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HPLC/UV quantitative analysis of capsaicinoids: insights into antioxidant potential of various *Capsicum* cultivars

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Abstract This study investigated capsaicin (CAP) and dihydrocapsaicin (DHC) contents in different *Capsicum* cultivars using high-performance liquid chromatography (HPLC). Fourteen *Capsicum* cultivars ('SUNGIL-C', '26B', '26C', 'DELB', 'DELC', 'MIGP-C', 'MI4H', 'MI2F', 'MI2H', 'MI8H', 'CAPSI 23-10', 'CAPSI 23-4', 'CAPSI 23-8', and 'CAPSI 23-9') were dried, extracted with ethanol, and concentrated. Results from the HPLC analysis revealed notable differences in the capsaicinoid (CAP and DHC) levels. For instance, the cultivar 'SUNGIL-C' exhibited the highest CAP and DHC content of 1.92 mg/g extract and 0.88 mg/g extract, respectively. However, cultivars '26B', 'DELB', 'MIGP-C', and 'MI8H' showed trace amounts of capsaicinoids or were not detected at all. In addition to the capsaicinoid analysis, total polyphenol and flavonoid content analyses were performed by selecting extracts from only those cultivars in which CAP and DHC were detected, and the effects of CAP and DHC and phenolic compounds on antioxidant activity were discussed. This analysis provides insight into the potential pungency and physiological effects of different *Capsicum* cultivars and highlights the variability in capsaicinoid and DHC content. Understanding this variation can clarify the biological meaning and practical applications of capsaicinoids and warrants further research into their usefulness.

Keywords Antioxidant activity · Capsaicin · *Capsicum* cultivar · Dihydrocapsaicin · High-performance liquid chromatography/Ultra Violet

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Introduction

Peppers, also known as *Capsicum* sp., are integral to global culinary landscapes and contribute distinct flavors, colors, and heat levels to a myriad of dishes [1]. Peppers are used worldwide in various forms, such as fresh, dried, or ground, and add depth and spice to vibrant cuisines [2]. In traditional medicine, *Capsicum* sp. has been used for its potential medicinal properties [3] because of the presence of the active compound capsaicin (CAP), which is known to exhibit analgesic properties and serves as a key ingredient in topical pain-relief formulations. *Capsicum* sp. have also been used to promote digestive health and as a potential aid in weight management [4]. Furthermore, *Capsicum* sp. contains essential nutrients, including vitamins A and C, as well as antioxidants [5], and are associated with various health benefits, including supporting the immune system, promoting skin health, and preventing oxidative stress [6]. In addition to their traditional uses, *Capsicum* sp. plays a central role in culinary innovations, such as hot sauces, spice blends, and fermented pepper products [7]. Capsaicin (CAP)-rich extracts have also been used to create condiments and seasonings [8].

Capsicum can be annual or perennial [9]. It is cultivated worldwide, particularly in tropical and subtropical regions, and is important for its vibrant color, pungent flavor, and aromatic

qualities. Its oil extract has been used for medicinal purposes for centuries and is a key component of traditional Mayan therapeutic remedies [10]. Derived from sweet pepper, *C. annuum* L. is a rich source of phytonutrients that have been associated with mitigating cardiovascular diseases and type II diabetes [11,12]. Additionally, it serves as a major provider of essential vitamins, including A, E, and C, as well as β-carotene, α-carotene, zeaxanthin, lutein, lycopene, and cryptoxanthin [13-15]. Owing to its pharmaceutical and therapeutic properties, *Capsicum* extracts are used as a topical analgesic, antiseptic, carminative, tonic, and counterirritant. Historically, it has been used to treat various conditions, including arthritis, rheumatism, itching, neuralgia, lumbago, and spasms [16].

Therefore, this study provides information on newly developed *Capsicum* cultivars to understand their diversity and to improve the quality of agriculture and food production to enhance the productivity of agricultural produce and consumer satisfaction. Initially, the present study examined the detailed information on the 14 *Capsicum* cultivars. These cultivars have different names and characteristics, with the common feature of developing red fruits in their mature state. This helped us identify both the similarities and differences among the existing cultivars. This study aimed to elucidate the significance and possibilities of *Capsicum* breeding, focusing particularly on newly developed cultivars. This study can help to gain insights into potential enhancements in agriculture and meet the evolving demands of consumers in the food industry.

CAP is the primary compound responsible for the pungent taste of chili peppers and has recently gained attention beyond its role in pungency [17]. CAP is abundant in chili peppers and possesses distinctive physiological properties, such as antioxidative, anti-inflammatory, and analgesic effects. Measuring the CAP content in *Capsicum* sp. helps to evaluate its medical benefits and plant quality [18-21]. Moreover, its anti-inflammatory properties suggest its potential use for managing various inflammatory conditions. Several researches have shown CAP's analgesic effects at its potential application in chronic pain management [4,22-24]. Therefore, peppers rich in CAP and dihydrocapsaicin (DHC) have garnered the attention of researchers because of their unique properties, including flavor and potential physiological effects [25,26]. High-performance liquid chromatography (HPLC) is widely used to quantify and analyze CAP and DHC contents. Specifically, combining HPLC with Ultra Violet (UV) detection offers an efficient method for precisely quantifying these compounds and delivering rapid and accurate analyses [27,28].

In this study, we utilized the HPLC/UV methodology to quantitatively analyze the amounts of CAP and DHC in various *Capsicum* cultivars. In addition, the total polyphenol content (TPC), total flavonoid content (TFC), and antioxidant activity of the selected cultivars were investigated. The multiple analyses performed in the paper not only establish an approach for accurately determining the CAP and DHC content of *Capsicum* cultivars but also suggest different conditions for selecting cultivars in terms of the health-promoting aspects associated with these capsaicinoids. The integrated examination of TPC, TFC, and antioxidant data further highlights the multifaceted potential of *Capsicum*-derived compounds, encouraging their exploration for various medical applications.

Materials and Methods

Plant materials

Different cultivars ('SUNGIL-C', '26B', '26C', 'DELB', 'DELC', 'MIGP-C', 'MI4H', 'MI2F', 'MI2H', 'MI8H', 'CAPSI 23-10', 'CAPSI 23-4', 'CAPSI 23-8', and 'CAPSI 23-9') of *Capsicum* were cultivated at Araon Co. (Anseong, Republic of Korea), and freshly harvested samples were used for the analysis (Fig. 1). All the samples were deposited at Araon Co.

Instruments and reagents

Freeze-drying was conducted using the FDU-1200 (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). HPLC was performed using an Agilent 1260 Infinity II Quat Pump (Agilent, Santa Clara, CA, USA), autosampler, and a variable wavelength detector (Agilent) with the YMC Pack-Pro C18 column (4.6×250 mm, 5 μm) (YMC Co., Ltd., Kyoto, Japan). The HPLC-grade solvents, methanol (MeOH), water, trifluoroacetic acid (TFA), and acetonitrile (ACN), were purchased from J. T. Baker (Radnor, PA, USA). The reagents for the colorimetric method, 2N Folin-Ciocalteu phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^+$) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and the absorbance was measured using a microplate reader (Epoch, BioTek, Winooski, VT, USA). The standard compounds, CAP, DHC (Fig. 2), tannic acid, quercetin, and ascorbic acid, were provided by the Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea.

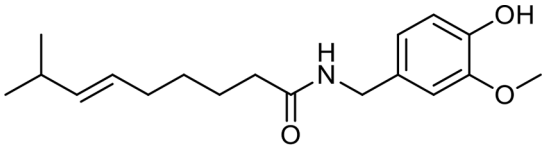
Sample extraction

Fourteen fresh *Capsicum* cultivars were freeze-dried to obtain powdered samples. These samples were subjected to triple extraction in ethanol (EtOH) using a reflux extractor for 3 h. The extracts were then filtered and concentrated using a vacuum concentrator to obtain the concentrated EtOH extracts.

Analysis of TPC

The TPC of *Capsicum* cultivars was determined using a method described in a previous study [29]. Initially, 60 μL of the extract was mixed with 40 μL of 2N Folin-Ciocalteu phenol reagent. Afterward, 100 μL 7.5% sodium carbonate solution was added to the mixture and incubated in the dark for 30 min. Finally, a calibration curve was constructed and TPC was quantified using tannic acid as a standard.

Fig. 1 *Capsicum* cultivars used in this study (1, 'SUNGIL-C'; 2, '26B'; 3, '26C'; 4, 'DELB'; 5, 'DELC'; 6, 'MIGP-C'; 7, 'MI4H'; 8, 'MI2F'; 9, 'MI2H'; 10, 'MI8H'; 11, 'CAPSI 23-10'; 12, 'CAPSI 23-4'; 13, 'CAPSI 23-8'; 14, 'CAPSI 23-9')



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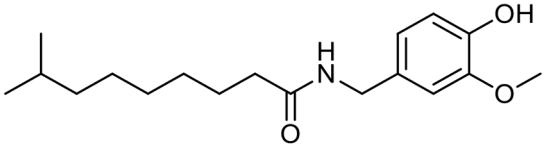

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Fig. 2 Chemical structures of capsaicin (**1**) and dihydrocapsaicin (**2**)

Analysis of TFC

The TFC of the *Capsicum* cultivars samples was analyzed using the method described in a previous study with some modifications [29]. Briefly, 100 µL of each sample was mixed with 100 µL 2% $AlCl_3$. The solution was then incubated for 10 min and the absorbance was measured at 430 nm using a microplate reader (BioTek Co.). A calibration curve was created using quercetin as the standard compound and TFC was determined.

DPPH radical-scavenging assay

The DPPH radical-scavenging activity was assessed following the methodology outlined in a previous study [29]. Ascorbic acid was diluted to generate a standard solution. Subsequently, 10 µL of both the standard and test solutions were added to a 96-well plate, followed by the addition of 200 µL of the DPPH working solution. Thorough mixing was ensured using a microplate shaker, and the mixture was incubated for 30 min at room temperature in the dark. After the reaction, the absorbance was measured at 514 nm using a microplate reader (BioTek Co.).

$ABTS^+$ antioxidant activity

Similarly, the antioxidant activity of the *Capsicum* cultivars was also analyzed using the $ABTS^+$ assay with a method mirroring that of a previous study [29]. The $ABTS^+$ and potassium persulfate solutions were mixed and diluted in distilled water to obtain an absorbance value of 1.0 mg/mL. Ten microliters of the standard and test solutions were added separately to a 96-well plate. Then, 200 µL of the $ABTS^+$ working solution was added. The samples were incubated in a dark room for 30 min and the absorbance was measured at 734 nm using a microplate reader (BioTek Co.).

Preparation of standard and sample solutions

The extracts of all samples (20 mg) and each standard compound (1 mg) were dissolved in 1 mL of MeOH. They were then sonicated

Table 1 Calibration curves equation for capsaicin (**1**) and dihydrocapsaicin (**2**)

Compound	t_R	Calibration equation [a]	Correlation factor, r^2 [b]
1	36.8	Y = 1.7344X + 1.0856	0.9999
2	39.3	Y = 3.5328X + 0.3463	0.9999

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

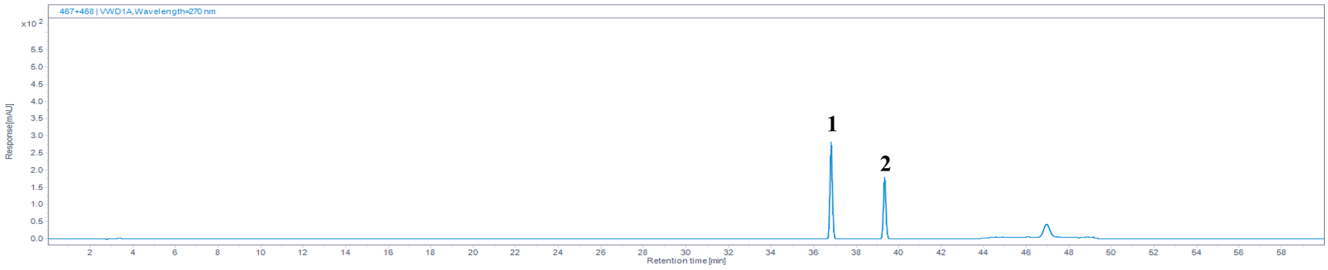

Fig. 3 HPLC/UV chromatogram depicting the peaks of capsaicin (**1**) and dihydrocapsaicin (**2**)

for 20 min and filtered using a 0.45 μm polyvinylidene fluoride membrane filter.

HPLC/UV conditions

The concentrated extracts were quantitatively analyzed using a reverse-phase HPLC system equipped with the YMC Pack-Pro C_{18} column (4.6×250 mm, 5 μm) (YMC Co.) using a gradient elution system. The mobile phase comprised 0.1% TFA in water (A) and ACN (B). The elution conditions were as follows: 17% B for 0-10 min, maintained at 17% B for 10 min, transitioned to 70% B for 40 min, returned to 0% B for 41-45 min, and reverted to 17% B for 50-60 min. The column temperature was held at 30 °C. Briefly, 10 μL of sample was injected, the flow rate was maintained at 1.0 mL/min, and the sample was monitored at a wavelength of 270 nm.

Calibration curve

Standard stock solutions of CAP and DHC were serially diluted to five different concentrations and were used to construct a 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 1).

Results and Discussion

Capsicum contains various phytochemical compounds, such as polyphenols, flavonoids, and capsaicinoids, which have potential health benefits. The main components of *Capsicum*, CAP, and DHC, regulate pungency and exhibit several physiological and pharmacological effects on the gastrointestinal tract and the respiratory, cardiovascular, sensory, and thermoregulatory systems. Furthermore, CAP exerts anticancer effects and can treat arthritis-related inflammation, pain, neurogenic inflammation, high cholesterol levels, and obesity [30].

Our study focused on analyzing the CAP and DHC levels. Using HPLC/UV, CAP and DHC concentrations in the samples were measured using the known concentrations of the standards (Fig. 3). We observed that in several instances in some samples, either the compounds were not detected or were only detected in trace amounts. Specifically, CAP and DHC were detected in and could be measured in samples ‘SUNGIL-C’, ‘DELC’, ‘CAPSI 23-10’, and ‘CAPSI 23-8’ (Fig. 4). Notably, the cultivar ‘SUNGIL-C’ exhibited the highest CAP and DHC content at 1.92 and 0.88 mg/g extract, respectively. In contrast, ‘DELC’ exhibited lower concentrations of CAP and DHC at 0.12 and 0.14 mg/g extract, respectively. ‘CAPSI 23-10’ displayed intermediate levels of CAP and DHC at 0.44 and 0.24 mg/g extract, respectively. ‘CAPSI 23-8’ had a lower CAP concentration of 0.10 mg/g extract, and trace amounts of DHC were detected (Table 2). Eight samples had no detectable amounts, including ‘26B’, ‘26C’, ‘DELB’, ‘MIGP-C’, ‘MI4H’, ‘MI2F’, ‘MI2H’, and ‘CAPSI 23-4’. Samples ‘MI8H’ and ‘CAPSI 23-9’ had trace amounts of CAP and DHC, but their concentrations could not be measured. Considering the adjusted concentrations from the standards and the levels detected in the samples, it was evident that there was variability in CAP and DHC concentrations among the detectable samples. The differences in the presence and concentration of these compounds among the samples can be attributed to sample diversity or variations in processing methods.

The CAP compositions of hot peppers from six *C. annuum* varieties have been published previously [31]. Through HPLC analysis, notable disparities in the CAP content among the varieties were identified. Notably, the ‘Vulcan’ and ‘Corno di capra’ (*C. annuum* var. *abbreviatum*) samples showed the highest

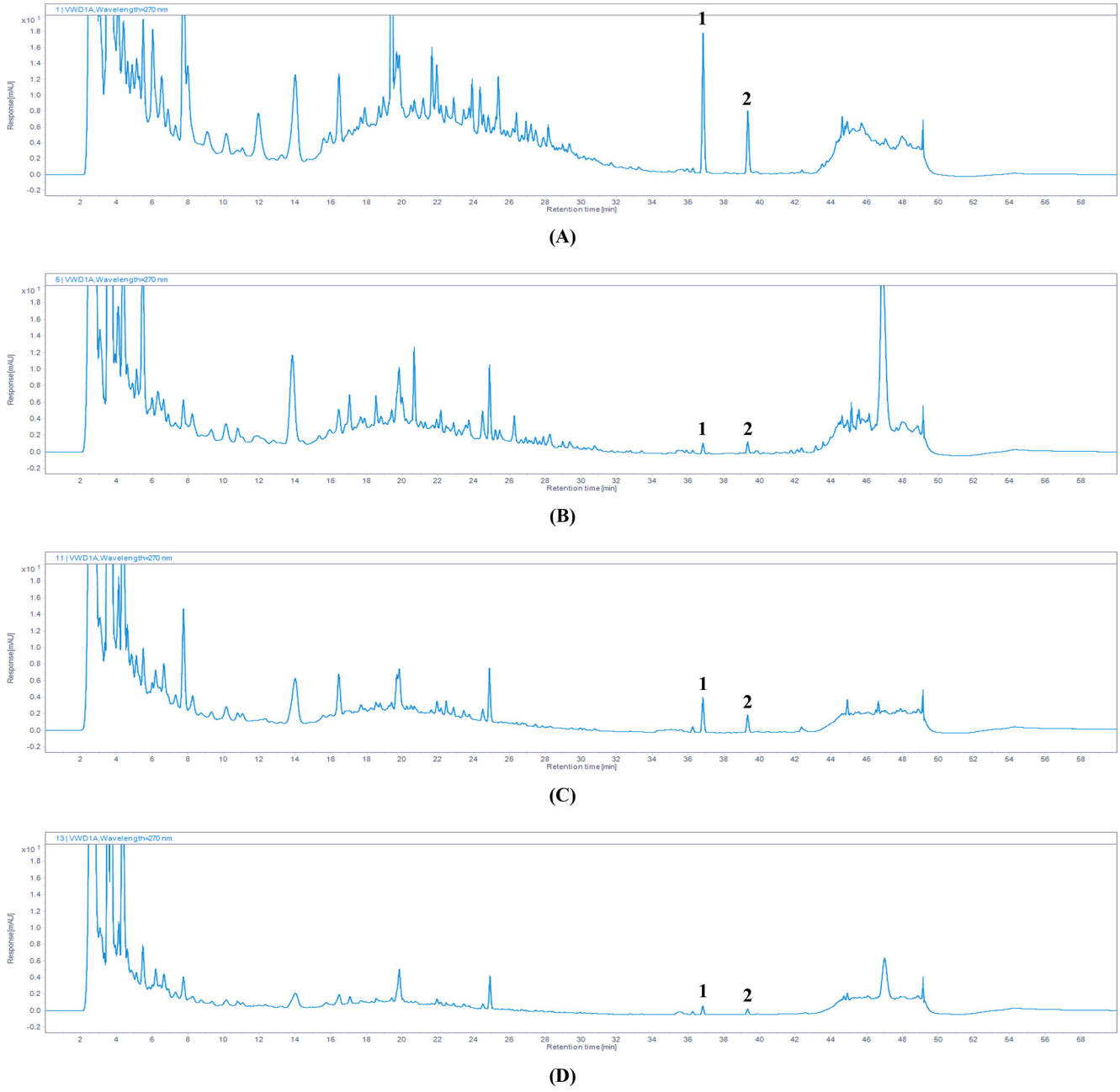

Fig. 4 HPLC/UV chromatograms of *Capsicum* cultivars (A, 'SUNGIL-C'; B, 'DELC'; C, 'CAPSI 23-10'; D, 'CAPSI 23-8')

concentrations of CAP, DHC, and nordihydrocapsaicin, whereas 'Ciliegino' (*C. annuum* var. *cerasiferum*) samples exhibited the lowest levels. Different concentrations of CAP and DHC were found in the different varieties. As documented in various studies, the CAP content of peppers, and consequently, their pungency, is primarily influenced by the species and variety. However, environmental factors, including fruit age, water stress, temperature, soil moisture, and fertilization level, also play significant roles in these variations [32,33].

Table 3 displays the TPC, TFC, DPPH, and $ABTS^+$ scavenging activities of *Capsicum* cultivar extracts. Among samples with detected CAP and DHC via HPLC/UV, 'SUNGIL-C' exhibited the highest TPC, indicating rich polyphenols. Elevated TPC and TFC were shown by 'DELC', while 'CAPSI 23-10' and 'CAPSI 23-8' had lower levels. The DPPH and $ABTS^+$ activities highlighted antioxidant potential. The sample 'SUNGIL-C' had the lowest DPPH IC_{50} (11.5 mg/mL), $ABTS^+$ IC_{50} (2.0 mg/mL), and the highest TPC (36.3 mg TAE/g ext.). Similarly, 'DELC' and 'CAPSI 23-10' showed substantial TPC, TFC, and antioxidant activities. Notably, 'CAPSI 23-8' had slightly lower activities,

Table 2 Content of capsaicin (**1**) and dihydrocapsaicin (**2**) in methanol extracts of *Capsicum* cultivars

Capsicum cultivars	Content (mg/g ext.)	
	1	**2**
'SUNGIL-C'	1.92±0.00	0.88±0.00
'26B'	ND	ND
'26C'	ND	ND
'DELB'	ND	ND
'DELC'	0.12±0.00	0.14±0.00
'MIGP-C'	ND	ND
'MI4H'	ND	ND
'MI2F'	ND	ND
'MI2H'	ND	ND
'MI8H'	tr	ND
'CAPSI 23-10'	0.44±0.00	0.24±0.00
'CAPSI 23-4'	ND	ND
'CAPSI 23-8'	0.10±0.00	tr
'CAPSI 23-9'	tr	ND

ND: not detected
tr: trace

resulting in lower TPC and TFC than the others. These results align with those of the HPLC/UV analysis, indicating 'SUNGIL-C' with the highest CAP, DHC, and TPC, contributing to superior antioxidant capacity, consistent with the capsaicinoid effects.

Considering these findings, it is evident that the concentrations of CAP and DHC exhibited considerable variability among detectable samples. These variations in capsaicinoid levels suggest potential differences in the genetic makeup of the samples or variations in the cultivation and processing methods. Furthermore, understanding the distinct concentrations of these compounds in specific samples, such as those in 'SUNGIL-C', 'DELC', 'CAPSI 23-10', and 'CAPSI 23-8', can provide valuable insights for our breeding program. Using this information, we can strategically select or develop pepper cultivars that align with consumer preferences for pungency, and potentially harness the health benefits of these compounds.

In a previous study, we obtained notable results for our breeding program regarding the contrasting concentrations measured in varieties such as *C. chinense, C. annuum, C. chacoense,* and *C. baccatum* which showed high levels of capsaicinoids. Here, the varieties with higher concentrations, such as *C. chinense* may appeal to consumers who prioritize both spiciness and health benefits [34]. However, varieties such as *C. annuum* may be suitable for consumers who prefer milder flavors. Overall, our study is expected to provide crucial guidance for strategic decisions regarding future cultivar development and consumer preference considerations [35]. We propose that selection of varieties can be expanded based on these factors.

Moreover, the measured concentrations of CAP and DHC extended beyond spiciness levels. They serve as a window into the potential physiological activities of the samples, paving the way for a more nuanced understanding of the biological effects and utility of these compounds. This prompted further investigation into their diverse applications, not only in the culinary domain but also in potential therapeutic avenues.

In conclusion, *Capsicum* is a versatile genus with global significance owing to its diverse culinary, medicinal, and nutritional contributions. The exploration of its uses not only reflects its cultural importance but also underscores its potential health benefits. Therefore, further studies are necessary to fully understand the mechanisms underlying the medicinal properties of *Capsicum* and to explore novel culinary applications in the evolving gastronomic landscape.

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Table 3 The TPC, TFC, DPPH and ABTS$^+$ assay in the representative extracts of *Capsicum* cultivars with detected capsaicin (**1**) and dihydrocapsaicin (**2**) from HPLC/UV results

Capsicum cultivars	Content			
	TPC (mg TAE/g ext.)	TFC (mg QE/g ext.)	DPPH IC_{50} (mg/mL)	ABTS$^+$ IC_{50} (mg/mL)
'SUNGIL-C'	36.3±1.9	12.4±2.9	11.5±0.4	2.0±0.5
'DELC'	32.7±1.3	14.7±0.3	13.1±1.3	4.7±0.3
'CAPSI23-10'	24.3±1.7	3.4±0.5	20.9±0.2	7.6±0.2
'CAPSI23-8'	16.1±0.8	10.0±0.1	21.3±1.1	12.0±0.3
Ascorbic acid	-	-	0.2±0.1	0.1±0.1

Ascorbic acid was used as a positive control

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