An HPLC-UV-based quantitative analytical method for *Chrysanthemum morifolium*: development, validation, and application

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Abstract A simple and reliable analytical method based on high-performance liquid chromatography-ultraviolet detection was established for the analysis of the flowers of *Chrysanthemum morifolium* (CM). Luteolin-7-O-glucoside (LU7G) was chosen as a target analyte considering its content, availability, and ease of analysis. Chromatographic separation of LU7G was achieved using a Phenomenex Gemini C_{18} column (250 × 4.6 mm, 5 μm) run with a mobile phase consisting of 0.5 % acetic acid in water and 0.5 % acetic acid in acetonitrile at a flow rate of 1.0 mL min^{-1}. The detection wavelength and column temperature were set at 350 nm and 40 °C, respectively. Method validation was performed according to the AOAC guidelines and the method was specific, linear (R^2 = 0.9991 for 50–300 μg mL^{-1}), precise (≤ 3.91 % RSD), and accurate (100.1–105.7 %). The limits of detection and quantification were 3.62 and 10.96 μg mL^{-1}, respectively. The established method was successfully applied to determine the contents of LU7G in various batches of bulk CM extracts and lab-scale CM extract. The developed method is a readily applicable method for the quality assessment of CM and its related products.

Key words: *Chrysanthemum morifolium*, quality control, marker compound, high performance liquid chromatography, method validation

1. Introduction

The flowers of *Chrysanthemum morifolium* (CM) have been widely used in food supplements, health beverages, and tea in many Asian countries, including South Korea, China, and Japan. CM flower is used as a traditional Chinese medicine by itself or in formulas for diaphoresis and antidotes for the common cold and eye diseases.[1,2] With CM and CM-containing products being demanded by consumers due to their beneficial health effects, it is important to guarantee their quality using an appropriate and reliable analytical method.

The analytical methods used for the routine quality control (QC) of plant-related substances are often based on the quantification of one or more marker compounds using high-performance liquid chromatography coupled with ultraviolet detection (HPLC-UV).[3-6] Various

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bioactive compounds have been identified in CM, including flavonoids,[1] phenolic compounds,[7,8] and caffeoylquinic acids (CQAs).[9] Wang *et al.*[10] developed an HPLC-UV method to quantify two flavonoids, luteolin-7-O-glucoside (LU7G) and rutin as indicative constituents of formulations of CM and *Sophora japonica* (SJ). However, their study focused on the manufacturing process to prepare total flavonoid fractions from CM and SJ. Their quality was assessed using both HPLC and colorimetric methods, with a fairly long analysis time. Huang *et al.* applied supercritical fluid chromatography (SFC) for the simultaneous determination of five flavonoids including LU7G in CM.[11] Although the chromatographic analysis was completed within 18 min, SFC is not readily available in many laboratories. Accordingly, the development of a simple, low cost, and reliable analytical method to ensure the quality of CM and CM-containing products is necessary.

The aim of this study was to develop and validate an analytical method that is readily applicable for QC of CM using HPLC-UV. Selection of a marker compound, establishment of a quantitative method for the selected marker, and rigorous validation of the developed method are presented.

2. Materials and Methods

2.1. Chemicals, reagents, and instruments
LU7G (purity > 98 %) was purchased from Biopurify Phytochemicals Ltd. (Chengdu, China). Luteolin (≥ 97 %) was obtained from Sigma Aldrich (St. Louis, MO). HPLC-grade methanol (MeOH), acetonitrile (ACN), and water were from Honeywell Burdick & Jackson (Ulsan, Korea). Acetic acid and formic acid were obtained from Sigma Aldrich.

A centrifuge 1580 NGR and a vortex mixer VM-10 were from Gyrogen (Incheon, Korea) and DAIHAN Scientific Co. Ltd (Seoul, Korea), respectively.

2.2. Preparation of the standard solutions and the CM extracts
The stock solution of LU7G was prepared at a concentration of 500 μg mL^{-1} in MeOH. To determine

linearity, the working solutions containing 50, 75, 100, 150, and 300 μg mL^{-1} of LU7G were prepared by diluting the stock solution with MeOH. A solution of 100 μg mL^{-1} of LU7G was then prepared as a QC solution. The stock and working solutions were stored at -45 °C until use.

The bulk CM extracts were prepared in three different batches (batch #1–3) and provided by Nutribiotech Co., Ltd (Seoul, Korea). The manufacturing process was as follows: 60 kg of finely pulverized CM was added to 900 L of 30 % (v/v) aqueous ethanol at 70 °C and extracted for 4 h. After filtration, the filtrate was mixed with maltodextrin at a 6:4 weight ratio of extract to maltodextrin and subjected to spray-drying. The CM extract was stored at < 4 °C until use.

A lab-scale CM extract was prepared in the manner as for the bulk preparation using 1.2 g of homogenized CM powder and 19 mL of 30 % (v/v) aqueous ethanol. The filtered extract was evaporated to dryness using a rotary evaporator.

2.3. Preparation of the sample solutions for HPLC analysis
To develop and validate the HPLC-UV method, sample solutions were prepared using the bulk CM extract powder. First, 400 mg of the extract was added to a 10 mL volumetric flask, which was filled to mark with MeOH. After brief vortexing, the mixture was subjected to ultrasonic irradiation at room temperature for 30 min, followed by centrifugation at 2898 *g* for 3 min. A 1 mL aliquot was filtered through a 0.45 μm PTFF syringe filter (Whatman, Piscataway, NJ, USA), then 10 μL of the filtrate was injected into the HPLC-UV system.

For the lab-scale CM extract, the entire residue dried was reconstituted with MeOH in a 10 mL volumetric flask and underwent the same procedure as in the bulk sample prior to HPLC-UV analysis.

2.4. HPLC-UV and HPLC-PDA conditions
The HPLC-UV system used for the quantitative analysis consisted of an Agilent technologies 1200 series (Santa Clara, CA, USA) equipped with a 1260

Infinity II quaternary pump, an autosampler, a column thermoeter, and a 1260 Infinity II multiple wavelength detector. A Phenomenex Gemini C_{18} column (250 × 4.6 mm, 5 μm) was used and mobile phases A1 (0.5 % v/v acetic acid in water) and B1 (0.5 % v/v acetic acid in ACN) were eluted at a flow rate 1 mL min^{-1}. The detection wavelength was 350 nm and column temperature was set at 40 °C. The linear gradient of the mobile phase was varied from 10 % B1 to 18 % B1 for 3 min, from 18 % B1 to 22 % B1 for 12 min, and from 22 % to 100 % B1 for 2 min. The column was equilibrated for 15 min before every run.

HPLC coupled to photodiode array detection (PDA) was used to evaluate method specificity. The HPLC-PDA system was Waters 996 (Millipore, MA, USA), combined with a detector (Model No. 996) and a separation module (Model No. 2695). The same operation conditions used for HPLC-UV analysis were applied to the HPLC-PDA system.

2.5. Ultra-high performance liquid chromatography-triple quadrupole tandem mass spectrometry (UHPL-QqQ/MS) conditions

The UHPLC-QqQ/MS system was used for identification and selection of a marker compound in the CM sample. It was composed of a Nexera X2 UHPLC system equipped with a pump (LC-30AD), an autosampler (SIL-30AC), a system controller (CBM-20A), a column oven (CTO-20AC), a UV-vis detector (SIL-30AC), and an LC-MS 8040 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan).. A Luna 1.8 μm C18 (2.1 × 100 mm) column was used at 30 °C. The mobile phase, 0.1 % formic acid in water (A2) and 0.1 % formic acid in ACN (B2) was used for elution at a 0.2 mL min^{-1} flow rate. Linear gradient elution was performed by increasing the %B2 from 10 % to 100 % over 20 min. The column was equilibrated for 5 min between runs and the m/z ranged from 100 to 1000.

2.6. Method validation

Bulk CM batch #1 was used for validation of the analytical method. Specificity, linearity, precision, and accuracy were assessed as described below.

2.6.1. Specificity, linearity, limit of detection, and limit of quantification

Specificity was evaluated by comparing the chromatograms and PDA spectra of the unspiked CM extract with those of the CM extract spiked with 100 μg mL^{-1} of LU7G. Linearity and sensitivity were evaluated based on a linear regression analysis. The calibration curve of LU7G was constructed by triplicate analyses of standard solutions at 50, 75, 100, 150, and 300 μg mL^{-1}. The limits of quantification (LOQ) and detection (LOD) were determined using Eqs. (1) and (2).

$$LOD = 3.3 \times \frac{SD}{S} \tag{1}$$

$$LOQ = 10 \times \frac{SD}{S} \tag{2}$$

where SD and S are the standard deviation of the intercept and slope of the calibration curve, respectively.

2.6.2. Precision and accuracy

Precision, expressed by the relative standard deviation (%RSD), was determined for intra-day, inter-day, and inter-person precisions. Intra- and inter-day precisions were measured using CM samples spiked with LU7G at three concentrations (0, 100, and 150 μg mL^{-1}) analyzed in five replicates on the same day and on three consecutive days, respectively. The inter-person precision was assessed using the same unspiked samples by two analysts in the same lab. The accuracy was determined as % relative recovery. LU7G was spiked into CM samples at three different levels (50, 100, 150 μg mL^{-1}) and the % recovery was calculated according to Eq. (3), where C_{found} is the real concentration of the sample spiked with standards; $C_{background}$ is the concentration of the unspiked solution; and C_{added} is the concentration of the added standard to the CM sample.

$$\% \text{ Recovery} = \frac{C_{found} - C_{background}}{C_{added}} \times 100 \tag{3}$$

2.7. Statistical analysis

Prism (GraphPad Software, San Diego, CA, USA)

was used for linear regression analysis and analysis of variance (ANOVA).

3. Results and Discussion

3.1. Selection of LU7G as a marker compound

Several factors were considered in the selection of the most appropriate marker compound(s) including specificity, content, ease of analysis, commercial availability, and price.[4,12] Bioactivities also can be a factor for consideration. In previous studies, flavonoids and volatiles were reported in the aqueous methanolic and ethanolic extracts of CM, as determined by HPLC[13] and GC-MS analyses.[14] Volatile compounds
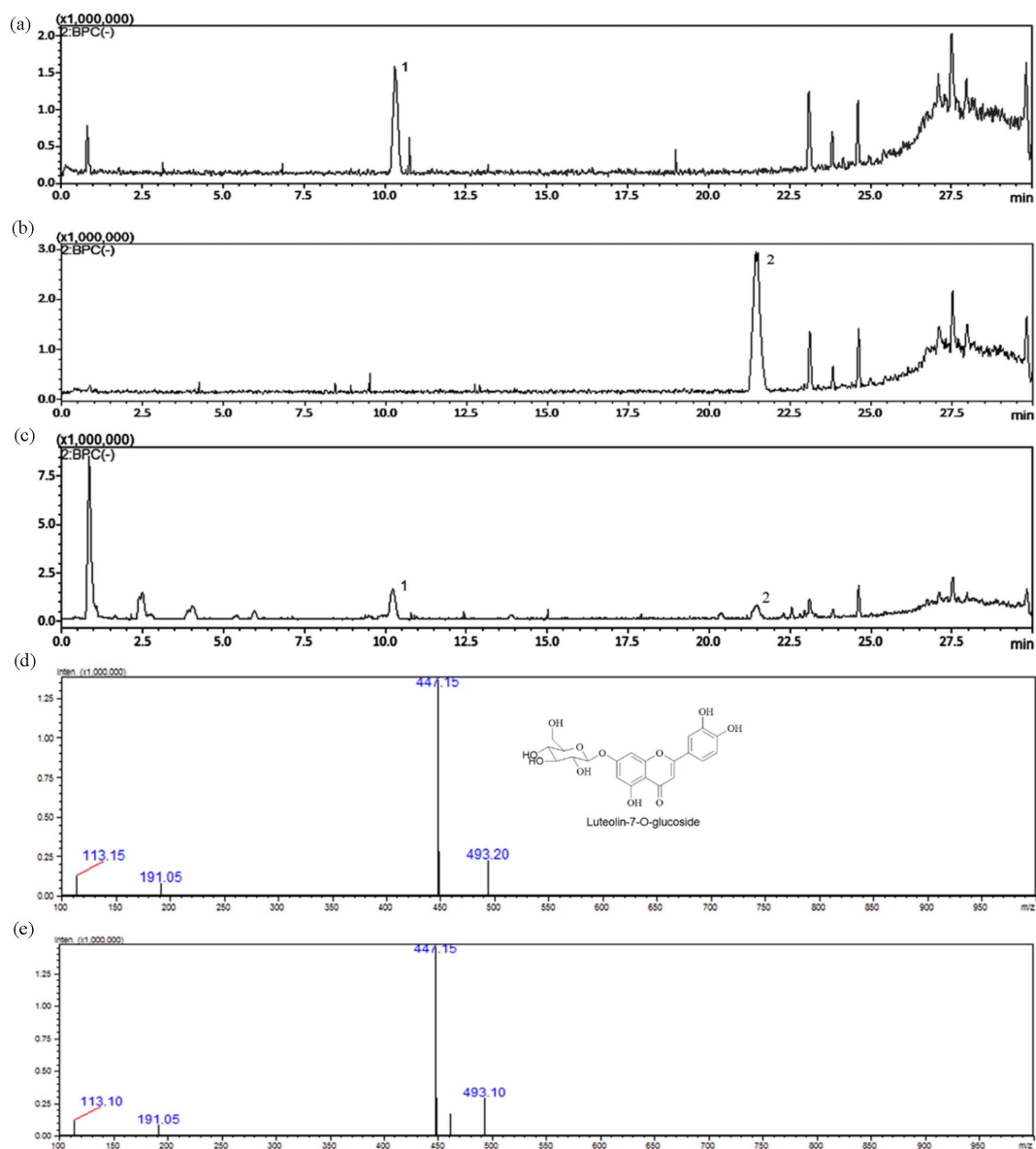
Fig. 1. Qualitative analysis of the CM extract using UHPLC-UV-QQQ/MS. Base peak chromatograms of luteolin-7-O-glucoside standard solution (a), luteolin standard solution (b), and the CM extract (c); mass spectra of peak 1 at 10.25 min of luteolin-7-O-glucoside standard solution (d) and the CM extract (e). Peak identification: 1, luteolin-7-O-glucoside; 2, luteolin.

are not preferred as QC markers due to possible changes in their content during storage and sample preparation.[15] Flavonoids including luteolin, LU7G, and apigenin were reported as the main constituents of CM.[2] In particular, LU7G and quercetin accounted for 85.7 % of the total flavonoids in CM.[14] Quercetin is prevalent in plants, leading to poor selectivity. Luteolin has been used as the marker compound for the verification of CM in the Korean Herbal Pharmacopoeia (KHP).[16]

In this study, the chemical profiles of CM extracts were acquired using UHPLC-UV-MS/MS. Analysis of chromatograms and mass spectra were compared to literature and standard compounds and confirmed that LU7G was the major component and that luteolin was present at a much lower level (*Fig*. 1). In terms of chromatographic separation using the HPLC-UV system, luteolin, which is relatively nonpolar and eluted later than polar compounds such as glycosides in reversed phase HPLC, was eluted closely with other compounds that appeared to be flavonoid aglycones (*Fig.* 1). In contrast, LU7G, eluted at earlier times than luteolin and could be easily baseline separated under relatively weak elution conditions. Being reasonably specific to CM and stable,[17] LU7G is also commercially available at a reasonable price and its quantification is likely faster than luteolin. It is well-known to exhibit anti-inflammatory,[18] anti-viral,[19] and anti-bacterial[20] activities. Therefore, LU7G was the most appropriate quantification marker of CM and an HPLC-UV method was established to quantify LU7G in CM as described below.

3.2. Establishment of an HPLC-UV method to determine LU7G in CM

The LU7G standard is soluble in water and several organic solvents, including MeOH. However, the CM extract in aqueous MeOH resulted in erratic mixture or emulsion formation during ultrasonic irradiation. Therefore, the LU7G standard and CM samples were dissolved and extracted using 100 % MeOH. For chromatography, the use of water and MeOH as the mobile phase resulted in significant peak tailing for LU7G, whereas water and ACN did

not (data not shown). Thus, water and ACN were adopted as the mobile phase. Detection was achieved at approximately 350 nm where LU7G exhibited the strongest absorbance based on HPLC-PDA analysis (data not shown).

The effects of pH of the mobile phase on the retention time, peak shape, and selectivity were examined by varying the types and concentrations of acid added to the water and ACN. For formic acid, both 0.1 % and 0.5 % concentrations yielded poor resolution between LU7G and neighboring interferences. Acetic acid was also tested at 0.1 % and 0.5 % concentrations, revealing that 0.5 % concentration effectively, but not completely, resolved the peaks. Finally, complete baseline separation was achieved when the 15 cm column was replaced with a 25 cm reversed phase column. Lastly, the column temperature varied from 25 to 40 °C. Although it did not affect the performance significantly in terms of peak shape and resolution, the higher temperature (40 °C) proved to efficiently shorten analysis time. The established method is described in the experimental section and was validated according to the Association of Official Analytical Chemists (AOAC) guidelines as follows.

3.3. Validation of the established analytical method
3.3.1. Specificity
Specificity was evaluated by comparing the chromatograms and spectra of the LU7G standard, CM sample, and CM sample spiked with the standard. No interferences were observed around the analyte peak at a retention time of approximately 13.5 min (*Fig.* 2). The PDA spectra of the LU7G peaks in all samples showed the same pattern with the maximum wavelength absorbance at 347.2 nm (*Fig.* 2). Accordingly, the method can be regarded as specific to LU7G.

3.3.2. Linearity, LOD, and LOQ
The linear regression equation for LU7G was $y = 25.75\ x + 24.22$ and its coefficient of determination (R^2) was 0.9991 with a linear range of 50 to 300 μg mL^{-1} (*Table* 1). According to the AOAC guidelines where R^2 should be greater than 0.99,[21] this was
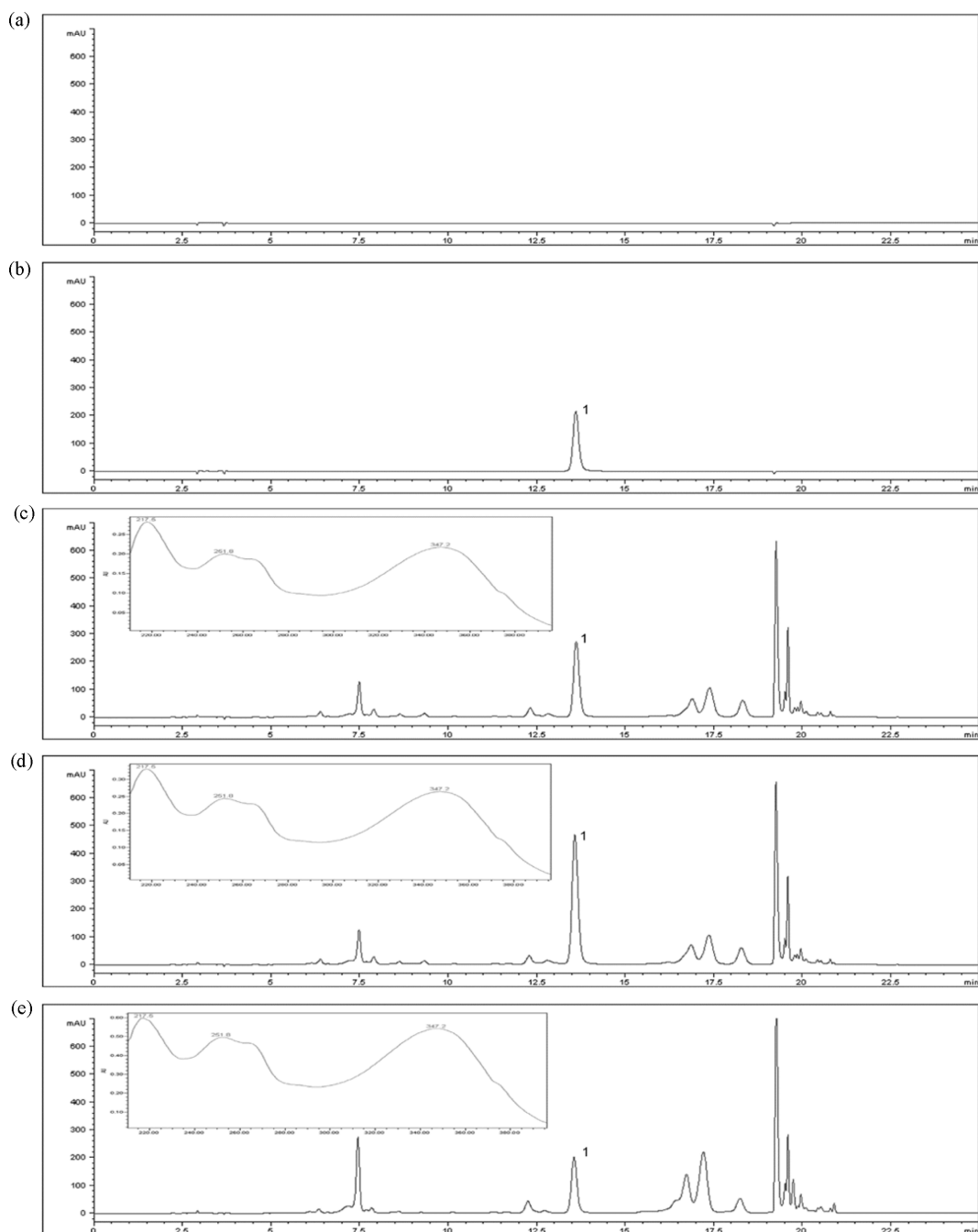
Fig. 2. HPLC-UV chromatograms of blank methanol (a), 100 μg mL^{-1} luteolin-7-O-glucoside (b), bulk CM extract (c), bulk CM extract spiked with 100 μg mL^{-1} of luteolin-7-O-glucoside (d), and the lab-scale CM extract (e). Inserted figures are the UV spectra of peak 1 in the chromatograms. Peak identification: 1, luteolin-7-O-glucoside.

acceptable. The LOD and LOQ values were calculated to be 3.62 and 10.96 μg mL^{-1}, respectively.

3.3.3. Precision and accuracy

Intra- and inter-day as well as inter-person precisions and accuracy were estimated at low, middle, and

Table 1. Linearity, LOD, and LOQ of the developed method

Range ($\mu g\ mL^{-1}$)	Linear regression equation		R^2	LOD ($\mu g\ mL^{-1}$)	LOQ ($\mu g\ mL^{-1}$)
	Slope (± SD, n=5)	Intercept (± SD, n=5)			
50 ~ 300	25.75 (± 0.39)	24.22 (± 28.22)	0.9991	3.62	10.96

Table 2. Intra- and inter-day precisions of the established method

Precision	Fortified concentration[a]	Measured concentration[a] (± SD, n=5)	% RSD
Intra-day	0	121.7 (±1.2)	1.00
	50	174.5 (±1.6)	0.94
	100	221.8 (±6.1)	2.74
Inter-day	0	121.2 (±1.8)	1.45
	50	174.6 (±1.7)	0.99
	100	227.5 (±6.5)	2.85

[a]$\mu g\ mL^{-1}$

Table 3. Accuracy of the established method   (n=5)

Background concentration[a] ($C_{background}$)	Fortified concentration[a] (C_{added})	Found concentration[a] (C_{found})	% Recovery	%RSD (n=5)
	50	174.5	105.7	3.16
121.7	100	221.8	100.1	6.13
	150	274.9	102.1	4.08

[a]$\mu g\ mL^{-1}$

high concentrations. From the AOAC guidelines,[21] repeatability and reproducibility, which correspond to intra- and inter-day precisions of this study, respectively, are acceptable at 3 % and ≤ 6 % RSD, respectively, at a concentration of 0.1 %, which is similar to the estimated concentration of CM extract samples (~0.3 % w/w). As shown in *Table* 2, the intra-day precisions were < 3 % RSD and the inter-day precisions were within 6 % RSD. The inter-person precision was 3.91 %, which is below the limit of 6 % prescribed by the AOAC guidelines. Accuracy values were measured as a relative recovery and were 100.1–105.7 % (*Table* 3), which are acceptable based on the AOAC guidelines (90–108 %). Therefore, precision and accuracy of the established method are satisfactory according to the AOAC guidelines.

3.4. Application of the developed analytical method to various CM samples

The current analytical method was established and

Table 4. Contents of luteolin-7-O-glucoside in three different batches of CM

Batch No.	Concentration[a] (±SD, n=3)	Contents[b] (±SD, n=3)	% RSD
1	118.05 ± 3.63	2.95 ± 0.09	3.07
2	119.20 ± 2.88	2.98 ± 0.07	2.42
3	119.73 ± 1.96	2.99 ± 0.05	1.64

[a]$\mu g\ mL^{-1}$.
[b]$mg\ g^{-1}$.

validated using bulk CM batch #1. The developed method was also applied to other CM batches and the three CM extract batches contained very similar levels of LU7G (2.95–2.99 mg g^{-1}) with no significant difference, as listed in *Table* 4. According to the protocol for the preparation of the bulk CM extract (see 'Preparation of CM extracts' for details), the production yield was ~20 %. This indicates that 1.2 g of raw CM flowers may result in the lab-scale CM extract containing LU7G at levels close to the 400 mg in the bulk CM batch. As a result of the analysis of 1.2 g raw flowers, the LU7G content of the lab-scale CM extract sample was 0.55 ± 0.04 mg g^{-1} (n = 3), which is approximately five-fold lower than that of the bulk extract. This discrepancy is likely because of the very different experimental scales. Nonetheless, the real flower sample could be readily analyzed using the developed method because the linear range of the method was wide enough to cover the low sample concentrations (*Fig.* 2).

4. Conclusions

In this study, a simple and reliable method to quantify LU7G in CM was established. LU7G was selected as a quantification marker for quality control of CM based on the consideration of various aspects. The established analytical method is based on HPLC-UV and was specific, linear, precise, and

accurate with reasonable sensitivity upon validation according to the AOAC guidelines. The method was applied to different samples of CM extracts prepared on the bulk and lab scales. Overall, the current method is a readily applicable method for the quality control of raw CM materials and its related products.

Acknowledgements

Conflicts of Interest

J. H. Kim and J. H. Geum are employed by COSMAX Inc. that might benefit from the results of the study. All other authors report no conflicts of interest relevant to this study.

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