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Validation of the analysis method for marker compound ellagic acid of ethanol extracts of *Sanguisorba officinalis* L. using HPLC-DAD

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Abstract

The validation of ellagic acid as a marker compound was attempted as an analysis method for the standardization of the extract from *Sanguisorba officinalis* L. for the development of healthy functional foods. The ellagic acid analysis using high-performance liquid chromatography with diode-array detection (HPLC-DAD) and retention time with standard compounds satisfied the stay time and specificity. Ellagic acid has confirmed limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, recovery rate, stability, and content analysis by HPLC for an 80% ethanol extract of *Sanguisorba officinalis* L. The correlation (R^2) at the concentration of ellagic acid of 9.5–95.0 μg/mL was above 0.999, showing high linearity. The LOD and LOQ were expressed as 1.5430 and 4.6757 μg/mL, respectively. The recovery rate was 99 to 110%. The precision of RSD intraday was 0.23–0.65% and interday was 0.18–2.13%. In addition, the average recovery rate of ellagic acid before and after 24 h was 98.79%, confirming a change in the content of 0.17%, and the content of ellagic acid was 22.48 μg/mL in 80% ethanol extract of *Sanguisorba officinalis* L.

Keywords: *Sanguisorba* officinalis L.; Validation; ellagic acid; HPLC-DAD.

Practical Application: Validation of the analysis method for marker compound ellagic acid of ethanol extracts of *Sanguisorba officinalis* L.

1 INTRODUCTION

The verification of ellagic acid as an index component was attempted as an analytical method in the standardization of *Sanguisorba officinalis* L. seed and *Sanguisorba officinalis* L. extract for the development of healthy functional foods. Retention time (RT) and specificity were satisfied using an analysis of ellagic acid using high-performance liquid chromatography with diode-array detection (HPLC-DAD) and comparison with standard compounds. Ellagic acid was determined for limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, recovery rate, stability, and content analysis by HPLC for 80% *Sanguisorba officinalis* L. ethanol extract. At ellagic acid concentrations of 9.5–95 μg/mL, the correlation (R^2) was higher than 0.999, showing high linearity, and the LOD and LOQ were 1.5430 and 4.6757 μg/mL, respectively. The recovery rate was 99 to 110%. As for precision, the RSD in the intraday was 0.23–0.65%, and the RSD in the interday was 0.18–2.13%. In addition, the average recovery rate of ellagic acid before and after 24 h was 98.79%, showing a change in the content of 0.17%, and the content of ellagic acid was found to be 22.48 μg/mL in the 80% ethanol extract of *Sanguisorba officinalis* L.

Cucumber grass (*Sanguisorba officinalis* L.) is a perennial herb belonging to the Rosaceae family and is widely grown in China, Japan, and Korea, with a root called Sanguisorbae radix used in herbal medicines (Rhim, 2013). Slightly curved fusiform, 10–20 cm long and 5–20 mm in diameter, it has cold properties, is non-toxic and odorless, and has an astringent, bitter, and sour taste (Kim et al., 2011). It is known to grow a lot in the mountains and fields, especially on the slopes with moderate moisture conditions, and has been used in the private sector as a treatment for burns and internal bleeding (Cheng & Cao, 1992), and recent studies have reported various physiological activities such as antioxidants, anticancer, antibacterial, and antiviral (Rhim, 2013; Kim et al., 2001). As pharmacological components of *Sanguisorba officinalis* L., flowers contain Cyanin and Chrysanthemin, leaves contain vitamin C, roots contain Ziguglycoside I and II, gallic acid, and ellagic acid, and branches contain Qurcetin and Kaempferol glycosides (Ahn et al., 2004). In addition, components such as pomolic acids, sanguinis, triterpenoids, and triterpene glycosides have been reported to have been isolated from *Sanguisorba officinalis* L. (Cheng & Cao, 1992; Liu et al., 2005; Mimaki et al., 2001; Yokozawa et al., 2002). Among them, ellagic acid is a polyphenol compound known for its antioxidant, anti-fibrotic, anti-aging, anti-fibrotic, and cholesterol-suppressing effects. As a result of a study on certain cancers, ellagic acid was found to have anti-cancer effects on prostate cancer cells, cervical cancer cells, colorectal cancer cells, oral cancer cells, etc. Also, it has been reported to exhibit anti-cancer and anti-apoptotic effects in leukemia and

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leukemia cells (Lee et al., 2021; Mertens-Talcott & Percival, 2005; Saleem et al., 2002; Seeram et al., 2005). Although food materials derived from natural products have secured efficacy and safety through long experiences in consumption, there are differences in qualities depending on the soil, re-inhalation time, production area, and cultivation conditions. In order to develop and produce healthy functional food, standardization and normalization occupy very important parts, and in order to scientifically prove the functionality and safety of food materials derived from natural products, the quality control method through the content of index ingredients is useful (Ministry of Food and Drug Safety, 2019a).

In this study, an extract of *Sanguisorba officinalis* L. was prepared and analyzed. Then, ellagic acid was selected as an indicator component, and an analysis method for ellagic acid was established. By validating the effectiveness of this study, we intend to provide the basis for the development of healthy functional foods.

2 MATERIALS AND METHODS

2.1 Materials

Sanguisorba officinalis L. (Sanguisorba officinalis Linne.) sample was purchased from Geoherb (Yangju, Gyenonggi-do) at 600 g, and it was identified by Professor Hyunjeong Kim of the College of Pharmacy, Mokpo University.

2.2 Reagents and instruments

Acetonitrile, a mobile phase used for extraction and separation of *Sanguisorba officinalis* L., was an HPLC-grade solvent and was purchased from Daejeong Chemical (Siheung, Korea). The column used for HPLC analysis was C18 (YMC ODS-A 5 μm, 4.6 × 250 mm), and the mobile phases acetonitrile and methanol were purchased from Daejeong Chemical (Siheung, Korea). The equipment used for the test was a grinder (NFM-3561SN, NUC Co., Daegu, Korea) and a reflux extractor (MS-DM, MISUNG Co., Seoul, Korea), and the equipment used for HPLC-DAD analysis was an autosampler and an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a DAD.

2.3 Preparation of Sanguisorba officinalis L. extract

The *Sanguisorba officinalis* L. used in this study was ground using a grinder (NFM-3561SN, NUC Co., Daegu, Korea). A 100 mL of each 80% ethanol was added to 10 g of pulverized *Sanguisorba officinalis* L. samples, extracted under reflux at 100°C for 1 h, and a filtrate was obtained using 185 mm Whatman Filter paper (GE Healthcare Life Sciences, United States). The filtrate was stored for the experiment in refrigerated storage in a refrigerated reagent cabinet at 4°C.

2.4 Separation of ellagic acid as an indicator component

Ellagic acid was extracted under reflux by repeating 450 g of dried *Sanguisorba officinalis* L. with 4 L of 80% EtOH three times. This was concentrated under reduced pressure using a vacuum concentrator to obtain 84 g. For Perp-HPLC, Atlantis® Prep T3 OBD™ (5 μm, 19 × 250 mm) columns (Milford, MA, USA) were used in Waters™ (Milford, MA, USA) 600E multisolvent delivery system. And the mobile phase was 90:10 (5 min) → 40:60 (20 min) → 20:80 (10 min) with third distilled water (solvent A) containing 0.1% formic acid and 100% acetonitrile (solvent B) → 90:10 (5 min) → 90:10 (15 min) conditions. The amorphous compound 1 was separated at a wavelength of 254 nm at a flow rate of 10 mL/min. Compound 1 is a triple quadrupole mass spectrometer, LC/MS-8050 (Shimadzu, Kyoto, Japan), linked to 1H-NMR, 13C-NMR spectrum, and LC-30A (Shimadzu, Kyoto, Japan) liquid chromatography. As a result of analysis using spectroscopy (MS/MS), it was confirmed that ellagic acid was compared with the existing literature (Figure 1, Table 1) (Da Silva et al., 2008).

2.5 Preparation of test solutions and standard solutions

As a test solution, *Sanguisorba officinalis* L. extracted in 80% ethanol was diluted 5 times in methanol and filtered using a syringe filter (0.45 μm, Hyundai Micro Co., Ltd., Korea). To prepare a standard solution, ellagic acid (Sigma-Aldrich Co., Louis, MO, USA) was dissolved in 100% methanol at a concentration of 0.25 mg/mL and diluted to concentrations of 0.01,

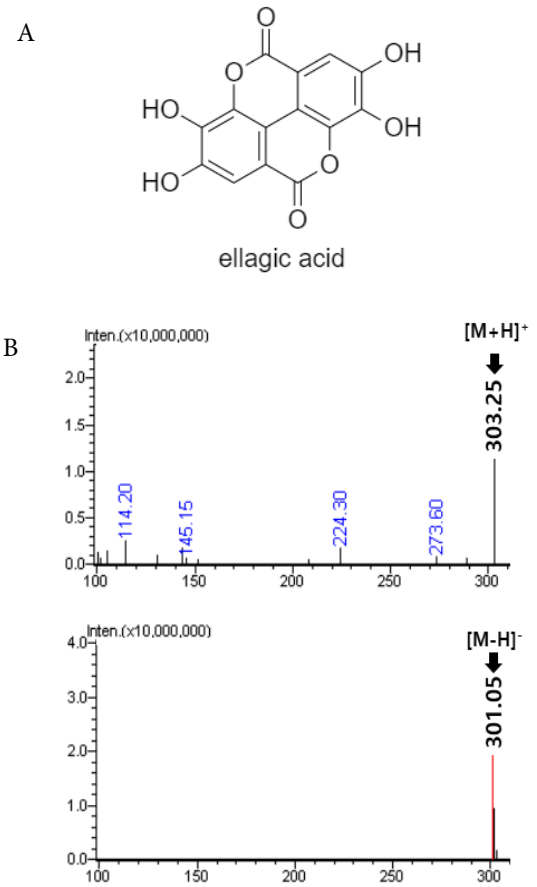

Figure 1. Structure of ellagic acid isolated and electrospray ionization (ESI) mass spectroscopy analysis from *Sanguisorba officinalis* L. (A) Ellagic acid, (B) positive mode, and negative mode.

Table 1. Conditions of HPLC for Sanguisorba officinalis Linne. and ellagic acid analysis.

Parameters		Conditions	
Column		SHISEIDO (UG120 5 μm, 4.6 mm I.D. × 250 mm)	
Flow rate		1 mL/min	
Injection volume		20 μL	
UV detection		254 nm	
Run time		40 min	
Gradient condition	Time (min)	A (%)	B (%)
	0	90	10
	5	90	10
	25	40	60
	30	20	80
	35	90	10
	40	90	10

0.02, 0.04, 0.06, 0.08, and 0.1 mg/mL. Then, it was filtered with a syringe filter (0.45 μm, Hyundai Micro Co., Korea) to prepare an ellagic acid standard solution.

2.6 HPLC analysis

Autosamplers and HPLC using Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a DAD detector were used for the analysis of oilseed milk extract. For the analytical column, SHISEIDO (UG120 5 μm, 4.6 × 250 mm, Osaka soda, Japan) was used, and for the HPLC mobile phase, third distilled water containing 0.1% formic acid (solvent A) and 100% acetonitrile (solvent B) were used. Mobile phase A:B ratio of 90:10 (5 min) → 40:60 (25 min) → 20:80 (5 min) → 90:10 (5 min) at a flow rate of 1 mL/min at 254 nm wavelength, and the ellagic acid content was measured by injecting 20 μL of the sample (Table 1).

2.7 Method validation

Method validation is a process to verify the validity of the corresponding method, and it proceeds by checking various parameters. In this study, specificity, linearity, LOD, LOQ, accuracy, and precision were decided according to the pharmaceutical validation guidelines notified by the Ministry of Food and Drug Safety The effectiveness of the analysis method was then verified (Ministry of Food and Drug Safety, 2019a).

2.8 Specificity

After analyzing the ellagic acid standard solution and the 80% ethanol extract of *Sanguisorba officinalis* L. by HPLC, they were compared by the RT on the chromatogram to confirm whether the ellagic acid peak could be separated.

2.9 Linearity, detection limit, and quantitation limit

The ellagic acid standard solution diluted stepwise was analyzed by HPLC, a calibration curve was prepared for the area value for the concentration, and the R^2 value was confirmed. The LOD and LOQ for each component were calculated based on the standard deviation and the slope of the calibration curve using the chromatogram of the standard solution (Equations 1 and 2).

$$LOD = 3.3 \times (\text{standard deviation/slope of calibration curve}) \quad (1)$$

$$LOQ = 10 \times (\text{standard deviation/slope of calibration curve}) \quad (2)$$

2.10 Accuracy and precision

Accuracy refers to the degree of proximity of a measured value to a known true value. The recovery was evaluated by the method of obtaining the recovery rate, and the index component of each sample was added in three different concentrations, and then the analysis was repeated three times. The recovery rate was calculated by adding an ellagic acid standard of known concentration (9.5, 57, or 95 μg/mL) to the sample to prepare a test solution and then converting the amount recovered by analysis into a percentage. Precision refers to the degree of dispersion of measured values when a sample is analyzed several times and is evaluated as intraday and interday.

2.11 Content evaluation

After analyzing the *Sanguisorba officinalis* L. extracted in 80% ethanol by the same analysis method, the average and standard deviation of the peak area values of each index component were obtained, and then the content was calculated by substituting it into the regression equation obtained from the linearity evaluation.

2.12 Statistical processing

All experimental results presented in this experiment are expressed as mean ± standard deviation (mean ± SD) measured three times. Statistical processing was performed using the Statistical Package for the Social Sciences (SPSS), and one-way analysis of variance (ANOVA) was performed for statistical significance tests between each experimental group. In case of any significance, the Turkey's test was conducted as a post-test, and when $p < 0.05$, it was determined that there was significance. As for the correlation, it was marked with significance using Pearson's correlation coefficient.

3 RESULTS AND DISCUSSION

3.1 Identification of indicator components and optimization of analysis methods

As a result of confirming the molecular weight of the *Sanguisorba officinalis* L. extract using ESI mass spectrometry, ion peaks were observed in positive mode [M+H] + m/z 303.25 and negative mode [M-H] - m/z 301.05 (Figure 1). As a result of comparing the mass information of the main peak of the *Sanguisorba officinalis* L. extract with the existing literature by Lee et al. (2005) and the HPLC analysis conditions of the standard product, it was confirmed as the structure of the ellagic acid compound.

Experiments were performed in various solvents and wavelength conditions to establish an analysis method for the ellagic acid contained in *Sanguisorba officinalis* L. In the case of water solvents, better resolution was obtained by using 0.1% formic acid, and acetonitrile was better than methanol as an organic solvent. At the measurement wavelength, more ellagic acid was absorbed at 254 nm than at 270 nm. In addition, when comparing the ellagic acid standard product and the *Sanguisorba officinalis* L. extract in Figure 2, it was confirmed that the RT of the main peak of the standard product and the extract was consistent.

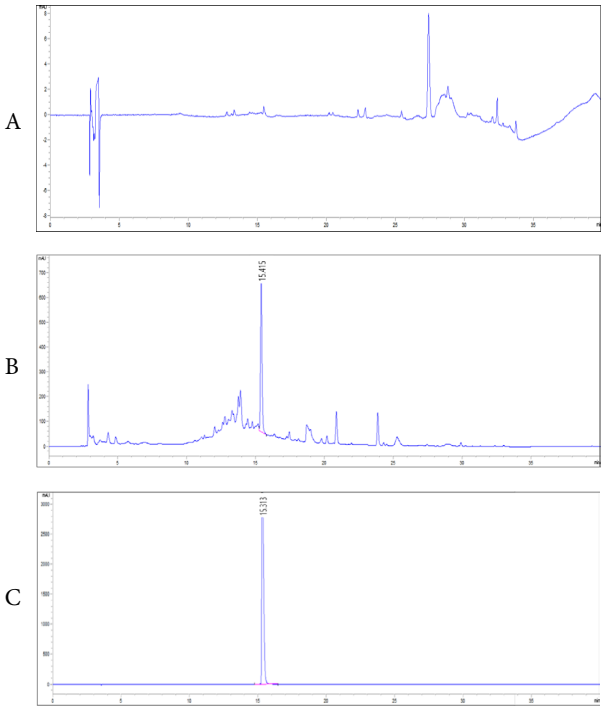

Figure 2. HPLC analysis pattern of *Sanguisorba officinalis* L. (A) HPLC analysis of methanol. (B) 80% EtOH extract of *Sanguisorba officinalis* L. (C) HPLC analysis of ellagic acid.

3.2 Specificity

As a result of confirming the specificity, the peak RT of the standard solution and the peak time of the test solution coincided with 15.31 min as a result of comparing the chromatograms of the standard solution and the test solution of the *Sanguisorba officinalis* L. extract to confirm whether the peaks of the contained standard substances were separated. In addition, it was confirmed that the solvent used through the solvent (methanol) chromatogram did not affect the standard material (Figure 2).

3.3 Linearity, detection limit, and quantitation limit

The ellagic acid standard solution was gradually diluted to a concentration of 9.5–95 μg/mL in order to check whether a linear measurement can be obtained for the amount of ellagic acid in a certain concentration range. Linearity was evaluated by writing HPLC measurements as a calibration curve. At a concentration of 9.5–95 μg/mL, the standard calibration curve showed high linearity with a correlation (R^2) of 0.999 or higher. As a result of the calculation using the y-intercept value, the detection limit of ellagic acid was 1.5430 μg/mL and the quantitation limit was 4.6757 μg/mL (Table 2).

3.4 Accuracy and recovery measurement

As a result of performing three sections of each indicator component to check the accuracy, the recovery rate of ellagic acid was 99.56 ± 2.12% at a concentration of 9.5 μg/mL, 104.26 ± 1.29% at a concentration of 57.0 μg/mL, and 101.63 ± 0.19% at a concentration of 95 μg/mL. It showed a high recovery rate in the range of ± 0.19%, and the relative standard deviation (RSD) was 0.18–2.13% (Table 3). The recovery rate of ellagic acid was 99–110%, which was less than 10% of the guideline standard

Table 2. Calibration curve, linearity, limit of detection (LOD), and limit of quantitation (LOQ) of ellagic acid.

Compound	Concentration (μg/mL)	Regression equation	R^2	LOD (μg/mL)	LOQ (μg/mL)
Ellagic acid	9.5–95.0	y = 234.64x - 553.12 y = 231.63x - 459.92 y = 234.96x - 677.75	0.9997 0.9996 0.9991	1.5430	4.6757

Table 3. Accuracy and precision of ellagic acid.

Compound	Spiked amount (μg/mL)	Measured amount (μg/mL)	RSD[1] (%)	Recovery[2] (%)	Recovery average
Ellagic acid	9.5	9.46 ± 0.20	2.13	101.08 97.14 100.47	99.56
	57.0	59.44 ± 0.74	1.24	104.91 105.14 102.80	104.26
	95.0	96.55 ± 0.18	0.18	101.84 101.58 101.48	101.63

[1]Relative standard deviation; [2]recovery (%) = (amount found-original amount)/amount spiked × 100%.

Table 4A. Precision of ellagic acid: interday and intraday precision of ellagic acid.

Compound	Concentration (μg/mL)	Interday		Intraday	
		Mean ± SD[1] (μg/mL)	RSD[2] (%)	Mean ± SD (μg/mL)	RSD (%)
Ellagic acid	9.5	8.84 ± 0.06	0.65	9.46 ± 0.20	2.13
	57.0	57.22 ± 0.26	0.46	59.44 ± 0.74	1.24
	95.0	94.01 ± 0.22	0.23	96.55 ± 0.18	0.18

[1]Value are mean ± standard deviation (n = 3); [2]relative standard deviation.

Table 4B. Precision of ellagic acid: repeatability of ellagic acid.

Compound	Concentration (μg/mL)	Mean ± SD[1]	RSD (%)
Ellagic acid	57.0	57.55 ± 0.17	0.29

[1]Value are mean ± standard deviation (n = 6).

value that has been provided by the Ministry of Food and Drug Safety. Also, the RSD value was 4% or less, and accuracy showed high reproducibility within 4%.

3.5 Precision measurement

The precision was evaluated by repeating the concentration of three sections at 9.5, 57, and 95 μg/mL in the interday and intraday analyses three times. The RSD was in the range of 0.23–0.65% and 0.18–2.13%, as shown in Table 4A, and was less than the standard value of the Ministry of Food and Drug Safety guidelines' standard value of 4% (Ministry of Food and Drug Safety, 2019a). In addition, repeatability was evaluated by repeating the analysis of each of the index components at a specific concentration six times. Also, as shown in Table 4B, the RSD of ellagic acid was 0.29%, which was less than the guidelines' standard value of 4%.

3.6 Stability

To determine the chemical stability of ellagic acid, an indicator component of the *Sanguisorba officinalis* L. extract, it was stored at room temperature for 24 h at a final concentration of 95 μg/mL. Within that, content change was measured. As a result, the average recovery rate of ellagic acid before and after 24 h was 98.79%, indicating a change in the content of 0.17%. This rate was within the Ministry of Food and Drug Safety's guideline of 2%. With the results, the chemical stability of ellagic acid for 24 h has been determined.

3.7 Analysis of ellagic acid content in 80% ethanol extract using HPLC

To establish and conduct verification of the HPLC analysis method, hyperin, gallic acid, and ellagic acid were set as indicator substances for quality control of *Sanguisorba officinalis* L. (Sha et al., 1998; Zhang et al., 2009).

4 CONCLUSION

In this study, standardization of the ellagic acid component contained in *Sanguisorba officinalis* L. was performed using HPLC-DAD. With that, validation of ellagic acid's content analysis and analysis method was performed, and its effectiveness was verified. The correlation (R^2) value of the calibration curve for the ellagic acid standard material was 0.999 or higher, showing very high linearity, the detection limit was 1.5430 μg/mL, and the quantitation limit was 4.6757 μg/mL. The recovery rate of ellagic acid was 99.56 ± 2.12% at the concentration of 9.5 μg/mL, 104.26 ± 1.29% at the concentration of 57 μg/mL, and 101.63 ± 0.19% at the concentration of 95 μg/mL. The RSD at day was 0.23–0.65%, and the interday RSD value was 0.18–2.13%. The average recovery rate of ellagic acid before and after 24 h was 98.79%, indicating a change in the content of 0.17%. With that, an indicator component of *Sanguisorba officinalis* L., ellagic acid's analysis method, was verified as an appropriate testing method. In addition, it was determined that the content of ellagic acid in the 80% ethanol extract of *Sanguisorba officinalis* L. was 22.48 mg/g.

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