

# Validation of Optimized HPLC Method for Determining Flavonoids in Jeju Native *Citrus* Fruits from Different Harvest Times

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## ABSTRACT

Jeju native *Citrus* have been used as functional ingredients due to their large quantities of flavonoids, and exhibit various biological activities such as antioxidant, antitumor, anti-inflammatory, and anti-obesity activities. In this study, we developed efficient quantification methods for the analysis of ten flavonoids in five types of Jeju *Citrus* fruits harvested at six different time points using HPLC. The parameter affecting the performance of HPLC method, such as column temperature, flow rate, detection wavelength, mobile phase buffer, were optimized. The established analytical method attained good linearity ( $R^2 \geq 0.9997$ ), precision (inter-day precision,  $RSD \leq 0.318\%$ ; intra-day precision,  $RSD \leq 0.034\%$ ), and accuracy (95.06-107.97%). Analysis of flavonoids in Jeju native *Citrus* species from different harvesting time revealed that the *Citrus* samples were mainly characterized by the species, showing higher contents of hesperidin and narirutin in *Citrus benikoji* Hort. ex Tanaka (Gamja), *Citrus pseudogulgul* Hort. ex Shirai (Sadugam), *Citrus junos* Sieb. ex Tanaka (Soyuja), and higher contents of naringin and neohesperidin in *Citrus grandis* Osbeck (Dangyuja) and *Citrus aurantium* L. (Jigak). PCA results represented that the harvesting period also influenced the changes in flavonoid contents within each *Citrus* species group. This study demonstrated that our analytical method is practical and reliable for the quantification of Citrus flavonoids. This method could be further utilized to evaluate the quality of various Jeju *Citrus* fruits.

## INTRODUCTION

### > Jeju *Citrus* Fruits

- ❖ Jeju native *Citrus* fruits have been utilized as traditional folk medicines due their health-promoting effects, including antioxidant, antitumor, anti-inflammatory, and anti-obesity activities.

### > Flavonoids

- ❖ Flavonoids are widely present in *Citrus* fruits and exhibit as strong antioxidants

## OBJECTIVES

- > Development of a simple, rapid, and reproducible method to analyze ten flavonoids in Jeju *Citrus* fruits harvested at different times

## MATERIALS & METHODS

### > Plant materials

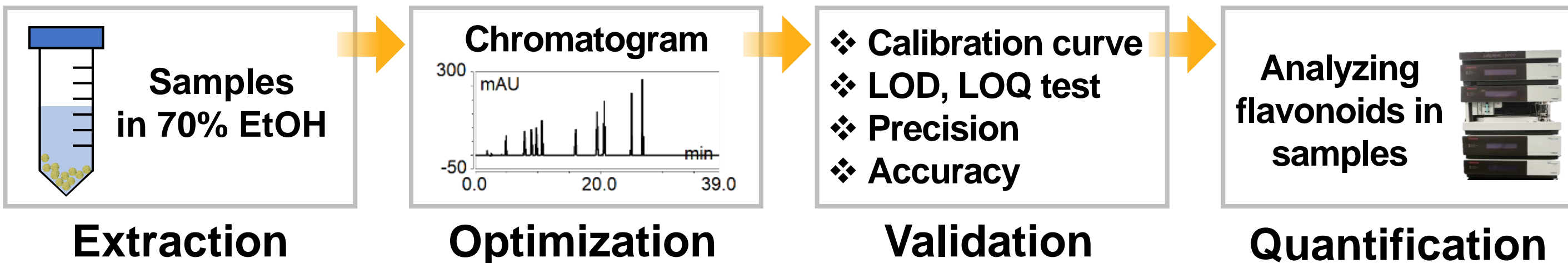
- ❖ Dangyuja (*Citrus grandis* Osbeck)
- ❖ Gamja (*Citrus benikoji* Hort. ex Tanaka)
- ❖ Jigak (*Citrus aurantium* L.)
- ❖ Sadugam (*Citrus pseudogulgul* Hort. ex Shirai)
- ❖ Soyuja (*Citrus junos* Sieb. ex Tanaka)

Harvested on

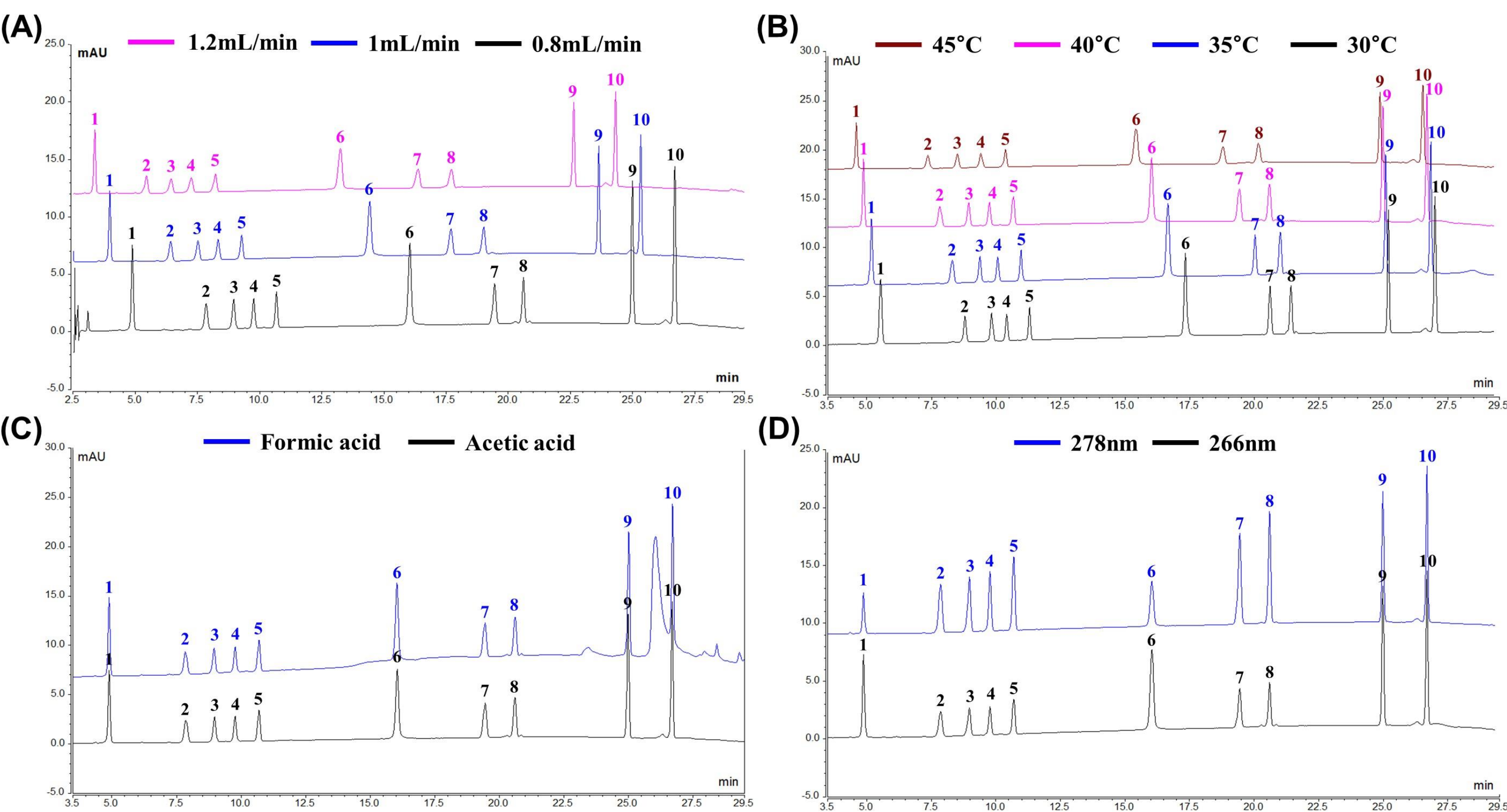
September 3<sup>rd</sup>  
September 18<sup>th</sup>  
October 4<sup>th</sup>  
October 21<sup>st</sup>  
November 6<sup>th</sup>  
November 21<sup>st</sup>



### > Experimental procedures



## RESULTS



**Figure 1.** Overlaid chromatogram of 10 flavonoids with different (A) flow rates (0.8, 1.0, and 1.2 mL/min), (B) column temperature (30, 35, 40, and 45 °C), and (C) mobile phase buffers (acetic acid and formic acid), and wavelengths (266 and 278 nm). Plot annotation: 1, Rutin; 2, Narirutin; 3, Naringin; 4, Hesperidin; 5, Neohesperidin; 6, Quercetin; 7, Naringenin; 8, Hesperetin; 9, Nobiletin; 10, Tangeretin.

**Table 1.** Calibration curves for limit of detection (LOD) and limit of quantification (LOQ) of 10 flavonoids

No.	Compounds	<sup>a</sup> RT (min)	<sup>b</sup> Linear regression equation	<sup>c</sup> R <sup>2</sup>	LOD (μg/mL)	LOQ (μg/mL)
1	Rutin	4.88	y = 0.3374 x + 0.1633	0.99979	0.0845	0.2560
2	Narirutin	7.86	y = 0.1563 x + 0.0451	0.99993	0.0527	0.1598
3	Naringin	8.97	y = 0.1567 x + 0.0278	0.99996	0.0713	0.2161
4	Hesperidin	9.77	y = 0.1458 x + 0.0182	0.99998	0.0610	0.1849
5	Neohesperidin	10.69	y = 0.1582 x + 0.0105	0.99998	0.0649	0.1965
6	Quercetin	16.04	y = 0.5322 x - 0.0979	0.99998	0.4395	1.3317
7	Naringenin	19.44	y = 0.2367 x + 0.0222	0.99999	0.1281	0.3882
8	Hesperetin	20.61	y = 0.2147 x + 0.0267	0.99999	0.1363	0.4130
9	Nobiletin	25.00	y = 0.5082 x + 0.0120	1.00000	0.0992	0.3005
10	Tangeretin	26.70	y = 0.5802 x + 0.0468	0.99999	0.0762	0.2310

<sup>a</sup> RT, retention time.  
<sup>b</sup> y, peak area; x, concentration of standard (μg/mL).  
<sup>c</sup> R<sup>2</sup>, correlation coefficient.

**Table 2.** Results of inter-day and intra-day precision and recovery test for 10 flavonoids

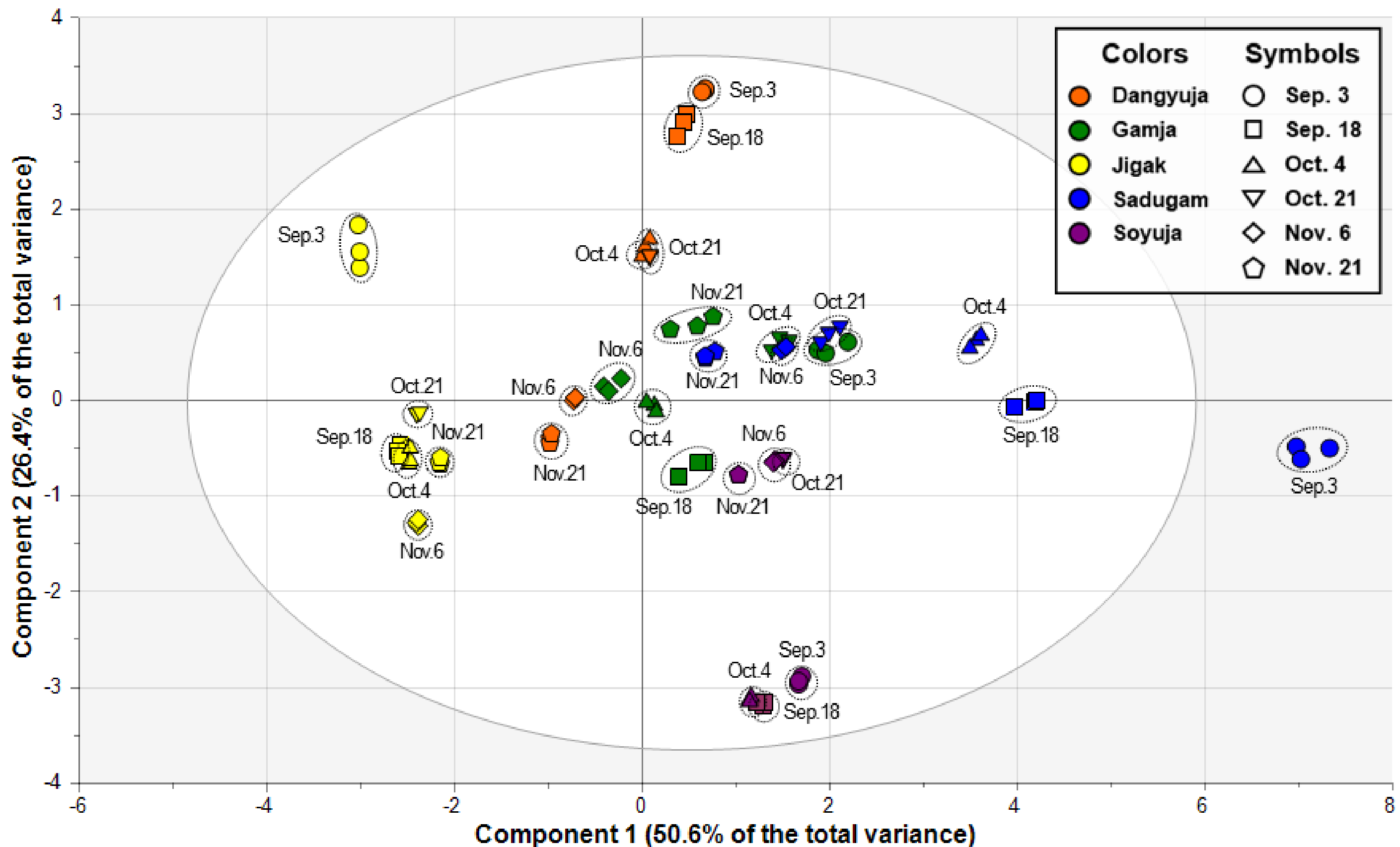
No.	Compounds	Inter-day precision (°RSD%)		Intra-day precision (RSD%)		Accuracy (%)	
		°RT	Area	RT	Area	Recovery	RSD
1	Rutin	0.116	0.318	0.012	0.026	105.82	2.709
2	Narirutin	0.093	0.263	0.010	0.020	102.19	1.521
3	Naringin	0.072	0.231	0.008	0.023	100.92	1.371
4	Hesperidin	0.055	0.236	0.007	0.019	107.97	1.480
5	Neohesperidin	0.045	0.221	0.006	0.020	100.02	1.391
6	Quercetin	0.036	0.321	0.006	0.034	102.45	2.820
7	Naringenin	0.024	0.241	0.005	0.023	95.62	1.221
8	Hesperetin	0.016	0.194	0.003	0.025	95.06	0.993
9	Nobiletin	0.010	0.221	0.002	0.025	96.61	1.737
10	Tangeretin	0.009	0.220	0.002	0.030	106.30	4.834

<sup>a</sup> °RSD, percent of relative standard deviation.  
<sup>b</sup> RT, retention time.

**Table 3.** Quantification results for five *Citrus* species harvested at six different time points

Species	Harvest Date	Compounds (mg/g)							
		Rutin	Narirutin	Naringin	Hesperidin	Neohesperidin	Quercetin	Nobiletin	Tangeretin
Dangyuja	September 3 <sup>rd</sup>	2.756 ± 0.021	5.902 ± 0.015	63.063 ± 0.027	7.029 ± 0.147	62.301 ± 0.049	4.053 ± 0.040	1.253 ± 0.006	1.197 ± 0.010
	September 18 <sup>th</sup>	2.423 ± 0.050	4.426 ± 0.077	60.059 ± 0.967	6.410 ± 0.113	62.342 ± 0.985	3.717 ± 0.042	1.216 ± 0.021	1.138 ± 0.020
	October 4 <sup>th</sup>	2.850 ± 0.042	4.645 ± 0.072	47.479 ± 0.759	5.214 ± 0.090	41.826 ± 0.662	2.661 ± 0.061	0.888 ± 0.014	0.924 ± 0.013
	October 21 <sup>st</sup>	2.778 ± 0.001	3.607 ± 0.004	42.156 ± 0.015	3.569 ± 0.010	39.650 ± 0.034	2.116 ± 0.029	0.904 ± 0.001	0.979 ± 0.003
	November 6 <sup>th</sup>	2.639 ± 0.012	2.566 ± 0.016	26.616 ± 0.190	2.791 ± 0.013	28.159 ± 0.201	1.527 ± 0.011	0.618 ± 0.004	0.536 ± 0.004
	November 21 <sup>st</sup>	2.609 ± 0.054	2.496 ± 0.040	27.054 ± 0.358	1.697 ± 0.026	22.972 ± 0.294	1.226 ± 0.036	0.478 ± 0.005	0.501 ± 0.004
Gamja	September 3 <sup>rd</sup>	3.572 ± 0.057	12.135 ± 0.221	N.D.	17.210 ± 0.814	0.159 ± 0.022	N.D.	0.935 ± 0.022	0.922 ± 0.033
	September 18 <sup>th</sup>	2.543 ± 0.114	8.787 ± 0.329	N.D.	15.101 ± 0.685	0.100 ± 0.006	N.D.	0.615 ± 0.021	0.662 ± 0.021
	October 4 <sup>th</sup>	2.520 ± 0.017	8.451 ± 0.042	N.D.	14.324 ± 0.627	N.D.	N.D.	0.612 ± 0.004	0.716 ± 0.006
	October 21 <sup>st</sup>	3.139 ± 0.045	8.215 ± 0.093	N.D.	16.903 ± 0.513	N.D.	N.D.	0.828 ± 0.017	0.973 ± 0.020
	November 6 <sup>th</sup>	2.866 ± 0.054	6.226 ± 0.093	N.D.	11.855 ± 0.362	N.D.	N.D.	0.537 ± 0.014	0.654 ± 0.020
	November 21 <sup>st</sup>	3.331 ± 0.102	5.482 ± 0.141	N.D.	11.999 ± 0.822	N.D.	N.D.	0.700 ± 0.027	0.847 ± 0.041
Jigak	September 3 <sup>rd</sup>	1.846 ± 0.091	1.211 ± 0.047	126.091 ± 4.586	2.330 ± 0.045	76.289 ± 2.738	0.743 ± 0.017	0.786 ± 0.034	0.424 ± 0.018
	September 18 <sup>th</sup>	1.250 ± 0.066	0.888 ± 0.010	76.538 ± 0.801	0.577 ± 0.018	38.794 ± 0.381	N.D.	0.409 ± 0.005	0.233 ± 0.006
	October 4 <sup>th</sup>	1.132 ± 0.043	0.645 ± 0.019	67.263 ± 1.527	0.611 ± 0.032	40.043 ± 0.919	N.D.	0.434 ± 0.011	0.237 ± 0.012
	October 21 <sup>st</sup>	1.322 ± 0.015	0.758 ± 0.004	76.695 ± 0.251	0.618 ± 0.015	41.622 ± 0.184	N.D.	0.527 ± 0.003	0.295 ± 0.004
	November 6 <sup>th</sup>	0.952 ± 0.021	0.560 ± 0.016	58.641 ± 0.463	0.591 ± 0.065	27.640 ± 0.180	N.D.	0.321 ± 0.006	0.210 ± 0.004
	November 21 <sup>st</sup>	1.103 ± 0.018	0.539 ± 0.016	56.356 ± 0.428	0.430 ± 0.029	36.403 ± 0.322	N.D.	0.491 ± 0.004	0.270 ± 0.006
Sadugam	September 3 <sup>rd</sup>	2.740 ± 0.054	126.41 ± 2.671	N.D.	30.666 ± 0.595	N.D.	N.D.	N.D.	N.D.
	September 18 <sup>th</sup>	2.417 ± 0.029	87.971 ± 0.952	N.D.	20.372 ± 0.777	0.158 ± 0.002	N.D.	N.D.	N.D.
	October 4 <sup>th</sup>	2.891 ± 0.046	73.851 ± 0.702	N.D.	17.574 ± 0.175	0.107 ± 0.018	N.D.	N.D.	N.D.
	October 21 <sup>st</sup>	2.765 ± 0.063	54.398 ± 0.949	N.D.	13.850 ± 0.257	N.D.	N.D.	N.D.	N.D.
	November 6 <sup>th</sup>	2.608 ± 0.017	48.014 ± 0.252	N.D.	12.663 ± 0.094	N.D.	N.D.	N.D.	N.D.
	November 21 <sup>st</sup>	2.414 ± 0.024	40.890 ± 0.623	N.D.	9.553 ± 0.133	N.D.	N.D.	N.D.	N.D.
Soyuja	September 3 <sup>rd</sup>	0.395 ± 0.032	21.344 ± 0.121	10.822 ± 0.057	24.063 ± 0.123	10.671 ± 0.052	N.D.	N.D.	N.D.
	September 18 <sup>th</sup>	0.245 ± 0.013	17.161 ± 0.283	8.177 ± 0.129	18.802 ± 0.544	8.211 ± 0.080	N.D.	N.D.	N.D.
	October 4 <sup>th</sup>	0.289 ± 0.021	15.529 ± 0.063	7.104 ± 0.046	16.940 ± 0.074	6.917 ± 0.039	N.D.	N.D.	N.D.
	October 21 <sup>st</sup>	N.D.	12.485 ± 0.026	6.317 ± 0.033	15.082 ± 0.064	6.457 ± 0.065	N.D.	N.D.	N.D.
	November 6 <sup>th</sup>	N.D.	11.755 ± 0.127	5.814 ± 0.093	13.753 ± 0.165	5.755 ± 0.082	N.D.	N.D.	N.D.
	November 21 <sup>st</sup>	N.D.	6.675 ± 0.020	3.816 ± 0.005	9.301 ± 0.024	4.242 ± 0.014	N.D.	N.D.	N.D.

\* N.D., Not Detected.



**Figure 2.** Principal component analysis (PCA) score plot for five *Citrus* species harvest at six different time points. Each *Citrus* species was represented on the plot by a unique color, and each harvest time was represented on the plot by a unique symbol: Dangyuja, orange; Gamja, green; Jigak, yellow; Sadugam, blue; Soyuja, violet; September 3<sup>rd</sup>, circle; September 18<sup>th</sup>, box; October 4<sup>th</sup>, triangle; October 21<sup>st</sup>, inverted triangle; November 6<sup>th</sup>, diamond; November 21<sup>st</sup>, pentagon.