 mg/g to 1.9257 mg/g.

It was also shown that there were significant different of the total flavonoid contents in different sample from different days (Figure 6), it had a increasing tendency from the first day to the eleventh day. The lowest flavonoid contents were detected in sample from the first day (1.0340 mg/g), while in the ninth day of flower bud white stages, the total flavonoids contents were nearly 2 times over those of the first day (2.0251 mg/g).

3.4.3. Iridoid Contents Analysis

Changes of iridoid contents of LJF Hua Jin 6 from different harvest time are shown in Figure 7. From the figure we can know that three iridoids had a slowly increase tendency and then reduced or kept relatively stable. Similarly, we calculated the total iridoid contents shown in Figure 7, form the figure we can know that there was an increasing tendency from the first day to the fifth day and then keep the relatively stable in other days.

By measuring 11 active components contents of LJF from different harvest

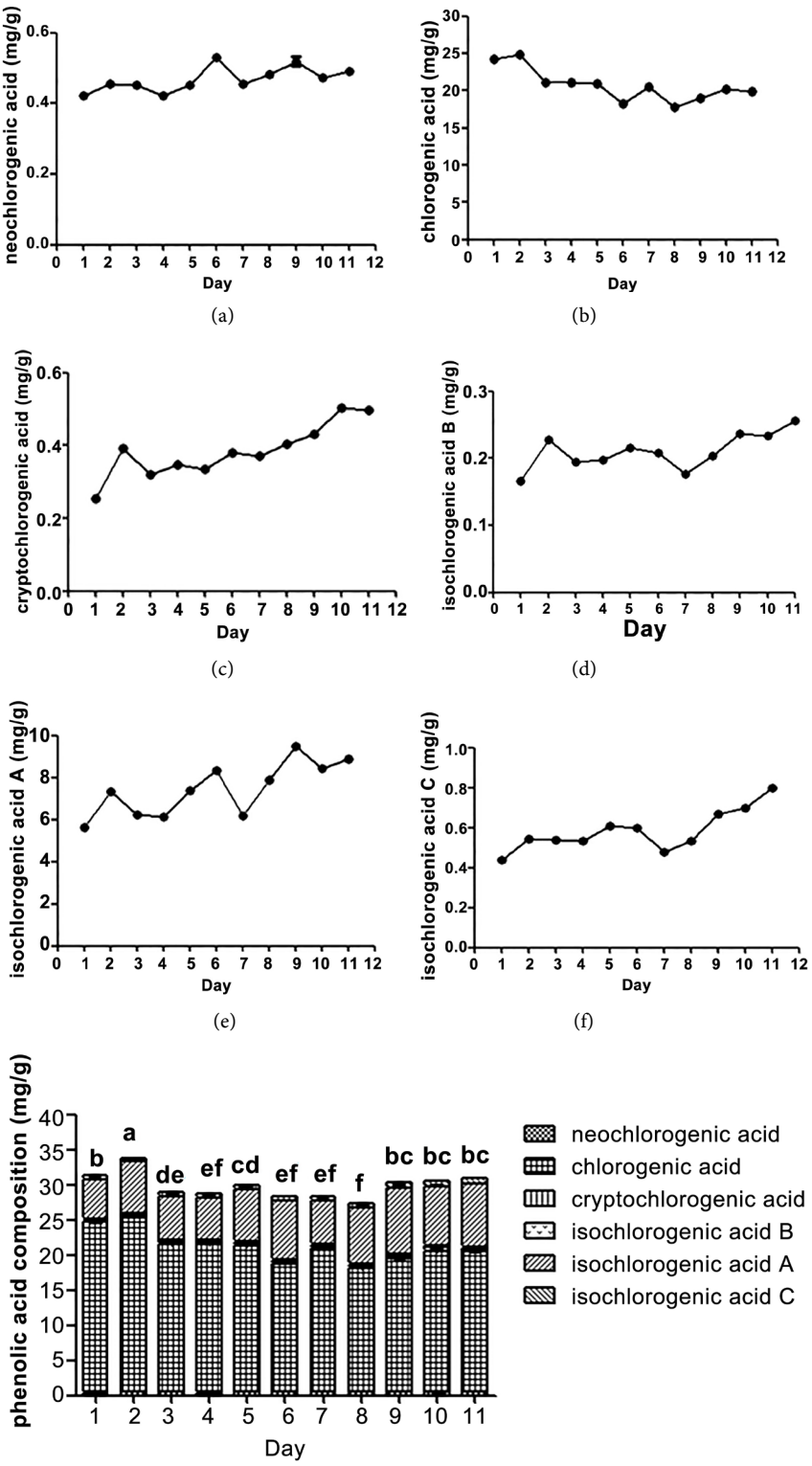


Figure 5. Changes of phenolic acid contents of LJF "Hua Jin 6" from different harvest time (a) NCA; (b) CA; (c) CCA; (d) ICA-B; (e) ICA-A; (f) ICA-C.

time, we known that the total phenolic acids contents were relatively stable in all bud white stages. However, the total flavonoids contents had an increasing tendency in all white bud stages, and flavonoids contents reached the highest in the

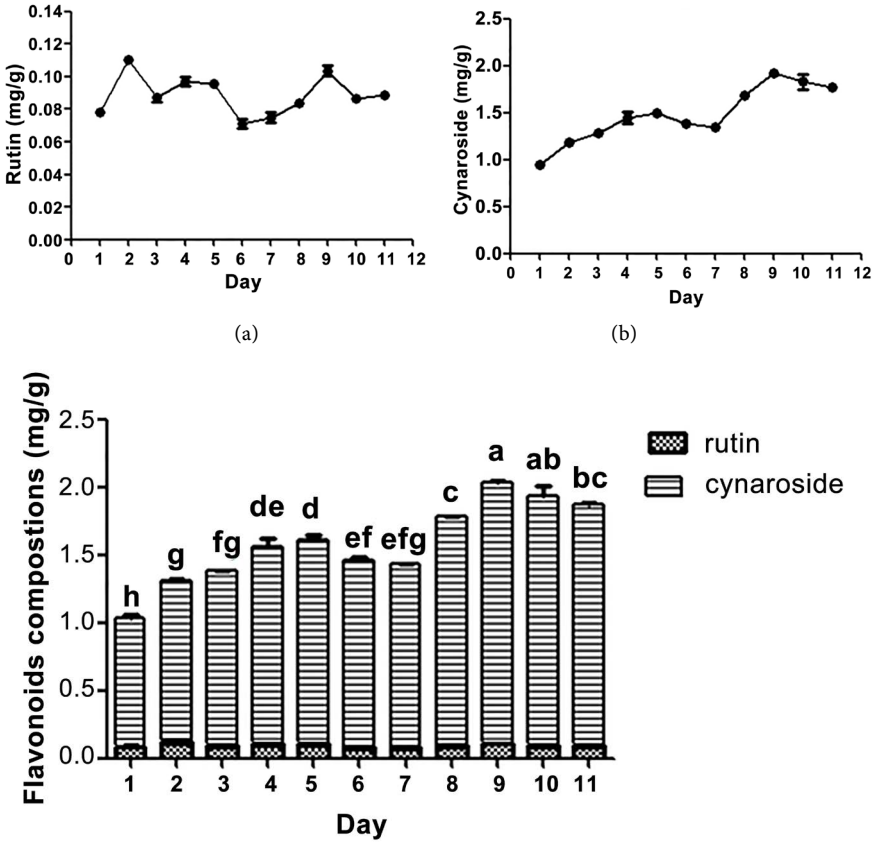


Figure 6. changes of flavonoid contents of LJF "Hua Jin 6" from different harvest time (a) RT; (b) CN).

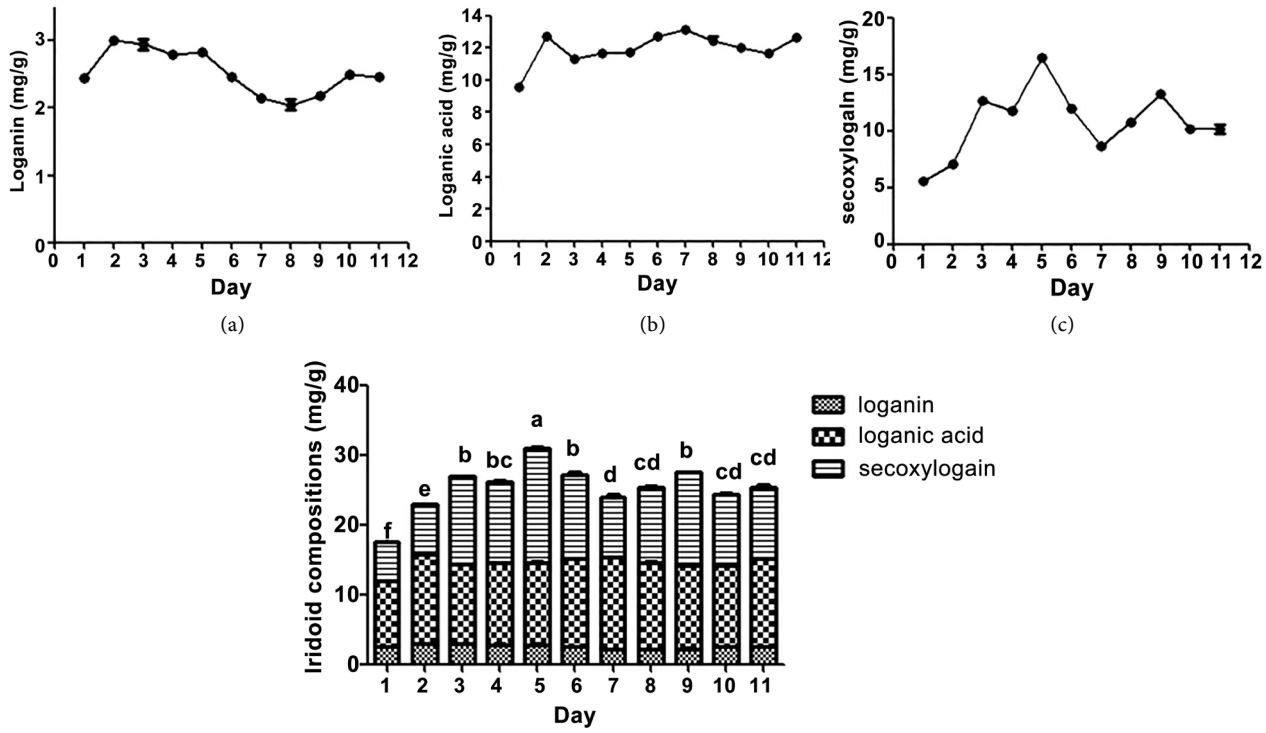


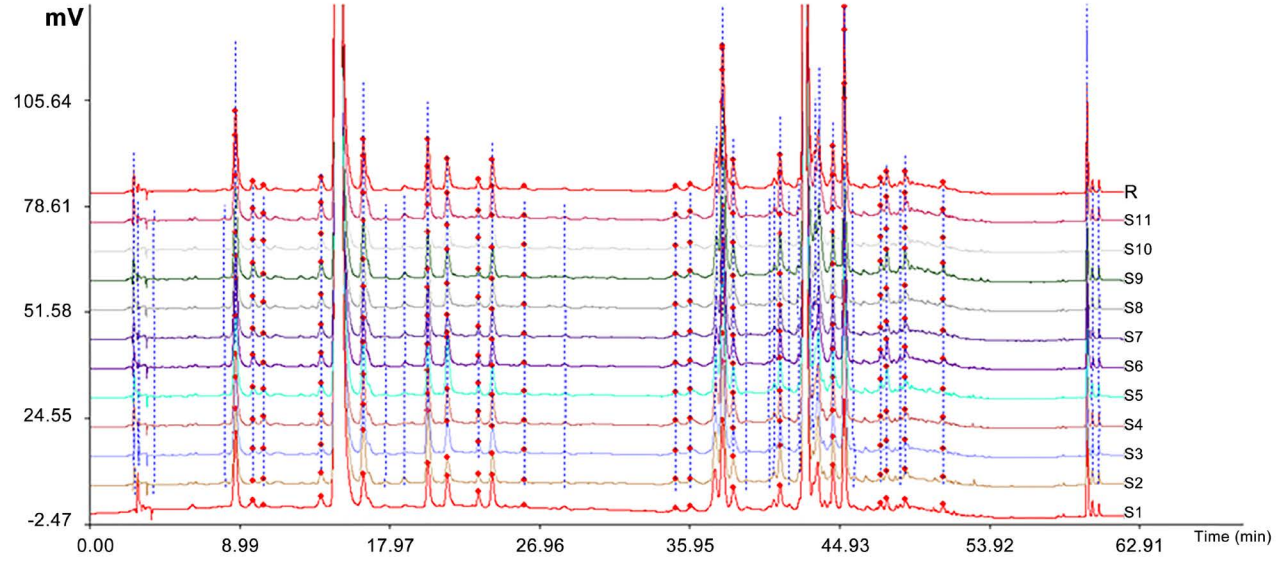
Figure 7. Changes of iridoid contents of LJF "Hua Jin 6" from different harvest time (a) LG; (b) LGA; (c) SCLG).

ninth day, which had no remarkably significant with the eighth, the tenth, the eleventh day. Meanwhile, the total iridoid contents had an increasing tendency from the first day to the fifth day, then slowly declined in the following day. Taking above mentioned consideration, we can select appropriate harvesting time according different harvest purpose.

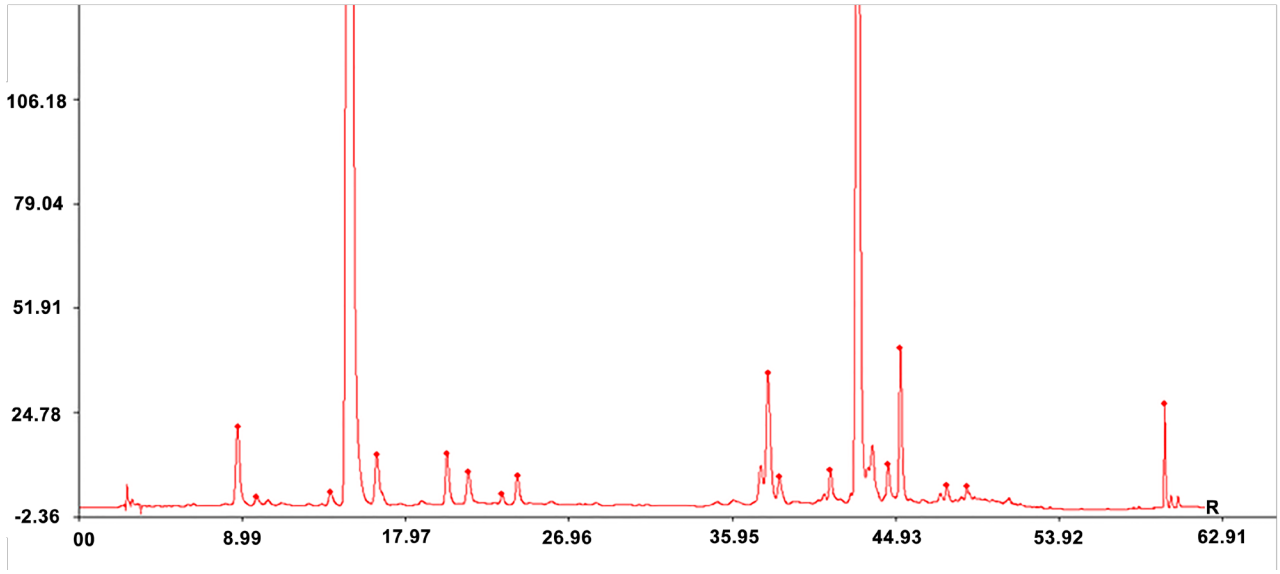
3.5. HPLC Fingerprints of LJF

3.5.1. HPLC Fingerprint Analysis

Based on the method of the samples determinated, HPLC fingerprints of LJF "Hua Jin 6" were obtained and are shown in Figure 8(a), which shown that 11



(a) Retention time (min)



(b) Retention time (min)

Figure 8. (a) HPLC fingerprint of 11 samples of LJF from different harvest time; (b) reference chromatogram generated from all LJF samples.

samples had generally similar spectra, indicated the similar chemical compositions of these samples. Meanwhile, the reference chromatographic fingerprint of LJF Hua Jin 6 was generated based on the 11 samples obtained from different harvest time and is shown in Figure 8(b). There were 18 common peaks (existing in all chromatograms of the 11 samples) which were marked (1 - 18) in referenced chromatographic. Peak 1 (tR = 8.727) was selected as the reference peak because it was a relatively stable peak in this chromatogram. The relative retention times (tR) and relative peak areas of these 18 common peaks are listed in Table 2.

3.5.2. Similarity Evaluation

It was necessary that chromatographic fingerprint of LJF from different harvest time should be evaluated by their similarities, which obtained from the calculation on the correlative coefficient of original date. The correlation coefficient between each chromatogram of LJF samples was shown in Table 3. The results indicated that the samples from different harvest time achieved a high similarity value with each other. Among of them, S2 and S9 had the lowest similarity value (0.956), and the highest similarity value achieved 1 between S1 and S2, S3 and S4, S5 and S7, S6 and S10, S8 and S11, which indicated that the total quality was stable from different harvest time. Furthermore, we calculated similarity between 11 samples and reference chromatogram, which was established through

Table 2. Relative peak areas and relative times of common peaks of LJF.

Peak no.	t_R (min)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	Average	RSD
Peak 1	8.727	1	1	1	1	1	1	1	1	1	1	1	1	0.086
Peak 2	9.785	0.150	0.047	0.083	0.082	0.109	0.138	0.112	0.144	0.174	0.132	0.140	0.120	0.357
Peak 3	13.847	0.169	0.153	0.133	0.152	0.160	0.166	0.167	0.184	0.194	0.223	0.216	0.175	0.197
Peak 4	14.837	50.246	61.900	46.547	43.020	40.802	37.149	40.210	34.170	31.274	37.895	37.128	41.421	0.159
Peak 5	16.39	0.527	0.728	0.647	0.685	0.637	0.625	0.686	0.707	0.736	0.883	0.847	0.702	0.175
Peak 6	20.253	0.613	0.872	0.691	0.667	0.583	0.514	0.534	0.438	0.454	0.530	0.565	0.582	0.173
Peak 7	21.422	0.514	0.352	0.432	0.483	0.804	0.425	0.273	0.305	0.331	0.329	0.314	0.412	0.357
Peak 8	23.278	0.171	0.086	0.106	0.123	0.104	0.080	0.083	0.091	0.067	0.171	0.171	0.171	0.216
Peak 9	24.123	0.419	0.372	0.316	0.357	0.328	0.269	0.284	0.308	0.282	0.374	0.342	0.329	0.077
Peak 10	37.913	1.222	1.470	1.400	1.740	1.617	1.252	1.401	1.669	1.706	2.090	1.822	1.581	0.182
Peak 11	38.539	0.300	0.259	0.244	0.293	0.271	0.230	0.221	0.250	0.244	0.293	0.263	0.259	0.067
Peak 12	41.348	0.328	0.412	0.357	0.377	0.389	0.324	0.321	0.351	0.391	0.382	0.435	0.370	0.137
Peak 13	42.823	14.071	16.487	14.767	14.371	16.005	15.990	14.757	16.734	19.347	16.436	18.375	16.209	0.175
Peak 14	44.518	0.425	0.431	0.410	0.452	0.457	0.355	0.336	0.379	0.349	0.374	0.407	0.396	0.084
Peak 15	45.193	1.101	1.758	1.348	1.359	1.522	1.204	1.184	1.216	1.450	1.382	1.696	1.386	0.179
Peak 16	47.749	0.145	0.122	0.129	0.130	0.266	0.194	0.108	0.138	0.183	0.149	0.163	0.158	0.327
Peak 17	48.841	0.113	0.092	0.072	0.078	0.083	0.077	0.148	0.182	0.117	0.104	0.115	0.108	0.334
Peak 18	59.728	0.658	0.570	0.422	0.502	0.443	0.399	0.486	0.534	0.474	0.438	0.469	0.487	0.116

tR is the retention time; RSD = Relative Standard Deviation.

Table 3. The similarity of 11 samples investigated.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	R
S1	1											
S2	1	1										
S3	0.999	0.999	1									
S4	0.999	0.998	1	1								
S5	0.995	0.993	0.998	0.999	1							
S6	0.991	0.989	0.995	0.996	0.999	1						
S7	0.997	0.996	0.999	0.999	1	0.998	1					
S8	0.983	0.98	0.989	0.991	0.997	0.999	0.994	1				
S9	0.96	0.956	0.969	0.973	0.984	0.989	0.979	0.995	1			
S10	0.99	0.988	0.994	0.996	0.999	1	0.998	0.999	0.989	1		
S11	0.982	0.98	0.988	0.99	0.996	0.998	0.994	1	0.996	0.999	1	
R	0.995	0.993	0.998	0.999	1	0.999	1	0.997	0.984	0.999	0.996	1

mean value method. The result shown that samples from different harvest time were of high similarity with reference chromatogram, most similarity ratio is at 0.993 upwards, which further revealed that the quality of 11 samples collected from different harvest time had similar chemical compositions and this reference chromatogram could be applied as a standard HPLC fingerprint. Consequently, we think the quality of LJF was relatively stable in different harvest time in term of HPLC fingerprints.

4. Discussion and Conclusion

Compared to the traditional varieties of LJF, "Hua Jin 6" has a broad development prospect for its longer picking time and higher yield. However, for every Chinese herbs, there is a different optimal stage of harvesting in order to have maximum amount of bioactive compounds or yield; the results indicated that the harvest time from the first day to the eleventh day had significant impact on the amount of the active components and yield, which can make it possible to select the suitable time for different harvest purposes. Our study demonstrated that: the yield of LJF was much higher after the seventh day harvesting; the total amount of flavonoids was much higher after the eighth day harvesting; the total amount of iridoids was much higher after the fifth day harvesting; and the total amount of phenolic acids was relatively stable in all flower buds white stage. Besides, we assumed that the quality of "Hua Jin 6" is relatively stable and suitable for harvesting in all flower buds white stage in term of HPLC fingerprints.

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Declaration of Interest

The authors report no conflicts of interest.

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