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Validation and Standardization of the Content of Three DCQAs (Di-caffeoylquinic acid) in Korean Ligularia fischeri by Region of Origin

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Article

Keywords: Ligularia fischeri, 3,4-DCQA, 3,5-DCQA, 4,5-DCQA

Posted Date: May 14th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-6576668/v1

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Scientific Reports on August 21st, 2025.
See the published version at https://doi.org/10.1038/s41598-025-10636-4.

Abstract

Ligularia fischeri is a perennial plant in the Asteraceae family, native to Japan, China, Eastern Siberia, and Korea. In general, it is said to be good for anti-aging, bronchial diseases, anti-cancer, and constipation. In this study, we obtained five regions (Hamyang, Hoengseong, Jeongseon, Nonsan, and Yangsan) of the Korean cultivated *Ligularia fischeri* and analyzed its compounds by HPLC-MS/MS and chromatograms with standards to confirm the absence of interfering substances and confirmed that it contains three types of DCQA (Di-caffeoylquinic acid): 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA. The linearity, precision, limit of quantification (LOQ) and limit of detection (LOD), and recovery were then measured by quantitative analysis to confirm the content of the three DCQAs. The results of the analysis of three types of DCQA content in *Ligularia fischeri* obtained from five regions (Hamyang, Hoengseong, Jeongseon, Nonsan, and Yangsan) using three different solvent concentrations (100% DW, 30% EtOH, and 50% EtOH) are as follows (5 g of raw material/50 mL of extraction solvent). In 100% distilled water, 3,4-DCQA was highest in Nonsan (9.29 mg/g), 3,5-DCQA was highest in Hoengseong (5.32 mg/g), and 4,5-DCQA was highest in Nonsan (3.38 mg/g). In 30% ethanol, 3,4-DCQA was highest in Nonsan (19.15 mg/g), 3,5-DCQA was highest in Hoengseong (9.98 mg/g), and 4,5-DCQA was highest in Nonsan (11.79 mg/g). In 50% ethanol, 3,4-DCQA was highest in Nonsan (21.52 mg/g), 3,5-DCQA was highest in Hoengseong (17.06 mg/g), and 4,5-DCQA was highest in Nonsan (11.25 mg/g).

1. Introduction

Ligularia fischeri is a plant of the Asteraceae family native to the high mountain regions of Northeast Asia, particularly Korea, China, and Japan. Its roots and leaves have been widely used traditionally for medicinal and edible purposes [1]. Recent studies have revealed that *Ligularia fischeri contains* various bioactive compounds and is attracting attention for its physiological effects, such as antioxidant, anti-inflammatory, and anti-cancer properties. [2].

The main bioactive compounds of *Ligularia fischeri* include polyphenolic compounds, flavonoids, terpenoids, and sesquiterpenoids, which have been also reported to contain various bioactive effects such as antioxidant, anti-inflammatory, and antibacterial activities [3–5]. In particular, polyphenols, flavonoids, and chlorogenic acid are known to have positive effects on the prevention of chronic diseases [6]. Additionally, some studies suggest that *Ligularia fischeri* extract may exhibit physiological functions such as inhibiting cancer cell proliferation, lowering blood sugar levels, protecting the liver, and treating rheumatoid arthritis [7–10]. However, scientific research on *Ligularia fischeri* is still in its early stages, and standardized methods are required to ensure reliable and reproducible experimental results, particularly in analytical procedures.

A search on PubMed using the keywords "*Ligularia* fischeri" and "DCQA" found a total of three papers. Among these, only one paper actually addressed the separation and identification of the compounds. Specifically, Shang et al. (2010) analyzed *Ligularia fischeri* collected from Daegwallyeong, Gangwon-do, South Korea, and identified three types of DCQA (dicaffeoylquinic acids), with 4,5-DCQA showing the

highest content. The three types of DCQA identified were 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA, with 4,5-DCQA having the highest content [11]. Kuroda et al. (2016) analyzed samples of *Ligularia fischeri* from two species in Sichuan Province and four species in Chongqing, totaling six species, and found differences in the distribution and composition of benzo[a]pyranoquinones and eremophilan compounds among the samples [12]. Additionally, according to a study conducted in Korea, leaf extracts of *Ligularia fischeri* collected in summer (June) had higher polyphenol and flavonoid content and stronger antioxidant and antibacterial activity compared to samples collected in winter (December) [13]. Thus, to date, there has been limited research on regional differences in the content of DCQA in Korea.

Therefore, in this study, five regions (Hamyang, Hoengseong, Jeongseon, Nonsan, and Yangsan) where *Ligularia fischeri* is cultivated in Korea and analyzed the compounds using HPLC-MS/MS and chromatograms, confirming the absence of interfering compounds. As a result, seven main peaks were identified, including three DCQAs (dicaffeoylquinic acid): 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA. After that, the content differences of three types of DCQAs (3,4-DCQA, 3,5-DCQA, and 4,5-DCQA) were confirmed using standard compounds, and linearity, precision, limit of quantification (LOD), limit of detection (LOQ), and recovery rate were measured for quantitative analysis. This study aims to scientifically confirm the potential value of *Ligularia fischeri* as a functional food ingredient, as well as regional and solvent-specific differences in content and validation methods, thereby providing foundational data for future industrial applications.

2. Materials and Methods

2.1. Chemicals and Reagents

The chemical standards of 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-MS/MS grade solvents (acetonitrile and triple deionized water) and methanol were obtained from Duksan Pure Chemical Co. Ltd. (Dongdaemun-gu, Seoul, Korea).

2.2. Preparation of Plant Materials

Ligularia fischeri was collected from Hamyang (Code No. 00951A) and Yangsan (Code No. 00952A), Gyeongsangnam-do; Hoengseong (Code No. 00953A) and Jeongseon (Code No. 00954A), Gangwon-do; and Nonsan (Code No. 00955A), Chungcheongnam-do. After obtaining permission from each local farm to collect and provided by Kick the Hurdle Inc. The samples were stored in the Animal Bio Resources Bank, Korea, a nationally designated research materials bank, and each source was assigned a code number (Fig. 1). The leaves and stems of the plants provided were washed with water, finely chopped, and dried in a drying oven at 56°C for 24 hours. They were then placed in sealed polyethylene bags containing silica gel and stored at -20°C until use.

2.3. Preparation of Sample and Standard Solutions

The preparation of plants and standards solution for LC-MS was performed using a modified technique [14]. *Ligularia fischeri* (5g) was extracted for 72 hours at 60°C in a water bath using three solvents: 100% DW, 30% EtOH, and 50% EtOH, each in 50 mL. The extract was centrifuged at 3000 rpm for 10 minutes. After centrifugation, the supernatant was filtered through filter paper (Whatman, Qualitative, Circles, 110mm Dia, Cat No. 1001 – 110). A rotary evaporator (N-1110, Eyela, Tokyo, Japan) was rotated at 100 rpm and used under reduced pressure at 45°C to completely remove any residual ethanol. Subsequently, the sample was freeze-dried to obtain a powder. Prepared *Ligularia fischeri* sample powder and standard of 3,4-DCQA, 3,5-DCQA, 4,5-DCQA were prepared at a concentration of 1 mg/mL in methanol. All samples were filtered through a 0.45 μm PVDF syringe filter prior to analysis and prepared in the same method as above prior to injection for HPLC-MS/MS analysis.

2.4. HPLC-MS/MS Instrumentation and Analysis

HPLC and LC-MS/MS was performed on a Shimadzu Nexera Lite LC-40D HPLC system (Shimadzu, Corp., Kyoto, Japan) and Ultra Quadrupole Time of flight LC/MS/MS System (X500R) operated in positive ion mode (spray voltage set at − 4.5 kV). The solvent used was DW and Acetonitrile containing 0.1% formic acid, a gradient system was used at a flow rate of 0.5 mL/min for analysis, and a Prontosil C18 column (length, 250 mm; inner diameter, 4.6 mm; particle size, 5 μm; Phenomenex Co., Ltd., California, USA, Biochoff Chromatography) was used. The solvent conditions used in the mobile phases were 0–10 min at 10–15% B, 10–20 min at 20% B, 20–30 min at 25%, 30–40 min at 40%, 40–50 min at 70%, 50–60 min at 95%, and 60–70 min at 95%. The analysis was performed at a wavelength of 284 nm, and temperature of 35°C.

The mass spectrometry conditions for qualitative analysis of peaks identified by HPLC were performed in positive mode using electrospray ionization (ESI) and multiscan between m/z 100–2000, with a desolvation temperature set at 500°C, spray voltage at 5500 V, ion source gas at 50 psi, curtain gas at 30 psi, and declustering potential (DP) at 80 V; MS/MS spectra were set with a collision energy of 35 ± 15V. The obtained ion chromatogram data were generated using SCIEX OS software (3.0.0).

2.5. Quantification and Validation of the Analytical Method

The analytical method validation was conducted in accordance with the ICH and U.S. Food and Drug Administration bioanalytical method validation guidelines [15, 16]. The quantification of compounds detected in *Ligularia fischeri* extract was performed at 284 nm, and the concentrations of the three DCQAs were calculated using the following formula: (Calibration curve results (ug/mL) × Final volume (mL) × dilution factor × standard solution purity) / (sample weight (g) × 1000 (ug/mg)). Specificity, linearity, detection limit (LOD), quantification limit (LOQ), precision, and recovery rate were measured to evaluate the performance of this method.

2.5.1. Specificity

The specificity of the chromatogram and PDA spectrum patterns of *Ligularia fischeri* extract was confirmed as follows. It was confirmed by comparison with standard solutions based on specificity

derived from the selective quantification of various compounds in complex mixtures.

2.5.2. Linearity and Range

Standard solutions of 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA at concentrations of 100, 250, 500, 750, and 1000 μg/mL (n = 5) were prepared and analyzed to confirm the linearity of the calibration curve. The standard solution analysis was repeated five times for each concentration. The eluted peaks were subjected to linear regression (n = 5) and measured as the ratio of peak area to analyte concentration. The linearity of the association was assessed using the correlation coefficients (R^2) that were computed from the calibration curves.

2.5.3. LOD and LOQ

The minimum quantity of 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA that may be found in the *Ligularia fischeri* extract sample is known as the LOD. The LOQ is the minimum quantity that can be accurately and quantitatively suitable for precision. The standard deviations of the y-intercepts and slopes of the calibration curves were used to determine the LOD and LOQ values. The calibration curve through standard deviation and slope was used to verify linearity.

2.5.4. Precision and Recovery

The precision of the validation method was determined using standard solutions of 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA by inter-day and intra-day at five different concentrations (100, 250, 500, 750, 1000 μg/mL). Intra-day was five times a day to analyze precision, and tests were run five times a day for three days in a row to analyze inter-day precision. To determine precision, the relative standard deviation (RSD) was calculated. The accuracy of the suggested approach was verified by a recovery analysis. Recovery was assessed after measuring the *Ligularia fischeri* extract with five concentrations of 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA. Each analysis was repeated five times. Recovery was calculated based on the sample peak area value, standard peak area value, and peak area value.

3. Results

3.1. Separation and characteristic analysis of phenolic compounds in *Ligularia fischeri* extract

The qualitative and quantitative analysis of compounds contained in *Ligularia fischeri* by region (Hamyang, Hoengseong, Jeongseon, Nonsan, and Yangsan) and extraction solvent (100% DW, 30% EtOH, and 50% EtOH) was performed using HPLC-MS/MS. A total of seven peaks were identified by HPLC retention time and UV-vis spectra (Fig. 1). The seven compounds obtained at a wavelength of 284 nm were identified as chlorogenic acid [17], Caffeic acid [18], Hyperin [19], 3,4-Dicaffeoylquinic acid [20], 3,5-Dicaffeoylquinic acid, 4,5- Dicaffeoylquinic acid [21], and an unknown peak. Table 1 provides the analysis results of mass spectrometry data based on existing literature. This is followed by the predicted fragmentation of compounds obtained through LC-MS/MS (Fig. 2).

Table 1
The HPLC-MS/MS data of phenolic compounds from *Ligularia fischeri* extract with different origins.

Peak No.	Rt(min)	Formula	Compound	UV max	[M + H]$^+$	MS/MS	Reference
1	16.95	$C_{16}H_{18}O_9$	Chlorogenic acid	325, 250	355	181 ($C_9H_8O_4$) [M + H-$C_7H_{10}O_5$]$^+$ 163 ($C_9H_6O_3$) [M + H-$C_7H_{10}O_5$-H_2O]$^+$ 135 ($C_8H_6O_2$) [M + H-$C_7H_{12}O_6$-CO]$^+$	[17]
2	21.16	$C_9H_8O_4$	Caffeic acid	330	181	163 ($C_9H_6O_3$) [M + H-H_2O]$^+$ 145 ($C_9H_5O_2^-$) [M + H-H_2O-H_2O] 135 ($C_8H_6O_2$) [M + H-COOH]$^+$ 117 ($C_8H_5O^-$) [M + H-COOH-H_2O]	[18]
3	31.62	$C_{21}H_{20}O_{12}$	Hyperin	355, 255	465	303 ($C_{15}H_{10}O_7$) [M + H-$C_6H_{10}O_5$]$^+$ 153 ($C_8H_8O_3$) [M + H-$C_6H_{10}O_5$-RDA] 109 (C_7H_8O) [M + H- $C_6H_{10}O_5$-RDA-H_2O]	[19]

Peak No.	Rt(min)	Formula	Compound	UV max	[M + H]$^+$	MS/MS	Reference
4	33.18	$C_{25}H_{24}O_{12}$	3,4-Dicaffeoylquinic acid	325, 290	517	499 ($C_{25}H_{22}O_{11}$) [M + H-H_2O]$^+$ 337 ($C_{16}H_{16}O_8$) [M + H-H_2O-$C_9H_6O_3$]$^+$ 193 ($C_7H_{12}O_6$) [M + H-$C_{18}H_{12}O_6$]$^+$ 175 ($C_7H_{10}O_5$) [M + H-$C_9H_8O_4$-$C_9H_6O_3$]$^+$	[20]
5	35.01	$C_{25}H_{24}O_{12}$	3,5-Dicaffeoylquinic acid	330, 290	517	355 ($C_{16}H_{18}O_9$) [M + H-$C_9H_6O_3$]$^+$ 193 ($C_7H_{12}O_6$) [M + H-$C_9H_6O_3$-$C_9H_6O_3$]$^+$ 181 ($C_9H_8O_4$) [M + H-$C_9H_6O_3$-$C_7H_{10}O_5$]$^+$ 137 ($C_8H_8O_2$) [M + H-$C_{16}H_{16}O_8$-O]$^+$	[21]
6	38.31	$C_{25}H_{24}O_{12}$	4,5-Dicaffeoylquinic acid	330, 290	517	355 ($C_{16}H_{18}O_9$) [M + H-$C_9H_6O_3$] 193 ($C_7H_{12}O_6$) [M + H- $C_9H_6O_3$-$C_9H_6O_3$] 175 ($C_7H_{10}O_5$) [M + H-$C_{18}H_{18}O_6$-H_2O]	[21]

Peak No.	Rt(min)	Formula	Compound	UV max	[M + H]$^+$	MS/MS	Reference
7	42.84	-	Unknown	-	-	-	-

3.2. Optimization of HPLC–MS/MS Condition

Standard solutions of 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA (100 µg/mL) were used to optimize the HPLC-MS/MS conditions. A Pronto SIL column (250 × 4.6 mm, 5 µm, 120-5-C18 SH, Bischoff Chromatography, Leonberg, Germany) was used to identify detectable phenolic compounds, and the system was set up using a gradient method with a column temperature of 35°C and a mobile phase consisting of water and acetonitrile, each containing 0.01% formic acid. Based on a literature review, the maximum absorbance of chlorogenic acid was found to be around 290 nm, so the UV wavelength range of 200–400 nm was set to detect its derivatives, 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA [22].

For the identification of compounds in the sample, positive ESI conditions were applied because ion fragments showed higher safety than negative mode. Subsequently, when the sample was prepared using methanol, it exhibited sufficiently excellent peak shapes.

3.3 Method of Validation

3.3.1 Specificity

Through the chromatograms of the standard solution and sample solution, we confirmed that the peaks in the *Ligularia fischeri* extract were clearly separated and that there was no interference from other compounds. Specificity was confirmed by verifying that the retention time (RT) patterns between the standard solution and sample solution matched and that when the standard solutions were mixed, each standard solution was detected at different times (Fig. 3).

3.3.2. Linearity, Range and LOD, and LOQ

Linearity verification was performed five times at each concentration, and the linearity of 3,4-DCQA ($R^2 \geq 0.9998$), 3,5-DCQA ($R^2 \geq 0.9998$), and 4,5-DCQA ($R^2 \geq 0.9997$) were confirmed. Subsequently, we obtained the linear expressions for 3,4-DCQA (y = 4390.5x − 34206), 3,5-DCQA (y = 5093.5x − 106812), and 4,5-DCQA (y = 5549.9x − 143191). The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined, with the LOD and LOQ for 3,4-DCQA being 0.795 mg/L and 2.386 mg/L, respectively. The LOD and LOQ for 3,5-DCQA were 0.637 mg/L and 1.910 mg/L, respectively. The LOD and LOQ for 4,5-DCQA were 0.589 mg/L and 1.766 mg/L, respectively (Table 2).

Table 2
Calibration curve data for the quantification of 3 type of DCQAs.

Compound	Slopes of Calibration	Correlation Coefficient (R^2)	LOD (mg/L)	LOQ (mg/L)
3,4-DCQA	4390.5	0.9998	0.795	2.386
3,5-DCQA	5093.5	0.9998	0.637	1.910
4,5-DCQA	5549.9	0.9997	0.589	1.766
LOD: Limit of Detection; LOQ: Limit of Quantitation, (= 5)				

3.3.3 Precision

Table 3 shows the intraday precision (repeatability) values of the standard solutions of 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA, which were measured under the same conditions within a single day. The tests were conducted five times within a concentration range of 100–1000 μg/mL, and the relative standard deviation (RSD) values for each standard solution concentration were within 0.25%. Table 4 shows the interday precision (intermediate precision) values of 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA in *Ligularia fischeri* extract obtained from five regions, using the same equipment under the same conditions but on different days. This confirmed the reliability and reproducibility of the HPLC-PDA analysis method.

Table 3

Intra-day precision for 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA in Ligularia fischeri extract using the HPLC-PDA method.

Compound	Nominal Concentration (ug/mL)	Intraday	
		Observed Concentration	Precision RSD (%)
3,4-DCQA	100	98.0465 ± 0.2410	0.2458
	250	255.2384 ± 0.6584	0.2579
	500	498.5773 ± 0.8622	0.1729
	750	744.1814 ± 0.8564	0.1151
	1000	1003.9732 ± 1.0276	0.1024
3,5-DCQA	100	99.5182 ± 0.1927	0.1936
	250	255.2977 ± 0.4544	0.1780
	500	494.9713 ± 0.6239	0.1260
	750	745.8983 ± 0.7657	0.1027
	1000	1003.0994 ± 0.9077	0.0905
4,5-DCQA	100	103.1656 ± 0.1782	0.1727
	250	254.2724 ± 0.3253	0.1280
	500	489.4829 ± 0.5913	0.1208
	750	748.1448 ± 0.7020	0.0938
	1000	1004.3856 ± 0.8119	0.0808

Data were expressed as the mean SD (n = 5).

Table 4

Inter-day precision for 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA in Ligularia fischeri extract using the HPLC-PDA method.

Compound	Origin	Solvent ratio (%)	Interday	
			Observed Concentration	Precision RSD (%)
3,4-DCQA	Hamyang	100	400.9328 ± 0.7392	0.1844
		70	694.7095 ± 1.0489	0.1510
		50	628.7130 ± 0.6949	0.1105
	Hoengseong	100	799.1811 ± 0.4865	0.0609
		70	1675.8443 ± 1.2639	0.0754
		50	1876.1564 ± 1.1760	0.0627
	Jeongseon	100	782.4230 ± 0.5818	0.5818
		70	894.3677 ± 0.6668	0.0746
		50	952.0126 ± 1.0759	0.1130
	Nonsan	100	929.3770 ± 0.9992	0.1075
		70	1914.7634 ± 1.2271	0.0641
		50	2152.2849 ± 2.0497	0.0952
	Yangsan	100	194.0019 ± 0.7225	0.7225
		70	300.7707 ± 0.5716	0.1900
		50	381.5616 ± 0.4148	0.1087
3,5-DCQA	Hamyang	100	347.1160 ± 0.2238	0.0645
		70	637.3278 ± 1.1169	0.1752
		50	1147.2962 ± 0.9989	0.0871
	Hoengseong	100	532.2904 ± 0.9027	0.1696
		70	995.4297 ± 0.5177	0.0520
		50	1706.0361 ± 1.1197	0.0656
	Jeongseon	100	320.7918 ± 0.4125	0.1286
		70	550.6090 ± 0.4755	0.0864
		50	1070.2532 ± 0.5016	0.0469
Solvent; DW (with Ethanol), (= 5)				

	Nonsan	100	495.3014 ± 0.5545	0.1120
		70	987.9929 ± 0.7045	0.5158
		50	1470.6069 ± 1.0601	0.0721
	Yangsan	100	171.6034 ± 0.4994	0.2910
		70	373.1339 ± 0.5158	0.1382
		50	843.3912 ± 0.7800	0.0925
4,5-DCQA	Hamyang	100	180.3627 ± 0.6340	0.3515
		70	749.1779 ± 0.4227	0.0564
		50	1044.9344 ± 0.9620	0.0921
	Hoengseong	100	304.4347 ± 0.4813	0.1581
		70	950.7943 ± 0.3793	0.0399
		50	988.9631 ± 0.9003	0.0910
	Jeongseon	100	213.6833 ± 0.4025	0.1884
		70	705.7721 ± 0.7480	0.1060
		50	927.4137 ± 0.7327	0.0790
	Nonsan	100	338.2645 ± 0.4671	0.1381
		70	1179.4910 ± 0.8460	0.0717
		50	1125.3682 ± 0.6967	0.0619
	Yangsan	100	86.7630 ± 0.4404	0.5076
		70	435.0737 ± 0.3509	0.0807
		50	466.1191 ± 0.6737	0.1445
Solvent; DW (with Ethanol), (= 5)				

3.3.4. Recovery

The recovery study was conducted using HPLC-PDA analysis with a mixture of *Ligularia fischeri* extract and standard solutions of 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA. The overall recovery rates for 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA were 96.9675–99.1368%, with RSD values all below 1% (Table 5).

Table 5
Recovery study of 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA in Ligularia fischeri extract

Compound	Concentration			Recovery (%)	SD	RSD (%)
	Extract	Standard	Extract with Standard			
3,4-DCQA	1079.5395 ± 1.2749	508.7541 ± 4.1856	1540.1124 ± 6.4529	96.9675	0.6123	0.631472
3,5-DCQA	745.7905 ± 3.0325	514.3746 ± 2.4918	1228.5486 ± 9.4095	99.1368	0.7146	0.720803
4,5-DCQA	576.4081 ± 5.8836	516.3915 ± 6.3605	1126.8748 ± 11.7163	97.4385	0.8892	0.912598

3.3.5 Quantitative Analysis of the 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA in Ligularia fischeri extract by solvent ratio and origin.

Analysis of the content of three types of DCQA by the concentration of extraction solvent in *Ligularia fischeri*. from five regions showed that in 100% distilled water, 3,4-DCQA was highest in Nonsan (929.3770 ± 0.9992 ug/mL), Hoengseong (799.1811 ± 0.4865 ug/mL), and Jeongseon (782.4230 ± 0.5818 ug/mL); 3,5-DCQA was higher in the order of Hoengseong (532.2904 ± 0.9027 ug/mL) Nonsan (495.3014 ± 0.5545 ug/mL) and Hamyang (347.1160 ± 0.2238 ug/mL); and 4,5-DCQA was higher in the order of Nonsan (338.2645 ± 0.4671 ug/mL), Hoengseong (304.4347 ± 0.4813 ug/mL), and Jeongseon (213.6833 ± 0.4025 ug/mL). In 30% ethanol, 3,4-DCQA was Nonsan (1914.7634 ± 1.2271 ug/mL), Hoengseong (1675.8443 ± 1.2639 ug/mL), and Jeongseon (894.3677 ± 0.6668 ug/mL); 3,5-DCQA was Hoengseong (995.4297 ± 0.5177 ug/mL), Nonsan (987.9929 ± 0.7045 ug/mL), and Hamyang (637.3278 ± 1.1169 ug/mL); and 4,5-DCQA was Nonsan (1179.4910 ± 0.8460 ug/mL), Hoengseong (950.7943 ± 0.3793 ug/mL), and Hamyang (749.1779 ± 0.4227 ug/mL). In 50% ethanol, 3,4-DCQA was Nonsan (2152.2849 ± 2.0497 ug/mL), Hoengseong (1876.1564 ± 1.1760 ug/mL), and Jeongseon (952.0126 ± 1.0759 ug/mL); 3,5-DCQA was Hoengseong (1706.0361 ± 1.1197 ug/mL), Nonsan (1470 ± 6069 ± 1.0601 ug/mL), and Hamyang(1147.2962 ± 0.9989 ug/mL); and 4,5-DCQA was Nonsan (1125.3682 ± 0.6967 ug/mL), Hamyang(1044.9344 ± 0.9620 ug/mL), and Hoengseong (988.9631 ± 0.9003 ug/mL) (Table 6).

Table 6
Changes in content of the 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA according to extraction by solvent ratio and region.

Compound Name	Solvent ratio (%)	Concentration (ug/mL)				
		Hamyang	Hoengseong	Jeongseon	Nonsan	Yangsan
3,4-DCQA	100	400.9328 ± 0.7392 ^{E}	799.1811 ± 0.4865 ^{A}	782.4230 ± 0.5818 ^{C}	929.3770 ± 0.9992 ^{B}	194.0019 ± 0.7225 ^{D}
	70	694.7095 ± 1.0489 ^{E}	1675.8443 ± 1.2639 ^{A}	894.3677 ± 0.6668 ^{C}	1914.7634 ± 1.2271 ^{B}	300.7707 ± 0.5716 ^{D}
	50	628.7130 ± 0.6949 ^{E}	1876.1564 ± 1.1760 ^{A}	952.0126 ± 1.0759 ^{C}	2152.2849 ± 2.0497 ^{B}	381.5616 ± 0.4148 ^{D}
3,5-DCQA	100	347.1160 ± 0.2238 ^{E}	532.2904 ± 0.9027 ^{C}	320.7918 ± 0.4125 ^{A}	495.3014 ± 0.5545 ^{D}	171.6034 ± 0.4994 ^{B}
	70	637.3278 ± 1.1169 ^{E}	995.4297 ± 0.5177 ^{C}	550.6090 ± 0.4755 ^{A}	987.9929 ± 0.7045 ^{D}	373.1339 ± 0.5158 ^{B}
	50	1147.2962 ± 0.9989 ^{E}	1706.0361 ± 1.1197 ^{C}	1070.2532 ± 0.5016 ^{A}	1470 ± 6069 ± 1.0601 ^{D}	843.3912 ± 0.7800 ^{B}
4,5-DCQA	100	180.3627 ± 0.6340 ^{E}	304.4347 ± 0.4813 ^{A}	213.6833 ± 0.4025 ^{C}	338.2645 ± 0.4671 ^{B}	86.7630 ± 0.4404 ^{D}
	70	749.1779 ± 0.4227 ^{E}	950.7943 ± 0.3793 ^{C}	705.7721 ± 0.7480 ^{A}	1179.4910 ± 0.8460 ^{B}	435.0737 ± 0.3509 ^{D}
	50	1044.9344 ± 0.9620 ^{E}	988.9631 ± 0.9003 ^{C}	927.4137 ± 0.7327 ^{B}	1125.3682 ± 0.6967 ^{A}	466.1191 ± 0.6737 ^{D}
Solvent; DW (with Ethanol), (□ = 5)						

All values are mean ± SD (n = 3). A-E Means with different superscripts in the same row were significantly different at $p < 0.05$ using Duncan's multiple range test. Also tested based on superscript A in the same row.

4. Discussion

Ligularia fischeri contains dicaffeoylquinic acids (DCQAs), one of the main bioactive compounds, which are promising substances that can contribute to health promotion through their antioxidant and anti-inflammatory effects [23, 24]. DCQA is a compound in which a caffeine acid residue is bound to a quinic acid structure and varies in form depending on its position, such as 3,4-, 3,5-, and 4,5-DCQA. Recent studies have shown that 3,5-DCQA inhibits NO and suppresses the expression of inflammatory mediators such as INOS, COX2, and TNF-α. Additionally, 4,5-DCQA has been found to regulate an anti-

inflammatory pathway mediated by the TRPV1 receptor and inhibit COX2 expression [23, 25]. Especially, in the case of *Ligularia fischeri*, 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA are evenly contained and exist at a certain concentration [26]. Therefore, there is a lot of research being conducted on the development of various functional foods and pharmaceuticals using DCQAs.

The study of analyzing and verifying three types of DCQA contained in *Ligularia fischeri* extract as marker compounds is already conducted. [27]. In addition, studies on the antioxidant and physiological activities of *Ligularia* fischeri have been conducted based on its extraction method, but these studies are limited to simple extracts [28]. However, in this study, we compared the concentrations of 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA, which act as marker compounds of *Ligularia fischeri* extract. Additionally, we compared and analyzed the concentrations of 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA in *Ligularia fischeri* extract using three different solvents and performed methodological analytical validity verification.

The results of the content analysis in Table 6 show that the concentration of 3,4-DCQA was highest at 2152.2849 ± 2.0497 ug/mL in the 50% ethanol extract from the Nonsan region, while the concentration of 3,5-DCQA was highest at 1706.0361 ± 1.1197 ug/mL in the 50% ethanol extract from the Hoengseong region, while the highest concentration of 4,5-DCQA was found in the 30% ethanol extract from the Nonsan region at 1179.4910 ± 0.8460 ug/mL.

This study examined the importance of validation procedures for ensuring data validity and standardization processes for enhancing data comparability, as well as practical application methods for these procedures. Through this, we confirmed that not only can data quality be improved, but the reproducibility and generalizability of research results can also be enhanced.

5. Conclusions

We screened six phenolic compounds contained in *Ligularia fischeri* and applied an HPLC-MS/MS system to perform quantitative analysis of 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA. A validation system was established to evaluate specificity, linearity, detection limits, quantification limits, precision, and recovery rates, and differences in content levels were confirmed based on extraction solvents and regional variations. This study contributed to the standardization of the three DCQA compounds as marker components of *Ligularia fischeri extracts*, providing valuable insights for analyzing compounds in related plants. Additionally, these findings are expected to facilitate the development of various health functional products and pharmaceuticals with potential applications in antioxidant, anti-inflammatory, blood sugar regulation, liver protection, and bone health improvement.

Declarations

Author Contributions

Conceptualization, H.H.K. and S.H.J.; methodology, H.H.K.; writing—original draft preparation, S.H.J. and H.H.K.; writing—review and editing, P.B.B., Y.G.M., and K.H.H,; investigation, S.H.J.; validation H.H.K. and

S.H.J.; project administration, T.Y.K., J.W.P. and G.I.K..; supervision, G.S.K. All authors have read and agreed to the published version of the manuscript.

Funding

This research received no external funding.

Informed Consent Statement

Not applicable.

Data Availability Statement

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Acknowledgments

This study was supported by the National Research Foundation of Korea, funded by the Ministry of Science and ICT (grant no. RS-2023-00243376, and RS-2024-00411709) and the Kick the Hurdle Co., Ltd.

Conflicts of Interest

The authors declare no conflicts of interest.

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Figures

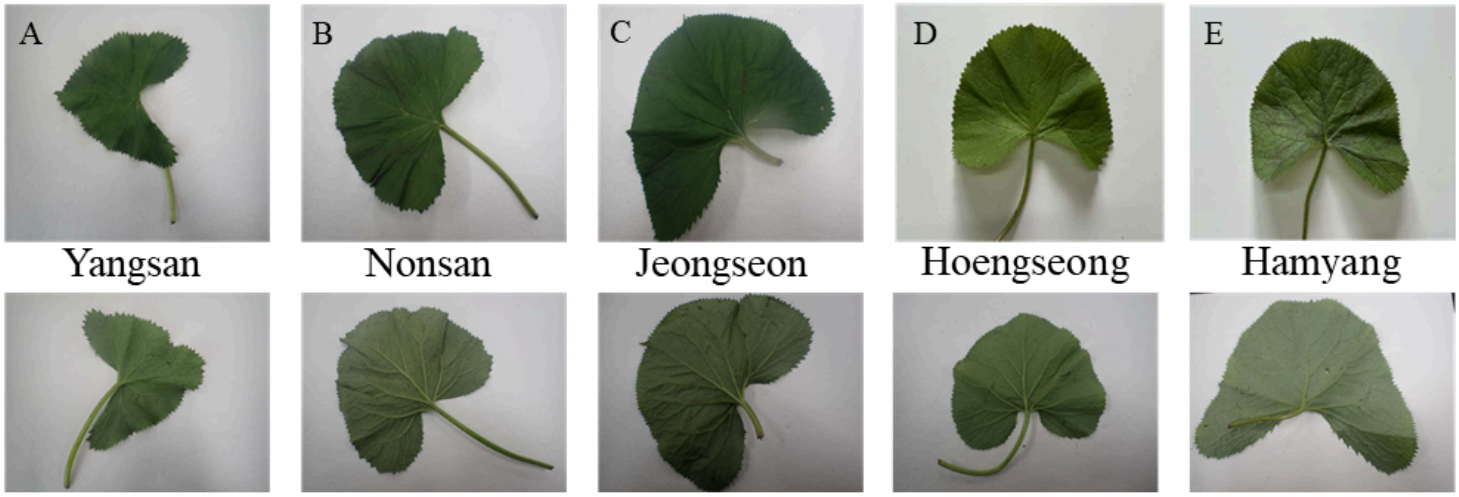

Figure 1

Leaf shapes of *Ligularia fischeri* in each cultivation area. (A) Yangsan, (B) Nonsan, (C) Jeongseon, (D) Hoengseong and (E) Hamyang.

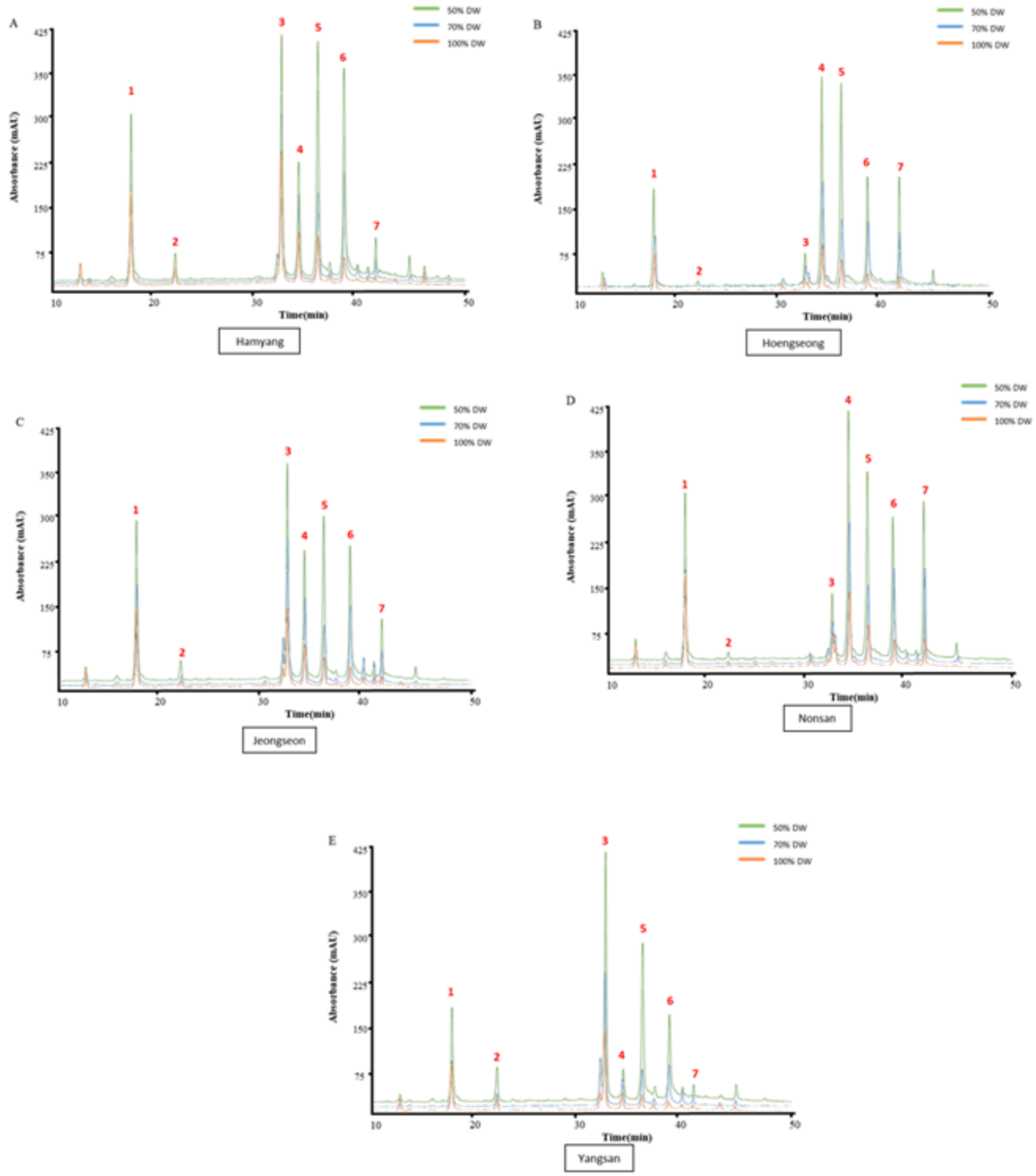

Figure 2

Chromatograms of *Ligularia fischeri* extract based on origin and extraction solvent. Orange represents 100% DW, blue represents 30% EtOH, and green represents 50% EtOH peaks. Each region is labeled as (A) Hamyang, (B) Hoengseong, (C) Jeongseon, (D) Nonsan, and (E) Yangsan. The compounds detected

at 284 nm are Chlorogenic acid (1), Caffeic acid (2), Hyperin (3), 3,4-Dicaffeoyl quinicacid (4), 3,5-Dicaffeoylquinic acid (5), 4,5-Dicaffeoylquinic acid (6), and unknown (7).

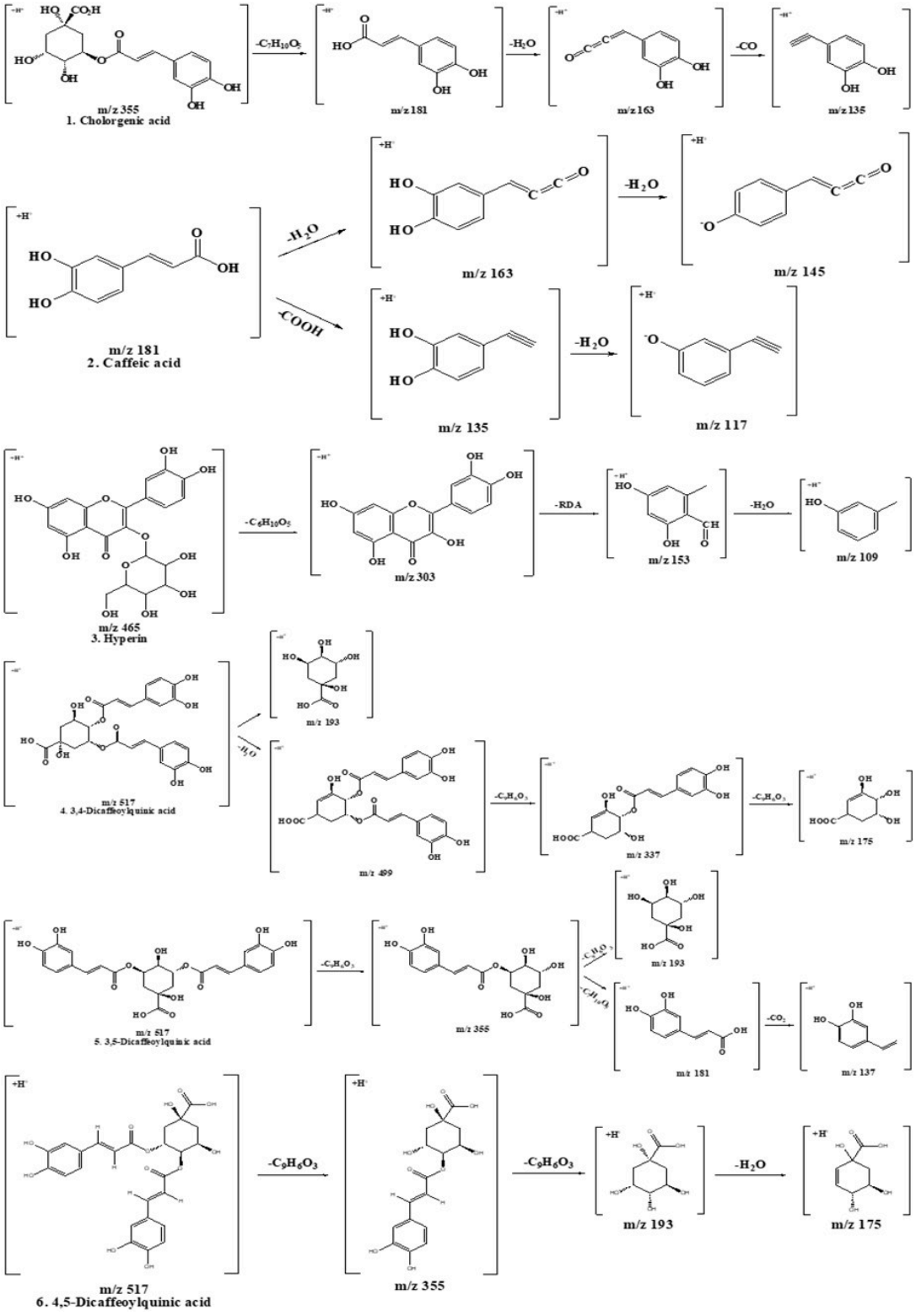

Figure 3

Fragmentation scheme of identified compounds in *Ligularia fischeri* extract.

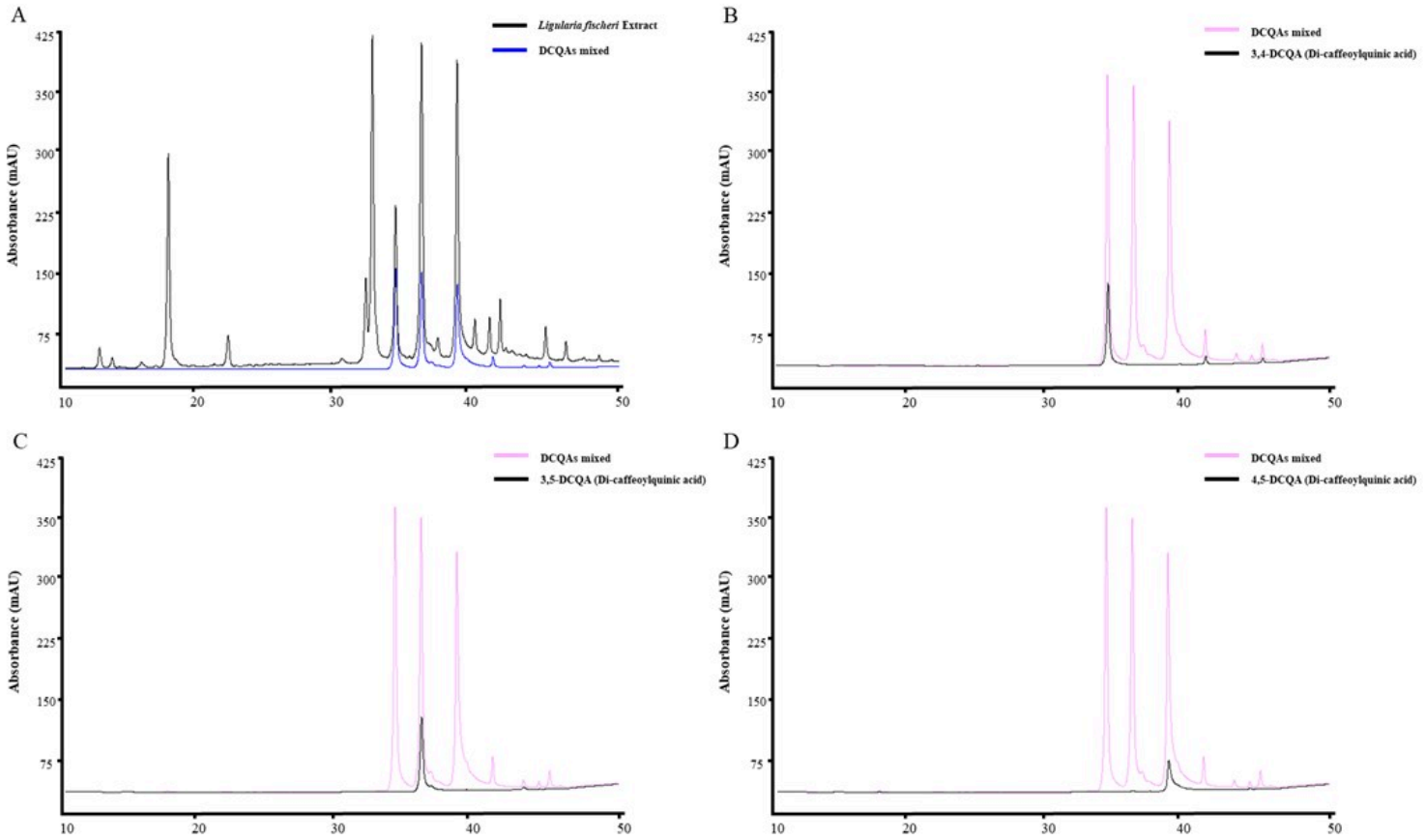

Figure 4

Chromatograms of *Ligularia fischeri* extracts and three DCQAs as potential functional compounds. (A) The black peak is from *Ligularia fischeri* extract, and the blue peak is from a mixture of three standard DCQAs. (B) The mixture of three standard DCQAs is shown as a pink peak, and 3,4-Dicaffeoyl quinic acid is shown as a black peak. (C) The pink peak represents the mixture of the three standard DCQAs, and the black peak represents 3,5-dicaffeoyl quinic acid. (D) The pink peak represents the mixture of the three standard DCQAs, and the black peak represents 4,5-dicaffeoyl quinic acid.