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Optimization of an analytical HPLC-DAD method for detecting hydroxycinnamic acid derivatives from mixtures of *Saussurea grandifolia* and *Taraxacum coreanum*

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Abstract

An analytical method was established to identify and quantify hydroxycinnamic acids, such as 1,5-dicaffeoylquinic acid (DCQA) and chicoric acid (CA), in mixtures of *Saussurea grandifolia* and *Taraxacum coreanum* (MST) by using reverse-phase high-performance liquid chromatography coupled with diode array detector (HPLC-DAD). Analyses were carried out by using an INNO C_{18} column with a gradient elution system, and different parameters were used to validate our optimized method. Results demonstrated limits of detection and quantification of 5.46×10^{-3} and 16.54×10^{-3} mg/mL for DCQA and 0.37×10^{-3} and 1.14×10^{-3} mg/mL for CA, respectively. The calibration curves for DCQA and CA showed good linearity over the concentration ranges of 0.025–0.4 and 0.00625–0.1 mg/mL, respectively, and both exhibited $r^2 = 1.0000$. In the accuracy test, high recovery rates were obtained ranging from 101.16–104.18% for DCQA and 97.55–108.49% for CA, while the precision values were $\leq 1.00\%$ for DCQA and $\leq 1.21\%$ for CA. The values obtained from our analyses support the use of this analytical method for the accurate identification and quantification of DCQA and CA from MST. Our methodology could be used further to determine the content of hydroxycinnamic acid derivatives in routine analyses and large-scale extraction processes.

Keywords: Chicoric acid, 1,5-Dicaffeoylquinic acid, HPLC-DAD, Method validation, *Saussurea grandifolia*, *Taraxacum coreanum*

Introduction

Dandelions are plants of the genus *Taraxacum* and are perennial herbs of the Compositae family that are widely distributed across the warm northern hemisphere, including Asia, Europe, and America. There are about 2000 species of dandelions in the world, which are often highly recognizable because they grow well-trafficked areas like by roads and in open grass fields. Plants in the genus *Taraxacum* have historically been used as

medicinal herbs because of their various bioactivities. For example, they have been used to treat inflammation and rheumatism [1]. Its medicinal effects include serving as an anti-oxidant and anti-cancer agent, as well as being hepatoprotective, neuroprotective, and useful in treating atopy [2–8]. Among plants in the genus *Taraxacum*, *T. coreanum* is known as "white dandelion" and is native to Korea. Diverse phytochemical compounds in *T. coreanum*, such as sterols, terpenoids, and phenolics among others, have been reported [8–10].

Plants in the genus *Saussurea* of the Compositae family have been broadly consumed as "edible wild plants" in Korea. The genus *Saussurea* is widely distributed throughout the northern hemisphere, but mainly in Asia.

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They are also well-diversified in Korea where 32 species have been identified [11], 16 species of which have been reported as endemic [12]. In China, some species have been used as traditional medicines to treat rheumatism, bleeding, and gynecological diseases [13]. They contain various phytochemicals that include flavonoids, sesquiterpenoids, triterpenes, lignans, and steroids [14, 15]. This imparts the plants in the genus *Saussurea* with health-promoting properties. Such plants can exhibit anti-inflammatory, anti-bacterial, and anti-fungal activities [16–19], as well as the capacity to regulate plant growth [20, 21].

Recently, the consumption of health functional foods has steadily increased due to individual's efforts and interest in self-health care, especially enhancing immunity due to aging and epidemic trends. Health functional foods can have a direct impact on promoting health and preventing disease by increasing the intake of food ingredients that benefit overall health [22]. As a result of this trend, developing health functional foods from medicinal plants and natural product materials has emerged as a high value-added industry, drawing attention as an industrial area with the highly attractive market outlook among the various biological industries. Different plants are often combined into a mixture that integrates various functions according to consumer needs. This trend requires a certified method or a precise analysis method to evaluate functional and marker compounds to standardized distributions in health functional foods.

Plenty of hydroxycinnamic acid derivatives are found in the two genera mentioned above. There have been many studies where hydroxycinnamic acid derivatives were already determined and quantified [23–25], but studies on its method validation are insufficient. Therefore, it is meaningful and necessary to establish a validated analytical method for 1,5-dicaffeoylquinic acid (DCQA) and chicoric acid (CA) from mixtures of *S. grandifolia* and *T. coreanum* (MST). In order to utilize a plant derived material as a functional ingredient, at least one marker compound needs to be standardized, developed, and verified. Analytical method validation is a set of procedures for establishing whether an analytical method is reproducible and provides reliable results when used for the intended purpose [26]. These validation procedures have gained wide acceptance as a criterion for determining the validity of analytical methods used for the quality control of health-related functional foods and pharmaceuticals [27].

In this study, we established a practical and appropriate analytical method that uses HPLC-DAD to quantify marker compounds in MST. This proposed method is validated by analyzing hydroxycinnamic acids, such as DCQA and CA, in MST by using HPLC-DAD, which is useful as a basis for developing a high value-added health product derived from MST.

Materials and methods

Plant materials and extraction procedure

The plants *S. grandifolia* and *T. coreanum* were collected from Ulneung Island (Kyungpook Province, Korea) and Haman (Kyungnam Province, Korea), respectively. MST was composed of mixtures of *S. grandifolia* and *T. coreanum* (1:4, w/w) extracts. Voucher specimen of *S. grandifolia* (No. LEE 2011-02) and *T. coreanum* (No. LEE 2007-01) were identified by Dr. K. Choi, Korea National Herbarium, Korea. Dried aerial parts of the plants were pulverized and used for extraction. Each plant (100 g) was extracted three times with ethanol (80 °C, 2 L × 3 h) and concentrated *in vacuo* (50 °C).

Instrumentation, chemicals, and reagents

Analyses were performed by using an HPLC system (Agilent 1260 Infinity II Quat Pump, CA, USA) that consisted of an auto-sampler, pump, and photodiode array detector (PDA WR detector, CA, USA). HPLC-grade solvents such as water, methanol (MeOH), and acetonitrile (ACN) were purchased from J. T. Baker (Phillipsburg, PA, USA). Acetic acid (99.7%) and ethanol (95.0%) were purchased from Samchun Pure Chemicals (Pyeongtaek, Korea). DCQA (98.9%) and CA (97.5%) were commercially acquired from Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea (Fig. 1).

Preparation of samples and standard solutions for HPLC

MST (70 mg) was dissolved in 1 mL MeOH and filtered through a syringe filter (0.45-μm) to prepare sample stock solutions. One milligram each of DCQA and CA was dissolved in 1 mL MeOH to prepare the standard stock solutions. Calibration curves were obtained by diluting stock solutions to the desired concentrations to prepare the working solutions and subsequently analyzing these working solutions.

HPLC-DAD conditions

HPLC analysis was conducted with a reverse-phase HPLC system equipped with an INNO C_{18} column (25 cm × 4.6 mm, 5 μm). The injection volume was 10 μL and a wavelength of 330 nm was used for detection. Analyses were conducted at ambient temperature with a flow rate of 1.0 mL/min. A gradient elution system was used for analysis. The mobile phase was 0.5% acetic acid in water (A) and ACN (B). The elution was conducted as follows: 90% A at 0 min, followed by 80% A from 0 to 18 min, then 73% A from 18 to 30 min, 0% A from 30 to 35 min which, 0% A was maintained until 47 min, 90% A
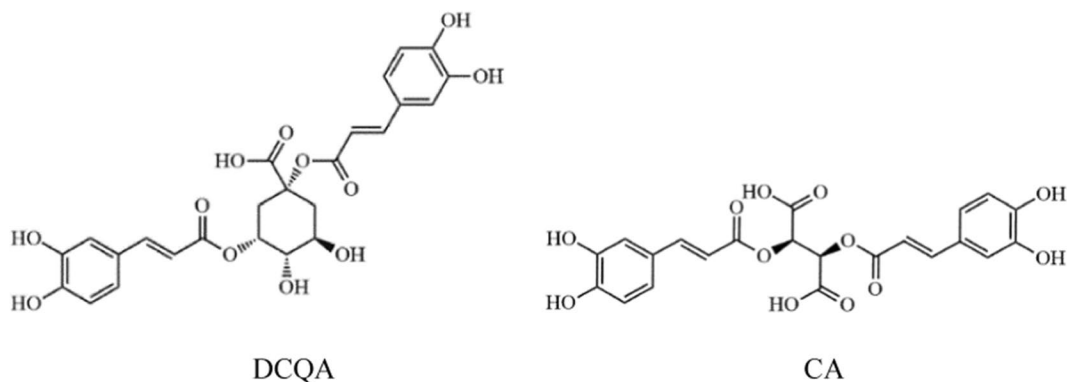
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Fig. 1 Chemical structures of 1,5-dicaffeoylquinic acid (DCQA) and chicoric acid (CA)

from 47 to 50 min, and 90% A was then maintained until 60 min.

Method validation

The specificity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and linearity of the new HPLC method were validated. The HPLC method was validated following the guidelines provided by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use [28]. The specificity of the method was tested to see whether there was any interference between the retention times and UV spectra of the standards and MST, and if the peaks indicated the presence of the same compounds. HPLC method accuracy was measured by conducting recovery tests in which sample extracts were spiked with four different standard mixtures at various concentrations and calculating the recovery (%) of the two compounds in the extract. To validate precision, three different concentrations of sample extracts were used for intra-day (repeatability) analysis, and only one concentration for the inter-day (intermediate precision) analysis in triplicate. Intra and inter-day precision were examined by calculating the percent of relative standard deviation (% RSD) of the standards on the same day and on three different days, respectively. Linearity was assessed by using five concentration levels of each standard solution (0.025–0.4 for DCQA and 0.00625–0.1 mg/mL for CA) with three injections. Using the peak areas measured at 330 nm on the chromatogram, a calibration curve was plotted against the known concentrations of the standard solutions. Linear least-squares regression was used to analyze the standard curves of DCQA and CA, and the correlation coefficient (r^2) of the regression formula were used to validate the linearity. The calibration curve of each standard compound was obtained by fitting, and the standard deviation values of the intercept (σ) and

the slope (S) were used to calculate the LOD and LOQ. The LOD is the lowest amount or concentration of analyte that can be reliably distinguished from the base line and the LOQ is the lowest amount of analyte that can be quantified with acceptable repeatability and accuracy. The standard deviation of the response and the slope of calibration were used to obtain the LOD and LOQ values. The LOD and LOQ were derived using the following formulas: LOD = 3.3 (σ/S) and LOQ = 10 (σ/S).

Calibration curves

Concentrations of the standard solutions were plotted with their respective peak areas to prepare the calibration curves. The correlation coefficient (r^2) of the calibration curve was used to determine the linearity of the fitted line, and the DCQA and CA concentrations in the samples were then calculated from the calibration curve. The calibration functions were determined based on the peak area (Y), concentration (X, mg/mL), and mean ± standard deviation (n = 3).

Results and discussion

An analytical HPLC method that quantifies DCQA and CA in MST was developed and validated. The two different compounds were analyzed by using HPLC-DAD, the results of which are shown in Fig. 2. The retention time was 16.0 min for DCQA and 19.6 min for CA, showing excellent separation. A wavelength of 330 nm provided the most effective response for quantifying DCQA and CA, including all impurities, in a single run. No impurity peaks were detected in quantifying DCQA and CA, suggesting that the method has high level of accuracy. The HPLC chromatograms in Fig. 2 showed adequate separation and the UV spectra showed good detection of DCQA and CA. Specificity of our method was evaluated by comparing the chromatograms of the standard versus the sample solution. From their
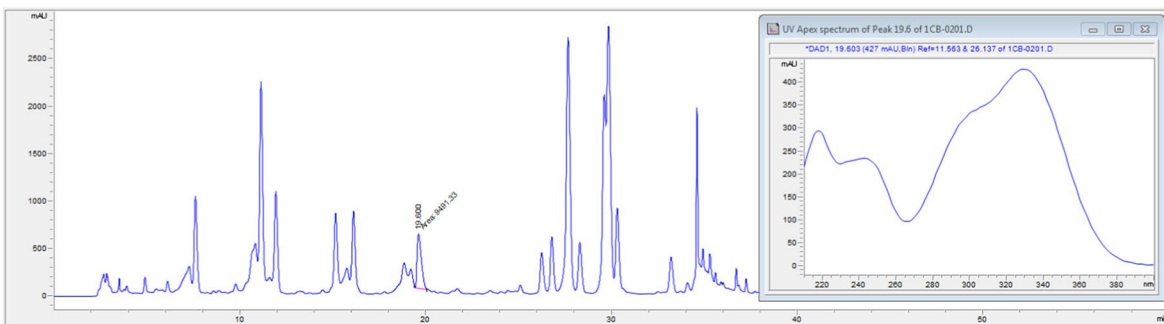
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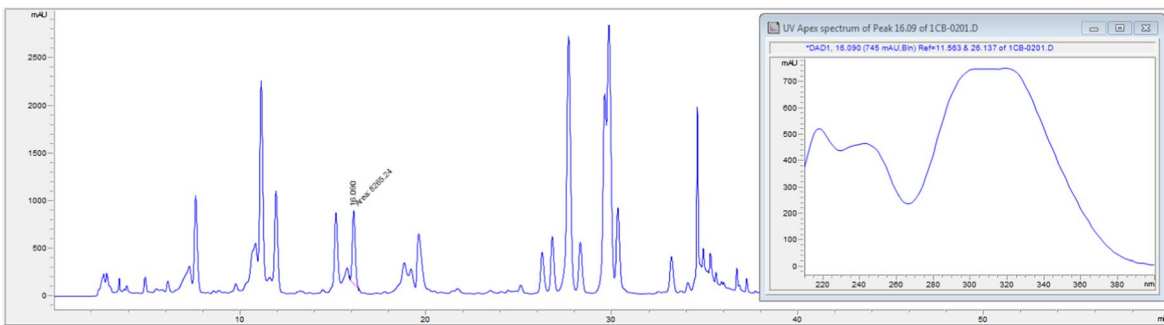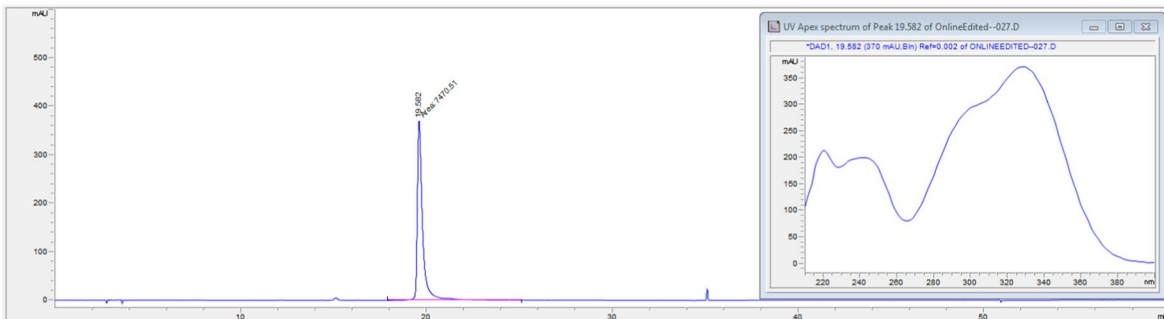
Fig. 2 HPLC chromatograms and specificity of 1,5-dicaffeoylquinic acid (**A**), 1,5-dicaffeoylquinic acid in MST (**B**), chicoric acid (**C**) and chicoric acid in MST (**D**)

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corresponding retention time and UV spectra in Fig. 2, we can see that DCQA and CA are present in MST. This indicates that our HPLC approach achieved specificity by exhibiting good resolution of chromatographic peaks associated with DCQA and CA.

Quantification parameters for DCQA and CA were examined under the HPLC conditions described above. Calibration curves for the isolated compounds were generated by mapping the peak areas obtained from HPLC analyses of solutions with various known concentrations of DCQA and CA. Linear regression was used to fit the data and generate the calibration curves, and additional statistics were used to assess the linearity. The linearity was evaluated by testing, five solutions with various concentrations of DCQA and CA over a range from 0.025–0.4 and 0.00625–0.1 mg/mL, respectively (n = 3). The regression equation was calculated by plotting the peak area (Y) versus the DCQA and CA concentrations (X) expressed in mg/mL. Correlation coefficients (r^2) of 1.0000 were obtained for both compounds indicating a strong linear relationship between peak area (Table 1) and DCQA and CA concentrations.

The LOD values obtained in our study were 5.46×10^{-3} for DCQA and 0.37×10^{-3} mg/mL for CA, whereas the LOQ values were 16.54×10^{-3} for DCQA and 1.14×10^{-3} mg/mL for CA. These values establish that the analytical method developed in this study has a high sensitivity for quantifying DCQA and CA derived from MST (Table 1).

Previous studies on the development of analytical method for quantifying DCQA and CA [29, 30] reported the r^2 values of DCQA and CA to be 0.9989 and 0.9997, respectively. LOD values were 11.21×10^{-3} mg/mL for DCQA, and 0.65×10^{-3} mg/mL for CA, and LOQ values were 13.12×10^{-3} mg/mL for DCQA, 2.17×10^{-3} mg/mL for CA. Comparing our results, the LOD and LOQ values for our method were much lower suggesting that our method can produce more accurate and reliable detection and quantification of DCQA and CA even in small amounts.

The accuracy of the analytical method was measured by spiking MST (50 mg/mL) with known concentrations of DCQA and CA standard solutions (0.125–1.0 mg). The recovery of each substance was established by comparing the detected amount and the original spiked amount of the compound. These accuracy tests were conducted in quadruplicate by using four injections, and analyses were carried out at least four times. Table 2 shows that the percent recovery ranges from 101.16–104.18% for DCQA and 97.55–108.49% for CA. All values obtained were within the acceptable range suggesting that the analytical method is highly accurate.

Precision was established by measuring the intra- and inter-day precision of the HPLC method, the results of which are shown in Tables 3 and 4. The coefficient of variance of the precision values for DCQA ranged from 0.29 to 1.00% for intra-day precision experiments and 0.42 to 0.62% for inter-day precision experiments. The variance in precision values for CA ranged from 0.15 to 1.21%

Table 1 Linearity, LOD, and LOQ of 1,5-dicaffeoylquinic acid (DCQA) and chicoric acid (CA)

Compound	t_R	Range (mg/mL)	Calibration equation[a]	$r^{2\,b}$	LOD (mg/mL)	LOQ (mg/mL)
DCQA	16.0	0.025–0.4	Y = 31569X + 41.782	1.0000	5.46×10^{-3}	16.54×10^{-3}
CA	19.6	0.00625–0.1	Y = 74846X + 21.402	1.0000	0.37×10^{-3}	1.14×10^{-3}

[a] Y = peak area, X = concentration of standards (mg/mL)

[b] r^2 = correlation coefficient for five calibration data points (n = 3)

Table 2 Accuracy for the determination of 1,5-dicaffeoylquinic acid (DCQA) and chicoric acid (CA) contents

Compound	Spiked amount (mg)	Recovery (%)				Average (%)	RSD (%)
		1st	2nd	3rd	4th		
DCQA	0.125	103.96	103.49	103.79	103.33	103.64	0.27
	0.25	103.83	103.04	103.51	103.66	103.51	0.33
	0.5	101.16	101.90	101.61	101.73	101.60	0.31
	1.0	103.96	103.52	103.90	104.18	103.89	0.27
CA	0.125	108.49	107.61	106.67	108.02	107.70	0.72
	0.25	106.51	104.16	106.48	107.01	106.04	1.20
	0.5	101.46	99.33	101.63	101.40	100.96	1.08
	1.0	97.60	99.64	99.66	97.55	98.61	1.22

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Table 3 Intra-day precision for the determination of 1,5-dicaffeoylquinic acid (DCQA) and chicoric acid (CA) contents

Compound	MST Concentration (mg/mL)	Intra-day (n = 3)	
		Measured content (mg/g)	RSD (%)
DCQA	50	1.238	1.00
	60	1.236	0.42
	70	1.233	0.29
CA	50	0.389	1.21
	60	0.389	0.15
	70	0.383	1.09

Table 4 Inter-day precision for the determination of 1,5-dicaffeoylquinic acid (DCQA) and chicoric acid (CA) contents

Compound	MST Concentration (mg/mL)	Inter-day (n = 5)	
		Measured content (mg/g)	RSD (%)
DCQA	50	1.229	0.43
		1.213	0.42
		1.242	0.62
CA	50	0.380	0.42
		0.384	0.60
		0.389	0.11

for intra-day precision experiments and 0.11 to 0.60% for inter-day precision experiments, respectively. These values are lower than 2% which meets the criteria recommended by the ICH guidelines. The results indicate excellent reliability of the analytical method for quantifying DCQA and CA derived from MST. Furthermore, when comparing the intra- and inter-day RSD mean values, a minor difference was observed between the two, but the latter values were significantly higher. It indicates that these compounds are relatively stable after being dissolved over time.

Several studies have investigated caffeic acid ester derivatives, such as DCQA and CA. These compounds are a type of hydroxycinnamic acid that has various bioactivities that include anti-oxidant [31], anti-inflammatory [32], anti-HIV [33], anti-nociceptive [34], and neuroprotective behaviors [35]. Moreover, our previous works were mainly focused on validating various marker compounds present in medicinal plant extracts [36–38].

In this study, we validated the HPLC analytical method for quantifying DCQA and CA as marker compounds for MST. To the best of our knowledge, this is the first study to establish an analytical method that utilizes HPLC-DAD to quantify these compounds in MST. The objective of our validation study was to establish a method for quantifying the compounds of interest. As described in the results section, our HPLC-DAD method proved to be an efficient and effective analytical technique that had satisfactory accuracy and precision to be used as a routine analytical method.

The consumption of bioactive compounds from plant-derived foods is a promising strategy for strengthening immunity and preventing illness. Hydroxycinnamic acids, such as DCQA and CA, have many health benefits as mentioned above. These bioactivities suggest that these compounds have great potential for treating an array of problems in the human body. This motivates additional studies into DCQA and CA as functional food ingredients, especially because our collective understanding of these compounds is still in its infancy. In this study, the analytical method was validated for quantifying DCQA and CA from MST. They were quantified from MST using a novel HPLC-DAD method. The validation parameters employed in our analytical methodology provide excellent results in terms of specificity, precision, and accuracy. The findings of our analysis support the use of the developed method for accurately identifying and quantifying DCQA and CA from MST in routine analyses and large-scale extraction processes. Moreover, our established optimized method can provide the first steps in developing health-related functional products and pharmaceuticals derived from MST.

Declarations

Competing interests
There is no conflict of interests.

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