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Simultaneous Determination of the Flavonoids and Limonoids in *Citrus junos* Seed Shells Using a UPLC–DAD-ESI/MS

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Abstract – *Citrus junos* seeds (CS) have been traditionally used for the treatment of cancer and neuralgia. They are also used to manufacture edible oil and cosmetic perfume. A large amount of CS shells without oil (CSS) are discarded after the oil in CS is used as foods or herbal remedy. To efficiently utilize CSS as a by-products, it needs to be studied through chemical analysis. Therefore, we developed an ultra-performance liquid chromatography (UPLC)–diode array detection (DAD) method for simultaneous determination and quantitative analysis of five components (two flavonoids and threes limonoids) in CSS. A Waters Acquity UPLC HSS T3 column C_{18} (2.1 × 100 mm, 1.8 μm) was used for this separation. It was maintained at 40 ºC. The mobile phase used for the analysis was distilled water and acetonitrile with gradient elution. To identify the quantity of the five components, a mass spectrometer (MS) with an electrospray ionization (ESI) source was used. The regression equation showed great linearity, with correlation coefficient ≥ 0.9912. Limits of detection (LOD) and limits of quantification (LOQ) of the five compounds were 0.09 – 0.13 and 0.26 – 0.38 μg/mL, respectively. Recoveries of extraction ranged from 97.45% to 101.91%. Relative standard deviation (RSD) values of intra- and inter-day precision were 0.06 – 1.15% and 0.19 – 0.25%, respectively. This UPLC–DAD method can be validated to simultaneously analyze quantities of marker flavonoids and limonoids in CSS.
Keywords – *Citrus junos* seed shells, flavonoids, limonoids, UPLC–DAD-ESI/MS, Simultaneous determination

Introduction

Citrus junos (Rutaceae), also called as 'yuja' in Korea, is cultivated in China, Japan, and Korea, particularly in the southern coast of Korea. It can prevent cardiovascular diseases,[1] osteoporosis,[1] and cough.[2] In Korea, the peel and pulp of *C. junos* fruits are consumed in large quantity as food like beverage called 'Yuja tea', which is used as a herbal remedy for common cold. Its seeds, which account for 15% of the whole fruit, are mostly discarded as waste.[3] Several recent studies have shown the physiological activity of its seeds, thus increasing their utilization. In addition, essential oil extracted from *C. junos* seeds (CS) is widely used in food, cosmetics, perfumery, and aromatherapy. However, this extraction process also produces a considerable amount of by-product, *C. junos* seeds shells without oil (CSS), causing a potential waste problem. Therefore, chemical analysis of CSS is essential to discover high-value materials.

CS is known to contain phytochemicals such as limonoids, flavonoids, and coumarin. Its major components, limonoids and flavonoids, play an important role in preventing chronic diseases.[4] Modern evidence has shown that citrus limonoids have various biological activities, including antialleric,[5] anti-inflammatory,[6] antiviral,[7] anti-mutagenic,[8] anticarcinogenic,[9] and antiproliferative[10] activities both *in vitro* and *in vivo*. Moreover, flavonoids are polyphenolics with health-promoting properties. They have been reported to have antiviral, anti-inflammatory, and anticancer[11] activities.[12] Especially, naringin was proven to have hypocholesterolaemic[13] and hypoglycaemic effects[14] as well as anti-inflammation.[15] Neoheperidin has been reported to have anti-proliferative effect[16] and neuroprotective activity.[17] Deacetyl nomilin exhibits anti-proliferative activity in colon carcinoma Caco-2 cells.[18] Limonin shows anti-cancer effect[19] and nomilin inhibits osteoclastogenic activity.[20] For this present study, we used CSS normally thrown away. Several studies on CSS are

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reported, however the simultaneous determination in CSS has not yet been studied. The aim of this study was to develop a method to quantitate active compounds in CSS and validate the method. Simultaneous quantitative analysis of five major compounds in CSS using ultra performance liquid chromatography (UPLC) coupled with photodiode array (PDA) detector (UPLC-PDA) and electrospray ionization/mass spectrometry (ESI/MS) was performed. We also developed a method for simultaneous separation of the five major compounds (two flavonoids: naringin and neohesperidin, and three limonoids: deacetylnomilin, limonin and nomilin).

Experimental

Plant materials – The CS was collected from Goheung. (Jungangno, Korea) in winter 2018. The CSS was obtained from Nano Bio Research Center, JBF, after removing oil from CS by supercritical fluid extraction. A voucher specimen (SCNUP 21) was deposited at the Laboratory of Pharmacognosy, College of Pharmacy, Sunchon National University, Suncheon-si, Jeollanam-do, Korea.

Chemicals and reagents – Naringin, limonin, and nomilin were purchased from Sigma (Sigma-Aldrich, MO, USA). Neohesperidin and deacetylnomilin were obtained from *C. junos* and identified by comparing the measured spectroscopic data with published values.[21, 22] The purity of neohesperidin was ≥ 94.6% and that of deacetyl nomilin was ≥ 99.1%. Chemical structures of these five components are shown in Fig. 1. HPLC-grade solvents, acetonitrile, and water were obtained from J.T. Baker (Phillipsburg, NJ, USA).

Preparation of standard solutions – A standard solution mix including analytes was dissolved in methanol and then diluted with methanol to obtain concentrations for establishing calibration curves.

Sample preparation – CSS (1 g) was extracted with 80% (v/v) denatured ethyl alcohol (10 mL) by sonication for 90 min twice. The extracted solution was filtered with filter paper. Its solvent was removed at room temperature (RT) using an evaporator under vacuum with condensation by a nitrogen generator (Claind, Italy). The amount of the dried 80% denatured ethanol residue was 107.6 mg (yield, 10.76%). For quantitative analysis of the CSS, the 80% ethanol extract (107.6 mg) was dissolved in 4 mL of methanol and extracted by sonication for 10 min.

UPLC and MS analysis – CSS sample was analyzed using a Waters Acquity UPLC-I-Class system (Waters, MA, USA) equipped with a binary solvent manager, an autosampler FTN, and a PDA detector. A Waters Acquity UPLC HSS T3 Column (2.1 × 100 mm, 1.8 μm) was used to carry out analysis at 40 °C. The mobile phase was

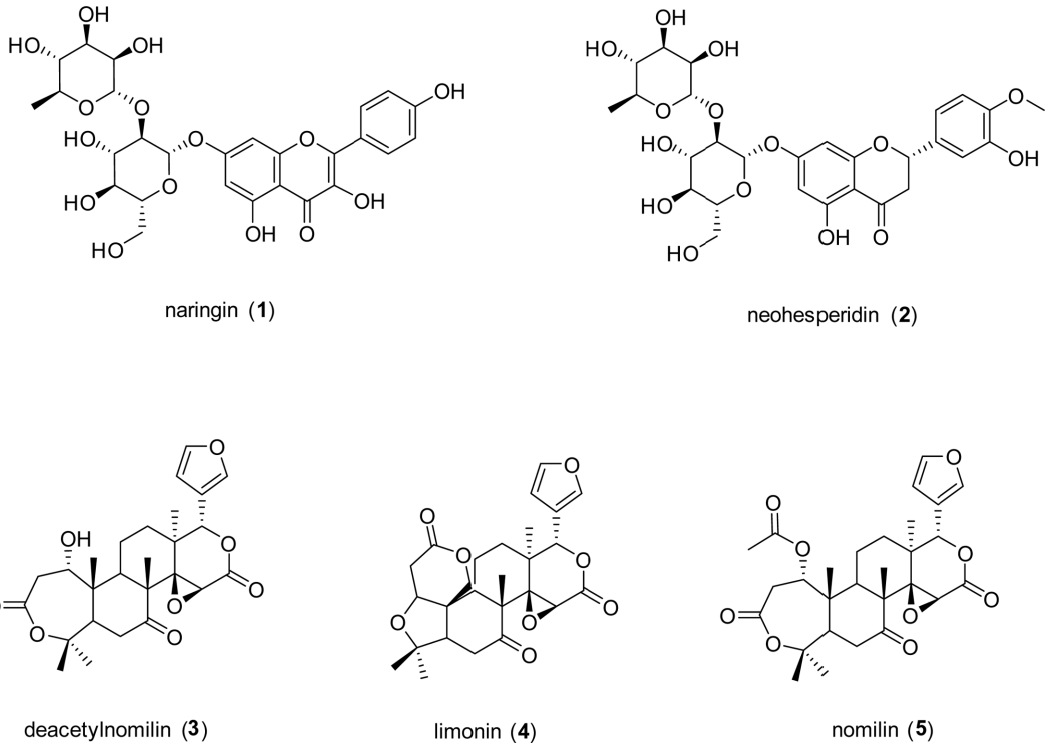

Fig. 1. Chemical structures of major five compounds in CSS.

acetonitrile (A) and distilled water (B). The gradient elution of mobile phase was conducted as follows: 0 - 1 min A; 20%, 1 - 6 min A; 20 - 27%, 6 - 6.1 min 27 - 40%, 6.1 - 14 min A; 40 - 65%, 14 - 14.1 min A; 65 - 100%. The column was then re-conditioned with 20% A isocratic for 3 min at a flow rate of 0.3 mL/min. The injection volume was 5 μL. The range of PDA detection wavelength was set at 200 - 400 nm. Of these, chromatographic data at 210 nm were recorded.

The mass spectrometer (MS) was operated using a Waters Quattro Micro Mass™ (MicroMass, Manchester, UK) equipped with an electrospray ionization (ESI) source. The instrument was operated in positive and negative ion modes. MS conditions were as follows: capillary voltage, 3.0 kV; extractor voltage, 3 V; cone voltage, 50 V; RF lens voltage, 0 V; source temperature, 100 °C; desolvation temperature, 300 °C; desolvation gas, 450 L/h; and cone gas, 40 L/h. All data acquisition and process were carried out using Empower 3 software and Waters MassLunx4.1 software (Waters, MA, USA).

Method validation – The UPLC-DA method was established and validated for linearity, precision, and recovery according to the guidelines described at the International Conference on Harmonisation.[23] Calibration graphs were plotted by the regression equation based on the peak area response (y) vs. corresponding concentrations (x, μg/mL) of five markers in standard solutions at five different concentrations. The limit of detection (LOD) was calculated using LOD = 3.3 × SD / S and the limit of quantification (LOQ) was calculated using LOQ = 10 × SD / S, where SD was the standard deviation and S was the slope of the calibration curve. Intra- and inter-day precision were assessed for each sample with six replicates during a day and by duplicating the experiments on six successive days. The relative standard deviation (RSD) was used for the evaluation of precision [RSD (%) = (SD × 100 / mean measured concentration)]. To prove the repeatability of this method, solution including five compounds was determined six times using established method repeatability at RT and assessed by the RSD value according to the above equation. To verify the accuracy, a recovery test was performed by spiked *C. junos* samples with three different concentrations (low, medium, and high). After evaporation of the solutions through nitrogen gas, the samples were extracted in described method in sample preparation. The recovery (%) was calculated using the following equation: recovery (%) = (detected concentration × 100 / (original concentration + spiked concentration).

Result and Discussion

To find the most suitable extraction efficiency, various factors were tested such as solvent (0, 20, 40, 60, 80, and 100% denatured ethyl alcohol extract), extraction time (0.5 h, 1 h and 1.5 h), and temperature (20, 30 and 35 °C).

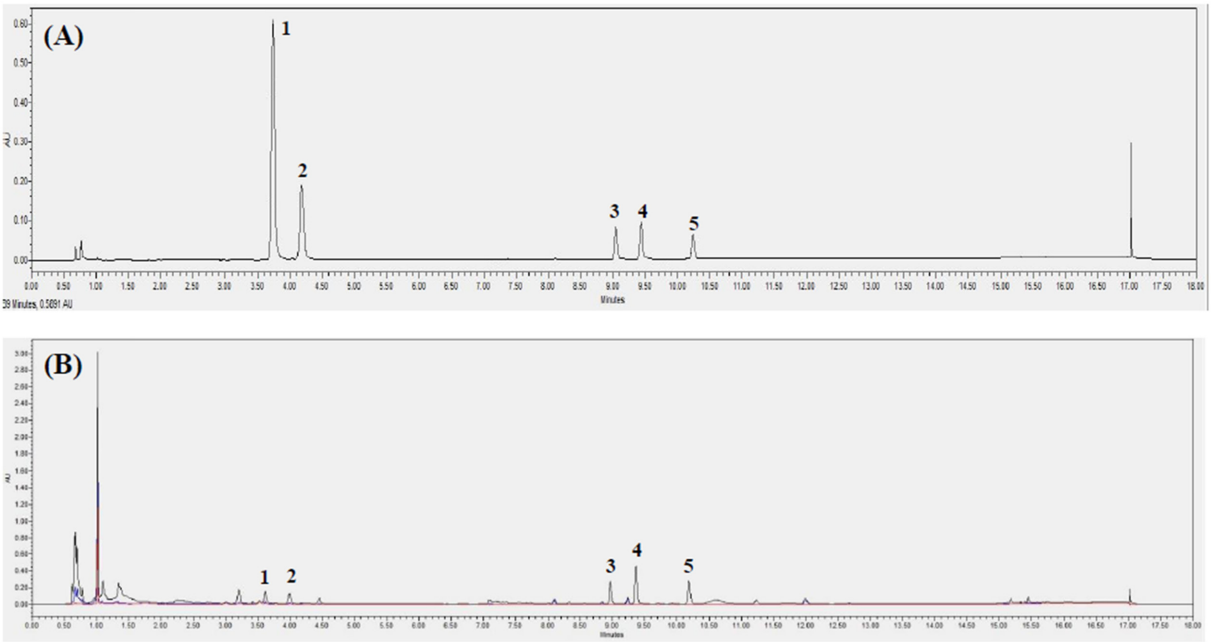

Fig. 2. UPLC chromatograms of the standard solution (A) and CSS extract (B) at 210 nm. Identified compounds are naringin (**1**; t_R 3.73 min), neohesperidin (**2**; t_R 4.17 min), deacetylnomilin (**3**; t_R 9.05 min), limonin (**4**; t_R 9.44 min) and nomilin (**5**; t_R 10.24 min).

To obtain the best appropriate chromatographic conditions, various column types [Waters Acquity UPLC HSS T3 Column C_{18} (2.1 × 100 mm, 1.8 μm) and Waters Acquity UPLC BEH column C_{18} (2.1 × 100 mm, 1.7 μm)] and temperatures of column (30, 35, and 40 °C) were used. After considering peak shapes, resolution, and baselines, we obtained an optimum separation condition with 1.5 h and 35 °C for extraction efficiency and Waters Acquity UPLC HSS T3 Column C_{18} (2.1 × 100 mm, 1.8 μm) at 40 °C for appropriate chromatographic conditions. An analytical method was established using UPLC-DAD. The five compounds (two flavonoids and three limonoids)

Table 1. Linear ranges, regression equation, LODs, and LOQs, of the five marker components in CSS

Compound	Linear range (μg/mL)	Regression equation	Correlation coefficient (r2)	LOD (μg/mL)	LOQ (μg/mL)
naringin	3.11 - 99.60	$y = 46152x - 47362$	0.9998	0.13	0.38
neohesperidin	1.48 - 47.29	$y = 36720x - 22387$	0.9998	0.09	0.26
deaceytylnomilin	3.04 - 97.30	$y = 5208.4x - 20314$	0.9962	0.09	0.29
limonin	1.52 - 48.65	$y = 12076x - 11150$	0.9994	0.13	0.38
nomilin	1.48 - 47.50	$y = 11810x - 36754$	0.9912	0.11	0.33

y: peak area (AU) of compounds; *x*: concentration (μg/mL) of compounds; LOD: 3.3 × SD/S; LOQ: 10 × SD/S. SD is the standard deviation.

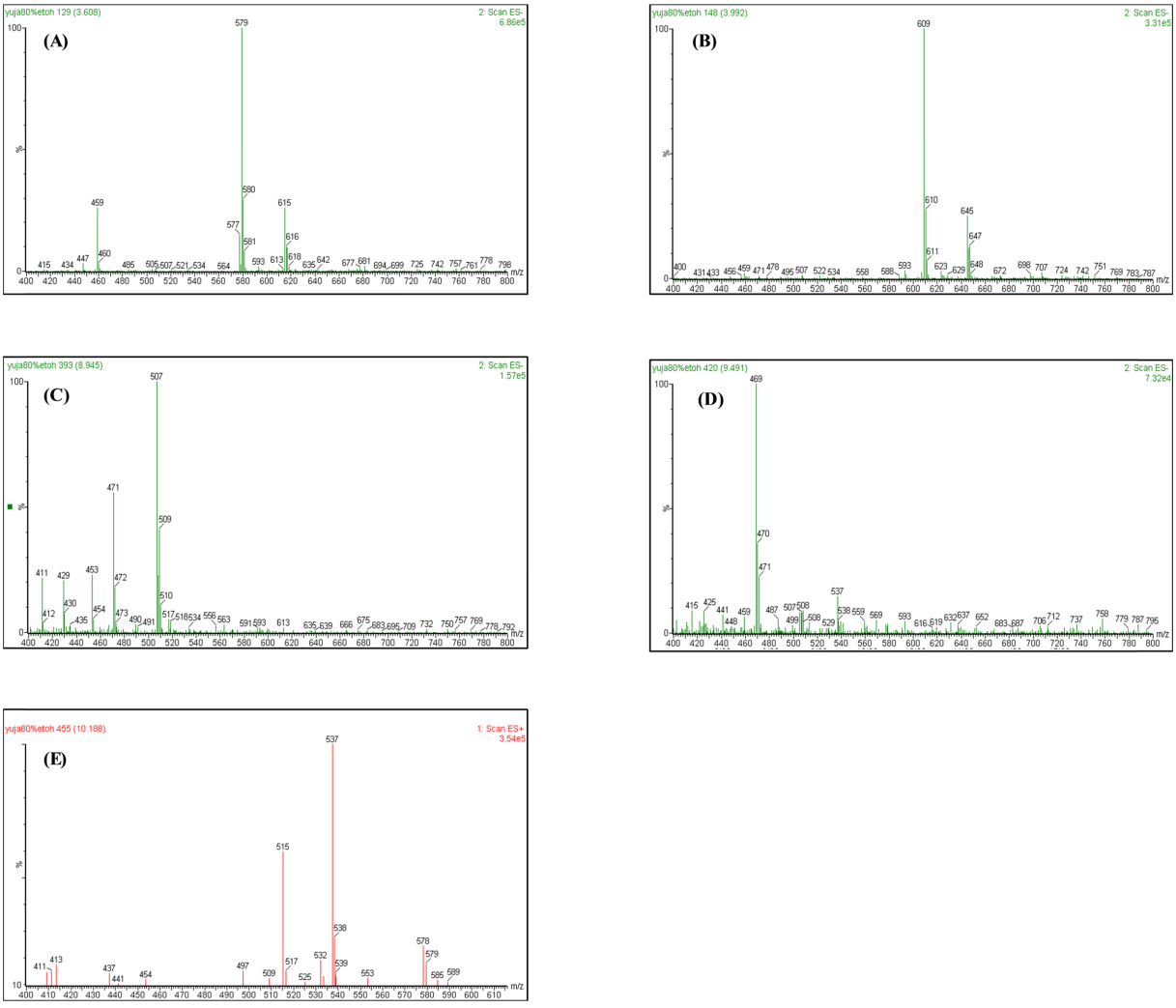

Fig. 3. Mass spectra of five reference standard compounds, naringin (**1**), neohesperidin (**2**), deacetylnomilin (**3**), limonin (**4**) and nomilin (**5**).

Table 2. Recovery data for the analysis of the five compounds in CSS

Analyte	Original conc. (μg/mL)	Spiked conc. (μg/mL)	Detected conc. (μg/mL)	Recovery (%)	RSD (%)
naringin	29.086	12.08	41.571 41.338 41.281	100.57	0.37
		24.15	52.729 52.252 53.301	99.10	1.00
		48.3	77.152 78.297 76.977	100.12	0.93
neohesperidin	10.639	5.91	16.163 16.598 16.432	99.07	1.34
		11.824	22.727 22.778 22.823	101.39	0.21
deacetylnomilin	17.065	23.643	34.267 34.645 34.116	100.18	0.79
		12.393	23.519 23.286 23.474	100.71	0.53
limonin	21.138	6.2	29.754 29.725 29.173	100.31	1.11
		6.081	41.603 41.202 41.505	99.01	0.5
nomilin	6.075	3.04	23.723 24.019 23.621	98.38	0.87
		9.543	27.038 27.522 26.817	99.66	1.33
		12.163	33.553 33.616 33.281	100.55	0.53
		6.5	12.431 12.336 12.3	98.42	0.55
		14.563	15.381 15.354 15.219	98.08	0.57
			20.824 20.621 20.568	100.16	0.65

Recovery (%) = (detected concentration × 100 / (original concentration + spiked concentration)

Table 3. Repeatability of retention times and peak area responses for the five analytes in CSS (n=6)

Compound	Retention time (min)		Peak area response (mAU)	
	Mean SD ± ($\times 10^{-2}$)	RSD (%)	Mean ± SD	RSD (%)
naringin	3.732 ± 0.2	0.06	453461.67 ± 8378.80	0.19
neohesperidin	4.176 ± 0.2	0.05	1700034.17 ± 3386.32	0.20
deaceytylnomilin	9.048 ± 0.2	0.02	487731.83 ± 918.82	0.19
limonin	9.438 ± 0.2	0.02	564202.17 ± 1393.10	0.25
nomilin	10.244 ± 0.2	0.02	532737.67 ± 1240.85	0.23

in the CSS were separated within 11 min at 210 nm. As shown in Fig. 2, retention times of these phytochemicals were detected as follows: naringin (**1**; t_R 3.73 min), neohesperidin (**2**; t_R 4.17 min), deacetylnomilin (**3**; t_R 9.05 min), limonin (**4**; t_R 9.44 min), and nomilin (**5**; t_R 10.24 min).

The linearity of the developed UPLC method was measured based on values of correlation coefficients (r^2) using calibration graphs of each compound. The linearity of the five compounds had the best r^2 values (≥ 0.9912) with the following concentration ranges: 3.11 - 99.60 μg/mL for naringin, 1.48 - 47.29 μg/mL for neoheperidin, 3.04 - 97.30 μg/mL for deacetylnomilin, 1.52 - 48.65 μg/mL for limonin, and 1.48 - 47.50 μg/mL for nomilin. The LOD and LOQ of these five compounds were 0.09 - 0.13 and 0.26 - 0.38 μg/mL, respectively. These results are shown in Table 1.

To evaluate the recovery, three different amounts (low, medium and high) were spiked to the CSS sample. Results of recovery are summarized in Table 2. Recoveries of these five compounds were in the range of 98.08-101.36% with relative standard deviation (RSD) values ≤ 1.33%, demonstrating that the developed method was suitable for the assessment of these flavonoids and limonoids in CSS. The repeatability was measured by analyzing six independently prepared samples using the same method. The sample repeatability was assessed by the RSD value of peak area responses and retention times. Results are shown in Table 3. RSD values of retention time and peak area responses were ≤ 0.06 and ≤ 0.25, respectively. To evaluate the precision of this method, we tested RSD values of intra- and inter-day. RSD values of intra-day and inter-day evaluations (n = 6) were 0.06-1.15 and 0.26-2.69%, respectively (Table 4).

The UPLC analytical method developed in this study was used to confirm the quantity of five components in CSS extract. Quantities of the five components were 5.34, 4.19, 21.24, 28.86, and 20.21 mg/g, respectively (Table 5).

MS spectra of the five compounds are shown in Fig. 3. Four components (naringin, neohesperidin, deacetylnomilin, and limonin) were detected using the negative ion mode $[M-H]^-$ with *m/z* 580, 610, 472, and 470, respectively.

Table 4. Precision data for the assay of the five analytes in CSS

analyte	Intra-day (n = 6)	Inter-day (n = 6)
	Precision (%)	Precision (%)
naringin	1.15	2.69
neohesperidin	0.51	2.04
deaceytylnomilin	0.06	0.26
limonin	0.22	0.32
nomilin	0.18	1.61

Precision is expressed as RSD (%) = (SD/mean) × 100.

Table 5. The amounts of the five compounds in CSS (n=3)

compound	Amount (mg/g)		
	Mean	SD × 10^{-1}	RSD (%)
naringin	5.34	0.69	1.28
neohesperidin	4.19	0.71	1.70
deaceytylnomilin	21.24	0.54	0.26
limonin	28.86	0.88	0.30
nomilin	20.21	0.14	0.07

Nomilin was detected in the positive ion mode $[M+H]^+$ at *m/z* 514 (Table 6).

In conclusion, in the present study, a UPLC-PDA method was established and applied to simultaneous determination of five marker components: naringin (**1**), neohesperidin (**2**), deacetylnomilin (**3**), limonin (**4**), and nomilin (**5**) at amounts of 4.19-28.86 mg/g. Quantities of limonoids were higher than those of flavonoids in the CSS. Suitable linear calibration graphs enabled simultaneous quantitative determination of the five marker molecules over various concentration ranges. The developed method was validated to have suitable sensitivity, repeatability, and precision. These five compounds were identified by UPLC-MS. These results indicate that the established UPLC-PDA method could be valuable for simultaneous quantitative assessment of CSS. The objective of this study was to develop analytical method on markers which have various biological activity for quality control of CSS. It can contribute to the development of this by-product into high-value materials for cosmetics, foods, and medicine.

Table 6. The mass data of two flavonoids and three triterpenoids acquired from CSS using LC-MS

Compound	Molecular formula	Molecular weight	Detected ion	Precursor ion	Production ion
naringin	$C_{27}H_{32}O_{14}$	580.18	[M-H]	579	615
neohesperidin	$C_{28}H_{34}O_{15}$	610.19	[M-H]	609	645
deacetylnomilin	$C_{26}H_{32}O_8$	472.21	[M-H]	471	507
limonin	$C_{26}H_{30}O_8$	470.19	[M-H]	469	-
nomilin	$C_{28}H_{34}O_9$	514.22	[M+H]	515	537

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