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Quantitative analysis of capsaicinoids in *Capsicum annuum* using HPLC/UV

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Abstract *Capsicum annuum* belongs to the Solanaceae family, crops of which are extensively cultivated worldwide. It is a food source containing various nutrients and vitamins and also serves as a medicine for treating ailments. The burning feeling experienced while consuming *Capsicum* fruits is due to the presence of capsaicinoids, particularly capsaicin and dihydrocapsaicin. This study aimed to assess the content of these two compounds in 34 varieties of capsicum and paprika. High-performance liquid chromatography with a gradient elution system and a reverse-phase YMC Pack-Pro column with UV detection at 280 nm was employed. The results revealed that, among the 34 samples, only six samples (samples 1, 15, 20, 29, 32, and 34) contained capsaicin and dihydrocapsaicin, and their highest contents were found in sample 1 - variety name: Sungil-c (capsaicin: 3.42 mg/g extract, dihydrocapsaicin: 1.20 mg/g extract). These findings suggest that the content of these two compounds is attributed to the variety and is influenced by geographical location and environmental factors. Additionally, this study provides a basis for establishing a *C. annuum* variety with high capsaicin and dihydrocapsaicin contents.

Keywords Capsaicin · *Capsicum* species · Dihydrocapsaicin · High-Performance Liquid Chromatography/Ultraviolet-Visible · Paprika · Quantitative analysis

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Introduction

The genus *Capsicum* (Solanaceae family) comprises approximately 30 species, typically *C. annuum* L., *C. frutescens* L., *C. baccatum* L., *C. chinense* Jacq., and *C. pubescens* Ruiz and Pav [1-2]. *C. annuum* L. (capsicum and paprika) is an annual or perennial plant [3]. It is among the most consumed spices, is cultivated worldwide (especially in tropical and subtropical regions), and is highly valued for its color, pungency, and aroma [4]. Capsicum (chili pepper) is an important ingredient for preparing spicy sauces in Mexican and Asian cuisines [5]. Its oil extract has been used as a medicine for centuries and is among the main components in Mayan therapeutic remedies [4]. Meanwhile, paprika (sweet pepper) contains a high level of phytonutrients, which prevent cardiovascular diseases and type II diabetes [6-7]. Both capsicum and paprika are abundant in vitamins A, E, and C and β-carotene, α-carotene, zeaxanthin, lutein, lycopene, and cryptoxanthin [8-10]. Owing to its pharmaceutical and therapeutic properties, *C. annuum* L. also serves as a topical analgesic, antiseptic, carminative, tonic, and counterirritant. Furthermore, it is used for treating arthritis, rheumatism, itching, neuralgia, lumbago, and spasms [11].

Capsaicinoids are produced by a condensation reaction between vanillylamine, a phenylalanine derivative, and a C9-C11 branched-chain fatty acid biosynthesized from valine and leucine [12]. Specifically, capsaicinoids are fatty acid amides linked to vanillylamine, whereas capsinoids are fatty acid esters with vanillyl alcohol [13]. Capsaicinoids are the alkaloids responsible for the spicy flavor of peppers [14]. The two main capsaicinoids (representing approximately 77-98% of the total capsaicinoids) found in peppers are capsaicin (CAP) and dihydrocapsaicin (DHC) [15]. Other minor compounds reported in *Capsicum* species include nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin, and nonivamide [16]. CAP and DHC are approximately twice as pungent as homocapsaicin and nordihydrocapsaicin. They are responsible for the hotness of the pepper [17]. These compounds have various properties and biological effects such as antimicrobial, anti-inflammatory, antioxidant, anticancer, antidiabetic, anti-arthritis, and analgesic effects [18-24]. However, the CAP and DHC contents can be affected by many factors, including the developmental stage of the fruits or the environmental growth conditions [25-26].

In this study, the CAP and DHC contents of 20 varieties of capsicum and 14 varieties of paprika were evaluated using high-performance liquid chromatography (HPLC) coupled with ultraviolet-visible (UV) spectroscopy analysis.

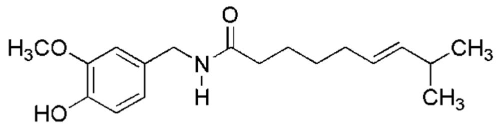
Materials and Methods

Plant materials

Different varieties of capsicum and paprika were cultivated at Araon Co., Anseong, Korea, and freshly harvested samples were used for the analysis (Table 1). All samples were air-dried for the

Table 1 List of the capsicum and paprika varieties examined

Sample		Classification	Variety name	Ripe fruit color
Capsicum (Chili pepper)	1	green pepper	sungil-c	red
	2	green pepper	migp-hf	red
	3	green pepper	migp-shf	red
	4	green pepper	migp-f	red
	5	green pepper	asag-i block 7	purple
	6	green pepper	asag-i block 24	purple
	7	green pepper	longmi-or	red
	8	green pepper	kkosungil	red
	9	green pepper	gp 32	red
	10	green pepper	gp 43	red
	11	green pepper	capsi 3	red
	12	green pepper	capsi 4	red
	13	green pepper	capsi 5	red
	14	cow-horn pepper	gms/azteca	red
	15	green pepper	capsi 10	red
	16	green pepper	capsi	red
	17	green pepper	capsi 11	red
	18	green pepper	capsi 21	red
	19	green pepper	capsi 23	red
	20	dry pepper	geum-b	red
Paprika (Sweet pepper)	21	paprika-blocky	mine-a	red
	22	paprika-blocky	mine-c	red
	23	paprika-blocky	spp	yellow
	24	paprika-blocky	vlti	orange
	25	paprika-tribelli	tribelli-r3	red
	26	paprika-mini	hal-a(gms)	red
	27	paprika-mini	midi-or	orange
	28	paprika-mini	red-one	red
	29	paprika-mini	imparmo	red
	30	paprika-mini	mibola	purple
	31	paprika-mini F1	hala/r-1	red
	32	paprika-mini F1	gp/r-1	red
	33	paprika-mini F1	gms/mibola	purple
	34	paprika-mini F1	imparmo/mibola	purple



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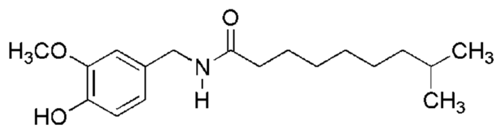

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Fig. 1 Chemical structure of CAP (**1**) and DHC (**2**)

Fig. 2 Capsicum and paprika samples

extraction and HPLC analysis.

Instruments and reagents

HPLC was performed on a Waters 1525 Binary HPLC MA 01757 USA, Quat with pump, autosampler, and Waters 2489 UV/Visible Detector MA 01757 USA. HPLC-grade solvents such as MeOH, water, trifluoroacetic acid (TFA) and acetonitrile (ACN) were purchased from J. T. Baker (Avantor, PA, USA). CAP and DHC (Fig. 1) were provided by the Natural Product Institute of Science and Technology, Anseong, Korea (www.nist.re.kr).

Sample extraction

Thirty-four samples of fresh capsicum and paprika (Fig. 2) were dried using a freeze-dryer to obtain powdered samples. The samples were extracted three times in ethanol (EtOH) under a reflux extractor for 3 h. Subsequently, they were filtered and evaporated using a vacuum concentrator to obtain concentrated EtOH extracts. The extraction yield is presented in Table 2.

Preparation of standard and sample solutions

The extracts of 34 samples (35 mg) and standard compounds (CAP and DHC) (1 mg) were dissolved in 1 mL methanol (MeOH). Subsequently, they were sonicated for 20 min and filtered using a 0.45 µm polyvinylidene fluoride membrane filter.

HPLC conditions

The extracts were quantitatively analyzed in a reverse-phase HPLC system using a YMC Pack-Pro C18 column (25 cm×4.6 mm, 5 µm) with a gradient elution system. The mobile phase was composed of 0.1% TFA in water (A) and ACN (B), and the elution conditions were 93% A from 0 min to 10 min, 70% A at 15 min, 30% A at 40 min, 0% A at 45 min, and 93% A from 50 min to 60 min. The column temperature was retained at 30 ºC. The injection volume was 10 µL, the flow rate was 0.8 mL/min, and the wavelength was monitored at 280 nm.

Calibration curve

The standard stock solutions of CAP and DHC were serially diluted to six concentrations, which were used to design the calibration curve. The linearity of the calibration curve was determined based on the correlation coefficient (r^2), and the CAP and DHC contents of the extracted samples were quantified. The calibration function of the two compounds was established based on the peak area (Y), concentration (X, µg/mL), and mean value (n =3) ± standard deviation (Table 3).

Results and Discussion

C. annuum L. (pepper) has many phytochemical compounds such as polyphenols, flavonoids, and capsaicinoids, which have potential health benefits [27]. Additionally, several studies have reported the acetylcholinesterase (an enzyme related to Alzheimer's disease) inhibitory and efficient antioxidative abilities of *Capsicum* species [28-29]. CAP and DHC are the main components of capsaicinoids that regulate the spiciness of peppers [30]. They have several physiological and pharmacological effects on the gastrointestinal tract and respiratory, cardiovascular, sensory, and thermoregulation systems [31]. Moreover, CAP has anticancer effects and can be used for treating arthritis-related inflammation and pain, neurogenic inflammation, high cholesterol levels, and obesity [32-35].

This study investigated the CAP and DHC contents of the fruits of 34 varieties of capsicum and paprika using HPLC/UV. CAP and DHC were well separated in the HPLC chromatogram and had retention times of 38.35 min and 40.99 min, respectively. The

Table 2 Extraction yield of the capsicum and paprika samples

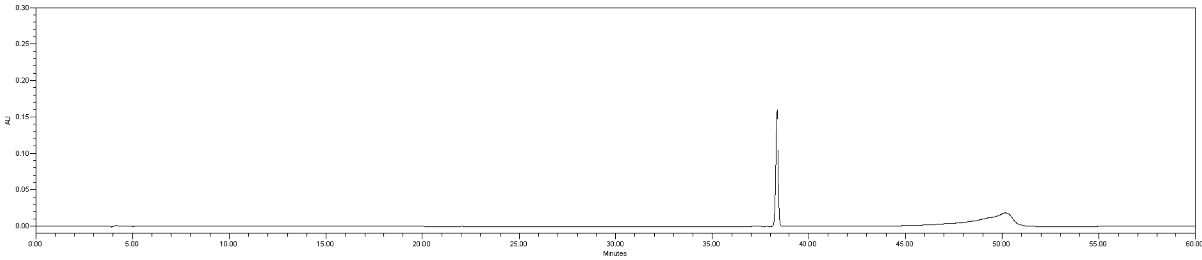
Sample		Fresh samples (g)	Dry sample (g)	Extract (g)	Yield (%)
Capsicum (Chili pepper)	1	50.6	5.41	1.6	29.58
	2	47.7	4.35	2.5	57.43
	3	49.2	4.23	2.7	63.83
	4	43.1	3.74	2.0	53.53
	5	52.4	5.74	2.8	48.75
	6	41.0	3.43	1.4	40.80
	7	76.4	7.24	4.3	59.39
	8	49.2	3.45	1.9	55.05
	9	66.4	5.69	3.8	66.77
	10	42.1	3.14	2.0	63.61
	11	64.4	5.21	2.7	51.82
	12	48.5	4.13	1.8	43.54
	13	53.4	4.74	2.4	50.64
	14	64.3	6.31	4.1	64.94
	15	61.1	6.21	2.9	46.73
	16	48.4	4.83	2.3	47.67
	17	57.6	6.04	3.1	51.33
	18	52.4	5.32	2.6	48.86
	19	51.6	5.30	2.6	49.06
	20	53.3	7.34	2.7	36.77
Paprika (Sweet pepper)	21	166.8	6.19	3.6	58.15
	22	212.5	5.66	3.9	68.88
	23	172.4	4.90	3.3	67.42
	24	150.7	5.83	3.9	66.93
	25	163.4	6.33	4.9	77.46
	26	52.4	6.11	2.9	47.44
	27	57.8	6.38	4.0	62.71
	28	74.3	7.52	5.7	75.76
	29	66.0	6.26	3.6	57.51
	30	52.6	5.09	2.9	56.96
	31	54.2	7.26	4.3	59.21
	32	42.6	5.45	3.1	56.88
	33	50.6	5.91	3.3	55.84
	34	57.1	7.13	4.5	63.11

Table 3 Calibration curve equation for CAP (**1**) and DHC (**2**)

Compound	t_R	Calibration equation[a]	Correlation factor, r^2 [b]
1	38.35	Y = 6178.2X + 27433	0.9996
2	40.99	Y = 6671.8X + 43993	0.9991

[a] Y = peak area, X = concentration of the standard (μg/mL)
[b] r^2 = correlation coefficient for five calibration data points (n =3)

HPLC results of these two compounds are shown in Fig. 3. The equations for the linear calibration of CAP and DHC were Y = 6178.2X + 27433 and Y =6671.8X + 43993, respectively, where Y represents a given peak area and X represents the compound concentration. The correlation coefficients (r^2) were all above 0.9991, illustrating that the quantification method had excellent linearity (Table 3). The peaks of CAP and DHC in all samples were determined based on the retention times of CAP and DHC and the experiment with the matrix spike samples. The content of each compound in the samples was determined using the



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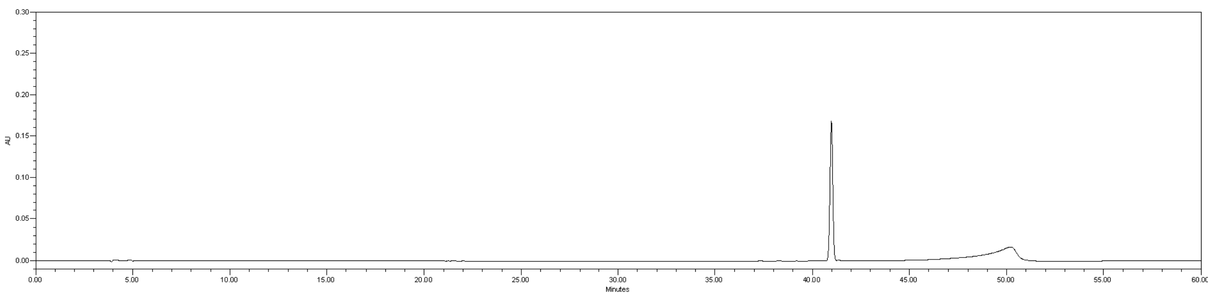

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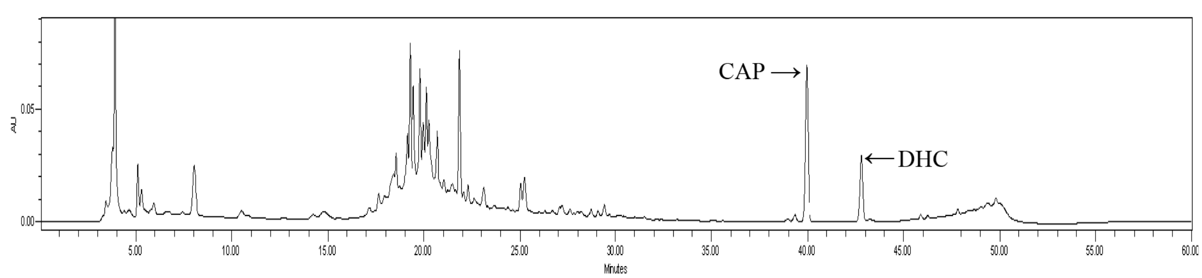
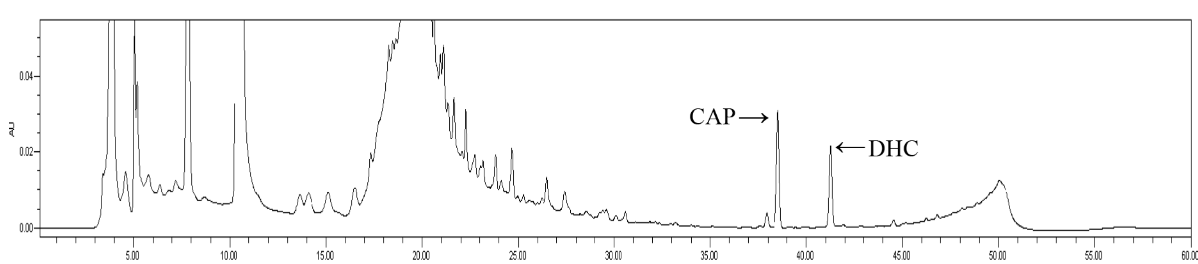
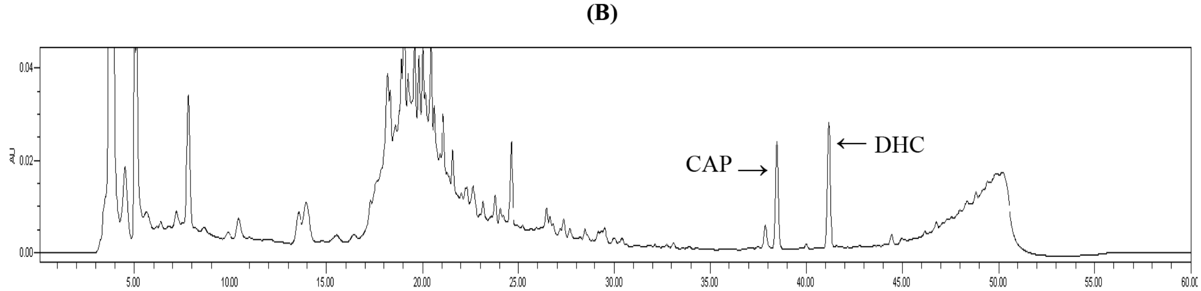
Fig. 3 HPLC chromatograms of CAP (**1**) and DHC (**2**)



(A)



(B)



(C)

Fig. 4 HPLC chromatograms of capsicum samples 1 (A), 15 (B), and 20 (C)

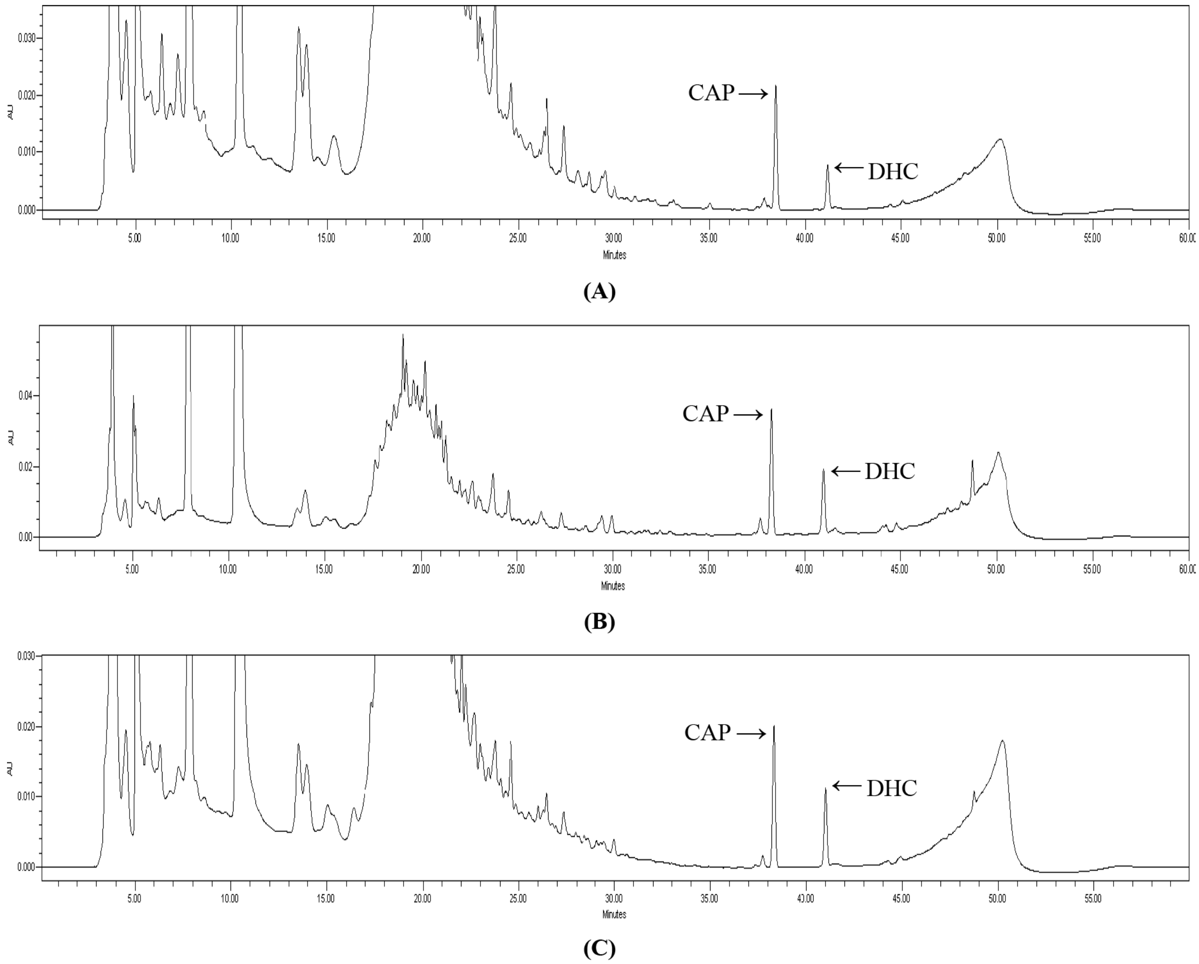


Fig. 5 HPLC chromatograms of paprika samples 29 (A), 32 (B), and 34 (C)

calibration curve equation. The chromatograms of the samples are shown in Figs. 4 and 5, and the results of the quantitative analysis are presented in Table 4.

The results revealed that, among the 34 samples, only six samples (capsicum: samples 1, 15, and 20; paprika: samples 29, 32, and 34) contained CAP and DHC. Sample 1 (variety name: sungil-c) had the highest CAP (3.42 mg/g extract) and DHC (1.20 mg/g extract) contents, whereas the lowest CAP (0.76 mg/g extract) and DHC (0.11 mg/g extract) contents were detected in sample 29 (variety name: imparmo).

Many researchers have described that the accumulation of capsaicinoids is mainly related to fruit age, size, and state of development. This process begins in the early stages of fruit development, reaches its maximum rate when the fruit is close to the end of its growth, and continues to increase slightly after reaching the maximum length [36-37]. Additionally, the CAP and DHC contents are influenced by environmental factors, including stress conditions such as drought, low light, high temperature, and pest and disease conditions [38-40]. This could be the reason for the absence of CAP and DHC in 28 samples.

Besides, these compounds are related to the levels of pungency, and most paprika fruits are non-pungent. Thus, as expected, among six samples, the CAP and DHC contents of capsicum samples were mostly higher than those of paprika samples. This trend was similar to that observed in a study investigating the

Table 4 Content of CAP (**1**) and DHC (**2**) in the MeOH extracts of capsicum and paprika samples

Sample		Content (mg/g extract)	
		1	**2**
Capsicum (Chili pepper)	1	3.42±0.22	1.20±0.08
	15	1.02±0.10	0.58±0.05
	19	tr	tr
	20	0.92±0.07	0.95±0.08
Paprika (Sweet pepper)	29	0.76±0.06	0.11±0.02
	32	1.44±0.06	0.58±0.03
	34	0.80±0.06	0.29±0.04

tr: trace
Noted: The content of CAP (**1**) and DHC (**2**) in other 27 samples were not detected

CAP and DHC contents in hot, red, and green chili peppers and green, red, and yellow sweet peppers [31]. The results showed that none of the sweet peppers contained DHC, and only green sweet pepper contained trace amounts (1.0 μg/g pepper) of CAP. In contrast, all three types of chili pepper contained both CAP and DHC, and their highest concentrations were observed in hot chili pepper (capsaicin: 4249 μg/g pepper, and dihydrocapsaicin: 4482 μg/g pepper).

In conclusion, the present study used HPLC/UV to examine the CAP and DHC contents in 34 varieties of *C. annuum* L. CAP and DHC were detected in six (samples 1, 15, 20, 29, 32, and 34) of the examined samples. The highest CAP (3.42 mg/g extract) and DHC (1.20 mg/g extract) contents were observed in sample 1 (sungil-c), whereas the lowest CAP (0.76 mg/g extract) and DHC (0.11 mg/g extract) contents were observed in sample 29 (imparmo). These results provide a basis for further experimentation, and the six varieties of *C. annuum* L. containing CAP and DHC could be used in the pharmaceutical industry for preparing potential health supplements.

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