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Determination of Capsaicin and Dihydrocapsaicin in Habanero Pepper (*Capsicum chinense* Jacq.) Hybrids Cultivated in Yucatán, México

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Abstract

The Habanero pepper (*Capsicum chinense* Jacq.) is the main crop of the Yucatán Peninsula and is recognized and distinguished from other Habanero peppers cultivated elsewhere in the world due to its aroma, flavor, and high pungency, which is conferred by a group of compounds called capsaicinoids. These compounds are in high demand by various industries due to their antioxidant, anti-inflammatory, analgesic, and antimicrobial properties. The present study aimed to quantify capsaicin and dihydrocapsaicin contents in 29 Habanero pepper hybrids cultivated under greenhouse conditions in Yucatán and to evaluate their pungency potential. Capsaicinoids were extracted from mature fruits using acetonitrile and quantified by HPLC with fluorescence detection (HPLC-FLD). Capsaicin concentrations ranged from 19.95 to 73.55 mg g^{-1} dry weight (DW), while dihydrocapsaicin varied from 4.57 to 14.36 mg g^{-1} DW. Total capsaicinoid content differed significantly among hybrids, ranging from 27.33 to 85.05 mg g^{-1} DW, corresponding to pungency levels between 439,979 and 1,369,360 Scoville Heat Units (SHU). Hybrids H14, H15, and H3 exhibited exceptionally high pungency, exceeding 1.1 × 10^6 SHU. The wide variability observed under uniform growing conditions indicates pronounced genotype-dependent differences in capsaicinoid accumulation and identifies promising hybrid materials for breeding programs and agro-industrial applications requiring elevated capsaicinoid content.

Keywords: *Capsicum chinense*; capsaicinoids; habanero pepper; scoville heat units; HPLC

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1. Introduction

Chilli peppers belong to the Solanaceae family and the *Capsicum* genus, comprising approximately 38 species, of which only five are of major economic importance: *C. baccatum*, *C. annuum*, *C. pubescens*, *C. frutescens*, and *C. chinense* [1,2]. These peppers exhibit remarkable diversity in shape, size, and colour. They are consumed worldwide, valued not only as a spice but also for their rich content of bioactive compounds, including capsaicinoids, carotenoids (provitamin A), flavonoids, vitamins (C and E), minerals, and essential oils [3–6].

Capsaicinoids are the compounds responsible for pungency, inducing a characteristic heat sensation. Twenty-three capsaicinoid analogues have been identified, including capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin, homocapsaicin, and norcapsaicin [7,8]. Among these, capsaicin and dihydrocapsaicin constitute up to 90% of the total capsaicinoid content in most peppers [9]. Traditionally, pungency levels in *Capsicum* species were assessed using the organoleptic Scoville Heat Test, measured in Scoville Heat Units (SHU) [10]. This method determines the degree of dilution required for the pungency to become undetectable to a human taster. However, due to its inherent subjectivity and lack of quantitative precision, the Scoville test has been largely superseded by more reliable and accurate chromatographic techniques. These modern methods include high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC-MS), gas chromatography–mass spectrometry (GC-MS), and UV spectrometry [11–16].

According to Fabela-Morón et al. [17], the selection of an appropriate solvent is a critical factor in achieving efficient extraction. The most used organic solvents include acetone, methanol, ethanol, and acetonitrile [18]. Conventional extraction techniques such as maceration, Soxhlet extraction, or hydrodistillation rely on the solvent's ability to dissolve capsaicinoids under prolonged heat application [19]. Chinn et al. [20] evaluated these methods in Habanero pepper samples and concluded that ethanol and acetonitrile were the most effective solvents. Several authors agree that acetonitrile, due to its intermediate polarity, provides higher recovery rates of capsaicin and dihydrocapsaicin [21–24].

The Habanero pepper (*C. chinense*) is renowned as one of the world's hottest chilli varieties, with capsaicinoid concentrations yielding pungency values typically ranging from 100,000 to 300,000 SHU [17]. In Mexico, its cultivation is primarily concentrated in the Yucatán Peninsula, where it holds significant cultural and economic value, underscored by its designation of origin ("Chile Habanero de la Península de Yucatán") granted in 2010 [25]. This certification guarantees authenticity, quality control, and global product identification, with a specific focus on capsaicinoid content [26]. Habanero peppers from Yucatán are distinguished by their unique flavour, aroma, and exceptionally high pungency, with some genotypes reported to reach levels between 145,950 and 892,719 SHU [27]. Furthermore, previous research has corroborated that pungency is a highly plastic trait strongly influenced by environmental factors, including climate and soil conditions [14,28]. The edaphoclimatic conditions of the Yucatán region appear to be particularly favourable, not only stimulating capsaicin production but also potentially potentiating it to significantly higher levels compared to other growing regions [29].

Habanero pepper serves as a raw material for a wide array of food products, such as fresh fruits, sauces, pastes, dried powder, condiments, and oleoresins, destined for both national and international markets [30,31]. Beyond their culinary use, capsaicinoids possess diverse industrial applications due to their recognised antioxidant [30] anti-inflammatory [32], analgesic [33], and antimicrobial properties [34]. However, capsaicinoid biosynthesis is highly influenced by genotype and its interaction with environmental factors, leading to substantial variation within and among *Capsicum* species and genotypes [14,28]. This study aims to determine and quantify the capsaicin and dihydrocapsaicin content in 29 Habanero pepper hybrids (*C. chinense*) cultivated in Yucatán, Mexico, in order to evaluate their pungency levels and describe variability among hybrids under uniform greenhouse conditions, providing preliminary information for the identification of materials with potential commercial and agro-industrial interest.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

A total of 29 Habanero pepper hybrids (*Capsicum chinense* Jacq.) were evaluated in this study (Figure 1). The plant material was cultivated in the greenhouse facilities of the Seed Production Unit at the Scientific and Technological Park of Yucatán, located in Sierra Papacal, Mérida, Mexico (21°07′20″ N, 89°43′41″ W; 9 m above sea level).

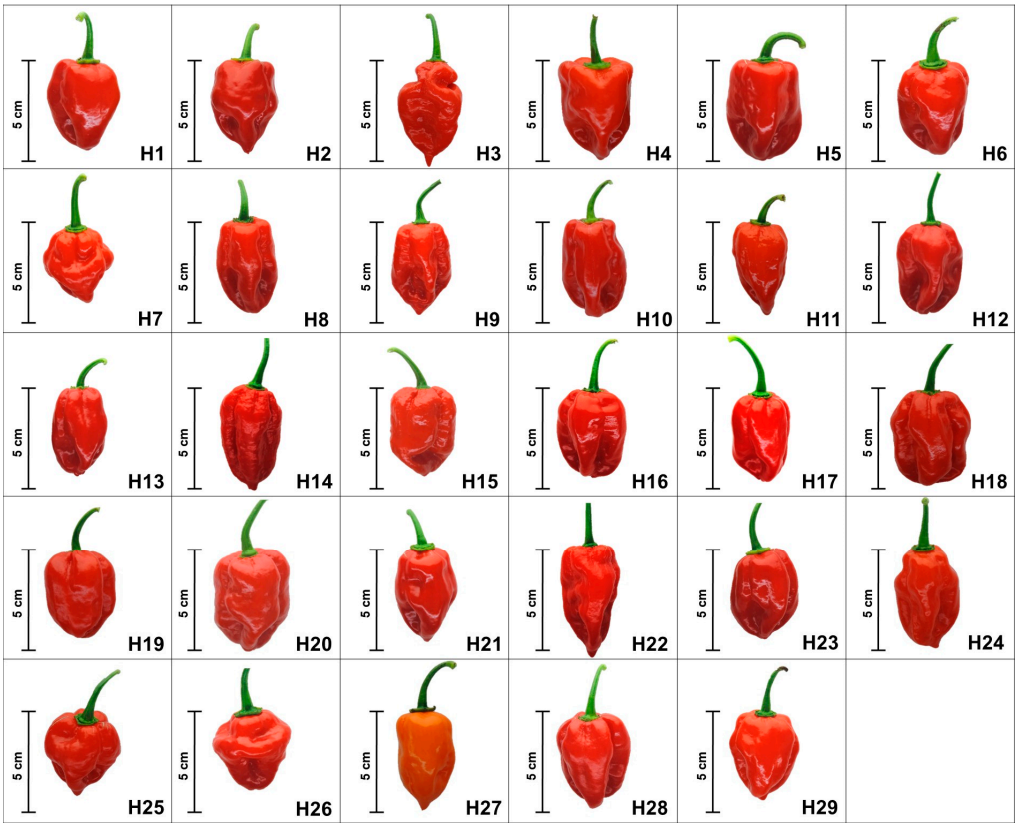


Figure 1. Morphological diversity of 29 hybrids (H1–H29) Habanero pepper (*C. chinense*) developed and grown at Yucatán, Mexico.

Seeds were germinated in 200-cavity polystyrene trays filled with a commercial PEAT MOSS® substrate. After 35 days, seedlings were transplanted into soil under greenhouse conditions, with a planting density of 30 cm between plants and 100 cm between rows. The soils used for cultivation correspond to the predominant edaphic types of the Yucatán Peninsula, characterized by a silty loam texture, low bulk density, high porosity, neutral to slightly alkaline pH, and a high organic matter content.

The cultivation was carried out over a single growing cycle under greenhouse conditions. During the experimental period, the average minimum and maximum temperatures were 18 and 37 °C, respectively, while the mean relative humidity was approximately 86%. Crop management practices, including irrigation, fertilization, and pest and disease control, followed standard regional protocols for Habanero pepper production [35]. Each hybrid was represented by 10 plants grown under the same greenhouse conditions. For capsaicinoid analysis, fruits were harvested at physiological maturity from multiple plants per hybrid during a single harvest event.

Fifty fruits per hybrid were collected and pooled to constitute one composite biological sample, which was considered the experimental unit for chemical analysis. This composite sampling approach was used to obtain a representative estimate of capsaicinoid content at the hybrid level under the evaluated conditions. The peduncle was removed from the fruits,

which were then cut longitudinally. The samples were placed on aluminum trays and dried in a forced-air circulation oven at 60 °C for 48 h. Subsequently, the dried fruits from each hybrid were ground using an electric coffee grinder (Hamilton Beach®, Innsbrook, VA, USA, #80350G) until a fine and homogeneous powder was obtained. Particle size distribution was not quantitatively measured; however, grinding continued until visual homogeneity was achieved. The resulting powder was stored at −80 °C until further analysis.

2.2. Extraction Procedure

This method was implemented following Collins et al. [13] with minor modifications. For capsaicinoid extraction, three technical replicates were prepared from each hybrid using aliquots of the same homogenized composite fruit powder. For most hybrids, 250 mg of dry fruit powder was mixed with 40 mL of acetonitrile. In the case of highly pungent hybrids, a reduced sample mass (100 mg) was used to ensure that extract concentrations remained within the validated calibration range of the analytical method.

The mixtures were maintained in a water bath at 80 °C for four hours with periodic agitation. After cooling to room temperature, the extracts were centrifuged (Sigma, Osterode, Germany, #2–16 KL) at 14,000 rpm for 10 min at 4 °C. The supernatants were then collected and filtered through 0.45 µm PTFE membrane filters (Thermo Scientific™, Waltham, MA, USA, #721-1345) into 2 mL amber glass vials and stored at 4 °C until chromatographic analysis.

2.3. Separation and Quantification of Capsaicinoids

The separation and quantification of capsaicinoids were performed using an HPLC system (Agilent, Santa Clara, CA, USA, 1200 series) equipped with an autosampler and a fluorescence detector. Capsaicinoids were separated on a Zorbax Eclipse Plus C18 (Agilent, Santa Clara, CA, USA, # 880975-914K) column (4.6 mm i.d. × 250 mm) at a column temperature of 25 °C, with an injection volume of 20 µL. The excitation and emission wavelengths for detection were set at 280 and 338 nm, respectively. The mobile phase was operated under isocratic conditions using a methanol–water mixture (73:27, v/v), delivered at a flow rate of 1 mL min^{-1}. The total run time was 15 min.

Quantification was based on an external calibration curve of capsaicin and dihydrocapsaicin standards, for capsaicin prepared in 100% methanol at five concentrations (20, 60, 100, 200 and 300 ppm) and for dihydrocapsaicin prepared in 100% methanol at five concentrations (10, 20, 60, 100 and 150 ppm). The calibration curve was constructed by plotting the peak area as a function of the analyte concentration. The concentrations of the major capsaicinoids were estimated using this curve and are reported in mg g^{-1} of dry weight (DW).

2.4. Analytical Perfomance of the HPLC-FLD Method

The analytical performance of the HPLC-FLD method was primarily assessed in terms of instrumental precision (repeatability), the main parameter supporting the reliability of the quantitative results, particularly in the absence of inter-day reproducibility testing. Instrumental precision was evaluated by six consecutive injections of the same sample extract and expressed as the relative standard deviation (%RSD) of the quantified capsaicinoid concentrations.

Linearity was evaluated using external calibration curves over the tested concentration ranges (20–300 ppm for capsaicin and 10–150 ppm for dihydrocapsaicin). As summarized in Table 1, excellent linearity was obtained for both analytes, with coefficients of determination (R^2) exceeding 0.998.

Table 1. Method validation parameters for capsaicin and dihydrocapsaicin.

Parameter	Capsaicin	Dihydrocapsaicin
Calibration range (ppm)	20–300	10–150
Number of calibration points (*n*)	5	5
Regression equation	$y = 41.996x + 255.29$	$y = 49.969x + 199.52$
Coefficient of determination (R^2)	0.9981	0.9986
LOD (ppm)	18.68	8.39
LOQ (ppm)	56.61	25.43
LOD (mg g^{-1} DW)	2.99–7.47	1.34–3.35
LOQ (mg g^{-1} DW)	9.06–22.65	4.07–10.18
Instrumental precision (%RSD, $n = 6$)	1.88	2.57

Note: LOD and LOQ were calculated as 3.3 σ/S and 10 σ/S, respectively. Values expressed in mg g^{-1} dry weight were calculated considering an extraction volume of 40 mL and the sample mass used for extraction (250 mg for most hybrids and 100 mg for highly pungent hybrids). Instrumental precision (repeatability) was evaluated by six consecutive injections of a representative sample extract.

The sensitivity of the method was assessed through the calculation of limits of detection (LOD) and limits of quantification (LOQ), which were determined according to ICH guidelines using the equations LOD = 3.3σ/S and LOQ = 10σ/S, where σ represents the standard deviation of the regression residuals and S is the slope of the calibration curve. LOD and LOQ values expressed in ppm were subsequently converted to mg g^{-1} dry weight by considering the extraction volume and the sample mass used (100 or 250 mg, depending on hybrid pungency). These sensitivity parameters confirm that the method is appropriate for the range of capsaicinoid concentrations analyzed in this study.

2.5. Conversion to Scoville Heat Units (SHU)

The pungency in Scoville heat units was calculated by multiplying the capsaicinoid concentrations in ppm units (1 ppm =1 μg/g dry weight) with the pungency coefficient of the pure compounds as given by Todd et al. [36], so as 1 ppm C/DHC = 16.1 SHU (Scoville heat units).

2.6. Statistical Analysis

Data analysis was performed using SPSS software, version 22 (IBM Corp., Armonk, NY, USA). Results are presented as mean ± standard deviation (SD) of three technical replicates obtained from a single composite biological sample per hybrid. One-way analysis of variance (ANOVA), followed by Tukey's post hoc test ($p < 0.05$), was used exclusively as a descriptive statistical tool to facilitate the comparative classification of hybrids under identical experimental and analytical conditions. Because the analysis is based on technical replicates derived from pooled samples, observed statistical differences should be interpreted as descriptive and comparative rather than as indicators of genetic stability or broad biological inference. Graphical representations and bivariate visualizations were used to support the comparison and relative ranking of hybrids based on their capsaicinoid profiles. All figures were generated using GraphPad Prism software, version 10.2.3 (GraphPad Software, San Diego, CA, USA).

3. Results and Discussion

Capsaicinoids are the compounds responsible for the characteristic pungency and distinctive flavor of pepper cultivars. Habanero pepper (*C. chinense*) is recognized as one of the most pungent varieties and represents a crop of major social, economic, and cultural

importance in the Yucatán Peninsula. Figure 1 illustrates the morphological diversity of the 29 Habanero pepper hybrids (H1–H29) and the reference varieties evaluated in this study, highlighting the phenotypic variability present within the analyzed germplasm.

The extraction and quantification of the major capsaicinoids, capsaicin and dihydrocapsaicin, in this collection of Habanero pepper hybrids cultivated in Yucatán were carried out using fruits at the mature stage, as this is the developmental phase during which a significant increase in these compounds occurs [37,38]. These studies have shown that both the degree of ripeness and the anatomical part of the fruit significantly influence capsaicin content, with the placenta being the tissue with the highest concentration. Therefore, the selection of an appropriate fruit ripening stage is a determining factor for obtaining representative and comparable pungency values among genotypes.

3.1. Identification and Chromatographic Separation of Capsaicinoids

The two major capsaicinoids, capsaicin and dihydrocapsaicin, were analyzed using high-performance liquid chromatography coupled with fluorescence detection (HPLC-FLD). Figure 2a shows a representative chromatogram obtained from hybrid H5, in which two dominant chromatographic peaks associated with the main pungency-related capsaicinoids can be observed. Capsaicin and dihydrocapsaicin were identified based on the comparison of their retention times with those of analytical standards analyzed under identical chromatographic conditions (Figure 2b).

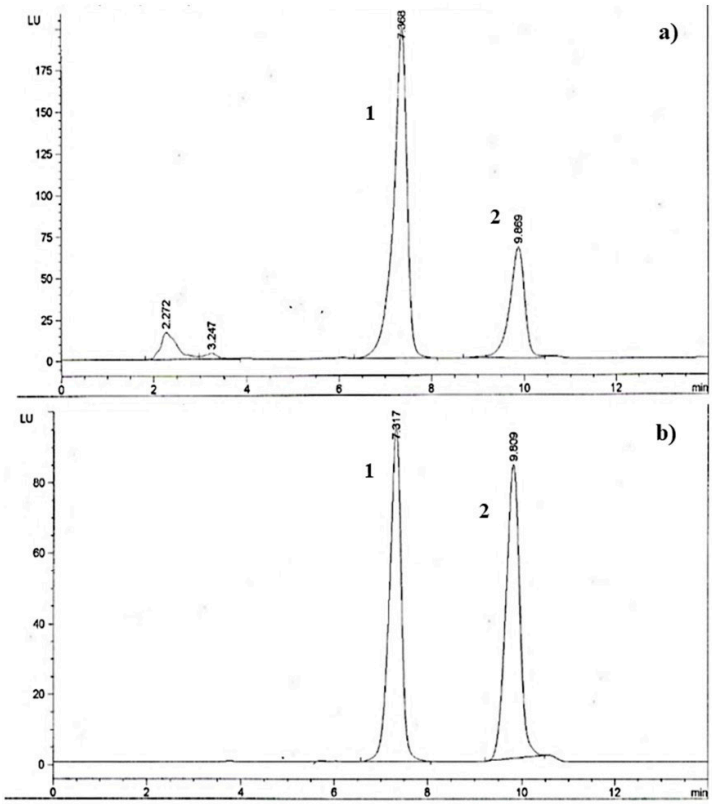


Figure 2. Representative HPLC-FLD chromatogram of a *Capsicum chinense* hybrid (H5) extract (**a**), showing the two dominant chromatographic peaks corresponding to the major pungency-related capsaicinoids. Capsaicin (1) and dihydrocapsaicin (2) were identified based on retention time matching with analytical standards analyzed under identical chromatographic conditions (**b**). The chromatographic method was optimized for the comparative quantification of the two major capsaicinoids; therefore, possible co-elution of minor capsaicinoid analogues cannot be excluded, and no claims regarding compound purity are made. Fluorescence detection: excitation 280 nm, emission 338 nm.

Under the applied chromatographic conditions, two major peaks with retention times of 7.31 and 9.80 min were consistently detected and corresponded to capsaicin and dihydrocapsaicin, respectively. The HPLC method employed was optimized for the comparative quantification of these two predominant capsaicinoids and not for the complete separation of all minor capsaicinoid analogues. Therefore, although reproducible chromatographic behavior was obtained, the possible co-elution of minor capsaicinoids cannot be fully excluded, and no claims regarding compound purity are made. The observed retention times are consistent with those reported in previous studies using reversed-phase C18 columns and methanol-based mobile phases for *Capsicum* fruit extracts [9,13,27], supporting the suitability of the method for comparative capsaicinoid analysis focused on the major pungency-related compounds in *C. chinense*.

3.2. Method Validation

The analytical performance of the HPLC-FLD method was primarily assessed in terms of instrumental precision (repeatability), which represents the main parameter supporting the reliability of the quantitative results, particularly in the absence of inter-day reproducibility testing. Instrumental precision, evaluated as the relative standard deviation (%RSD) from six consecutive injections of a representative sample extract, showed values below 3% for both analytes (1.88% for capsaicin and 2.57% for dihydrocapsaicin), confirming the repeatability and stability of the chromatographic system.

Linearity was evaluated using external calibration curves over the tested concentration ranges (20–300 ppm for capsaicin and 10–150 ppm for dihydrocapsaicin). As summarized in Table 1, excellent linearity was obtained for both analytes, with coefficients of determination (R^2) of 0.9981 for capsaicin and 0.9986 for dihydrocapsaicin.

The calculated limits of detection (LOD) and limits of quantification (LOQ) demonstrated the high sensitivity of the method. For capsaicin, the LOD and LOQ were 18.68 and 56.61 ppm, respectively, while for dihydrocapsaicin, the corresponding values were 8.39 and 25.43 ppm. When expressed on a dry weight basis, these limits corresponded to 2.99 and 9.06 mg g^{-1} DW for capsaicin and 1.34 and 4.07 mg g^{-1} DW for dihydrocapsaicin. These sensitivity parameters confirm that the method is suitable for the concentration ranges of capsaicinoids quantified in the analyzed samples.

3.3. Capsaicinoid Content in Habanero Pepper Hybrids

The capsaicinoid profiles of the evaluated Habanero pepper (*C. chinense*) hybrids exhibited a wide range of concentrations when cultivated under uniform greenhouse conditions. Capsaicin (C) and dihydrocapsaicin (DHC), the two major capsaicinoids responsible for pungency, were detected in all samples, confirming the inherently high pungency of Habanero peppers cultivated in the Yucatán Peninsula. This qualitative profile is consistent with previous reports for *C. chinense*, in which capsaicin is the predominant capsaicinoid, followed by dihydrocapsaicin [27,39].

The evaluated hybrids exhibited a wide range of total capsaicinoid concentrations, from 27.33 to 85.05 mg g^{-1} DW (Table 2), reflecting pronounced variability among hybrids evaluated at the same developmental stage and under identical cultivation and analytical conditions. The total capsaicinoid content found in the different varieties and hybrids evaluated in this study was higher than that reported by other research groups for Habanero peppers cultivated in various regions of the world [38–43].

Table 2. Capsaicinoid content and calculated pungency (SHU) of Habanero pepper (*C. chinense*) hybrids cultivated in Yucatán.

		C mg g^{-1} DW	DHC mg g^{-1} DW	Total Capsaicinoids mg g^{-1} DW	SHU
Hybrids	H1	41.36 ± 2.19 ^{f}	12.02 ± 0.47 ^{b}	53.38 ± 2.67 ^{d}	859,457
	H2	24.06± 1.09 ^{nop}	6.24 ± 0.27 ^{lmn}	30.30 ± 1.37 ^{pqr}	487,852
	H3	59.71 ± 0.22 ^{c}	12.14 ± 0.01 ^{b}	71.85 ± 0.23 ^{b}	1,156,762
	H4	35.88 ± 0.08 ^{g}	9.24 ± 0.16 ^{de}	45.12 ± 0.24 ^{f}	726,415
	H5	26.44± 1.87 ^{klm}	7.23 ± 0.69 ^{hijk}	33.67± 2.57 ^{mno}	542,026
	H6	32.84 ± 0.36 ^{hi}	6.69 ± 0.21 ^{jklm}	39.53 ± 0.57 ^{ijk}	636,497
	H7	40.27 ± 0.97 ^{f}	7.91 ± 0.14 ^{fg}	48.17 ± 1.11 ^{e}	775,592
	H8	35.42 ± 0.86 ^{g}	6.58 ± 0.14 ^{klm}	41.99 ± 1.00 ^{ghi}	676,117
	H9	46.47 ± 5.97 ^{d}	9.38 ± 1.54 ^{de}	55.85 ± 7.52 ^{cd}	899,128
	H10	22.00± 2.05 ^{opq}	8.17 ± 0.82 ^{f}	30.17 ± 2.87 ^{pqr}	485,651
	H11	24.83 ± 0.69 ^{mn}	6.90 ± 0.22 ^{ijkl}	31.73 ± 0.91 ^{opq}	510,838
	H12	35.04 ± 1.48 ^{gh}	8.98 ± 0.52 ^{e}	44.02 ± 2.00 ^{fgh}	708,699
	H13	27.74 ± 0.01 ^{jkl}	4.57 ± 0.08 ^{rs}	32.30 ± 0.09 ^{nop}	520,069
	H14	73.55 ± 0.50 ^{a}	11.50 ± 0.04 ^{b}	85.05 ± 0.55 ^{a}	1,369,360
	H15	63.18 ± 0.27 ^{b}	10.36 ± 0.02 ^{c}	73.54 ± 0.24 ^{b}	1,183,915
	H16	34.57 ± 0.10 ^{gh}	6.22 ± 0.08 ^{mn}	40.78 ± 0.19 ^{ij}	656,592
	H17	32.12 ± 1.04 ^{i}	6.68 ± 0.23 ^{jklm}	38.81 ± 1.27 ^{jk}	624,786
	H18	34.99 ± 3.33 ^{gh}	6.71 ± 0.81 ^{ijklm}	41.70 ± 4.14 ^{ghij}	671,336
	H19	28.43 ± 0.34 ^{jk}	4.89 ± 0.03 ^{qr}	33.33± 0.37 ^{mno}	536,549
	H20	40.32 ± 0.93 ^{f}	7.90 ± 0.24 ^{fgh}	48.22 ± 1.17 ^{e}	776,263
	H21	25.57± 1.98 ^{lmn}	9.75 ± 0.86 ^{cd}	35.32 ± 2.84 ^{lm}	568,665
	H22	44.00 ± 0.74 ^{e}	14.36 ± 0.06 ^{a}	58.36 ± 0.81 ^{c}	939,608
	H23	25.03 ± 0.24 ^{mn}	10.38 ± 0.13 ^{c}	35.41 ± 0.38 ^{lm}	570,083
	H24	29.28 ± 0.23 ^{j}	7.91 ± 0.06 ^{fg}	37.18 ± 0.30 ^{kL}	598,651
	H25	28.38 ± 0.77 ^{jk}	6.53 ± 0.18 ^{lm}	34.91 ± 0.96 ^{lmn}	562,021
	H26	24.31± 0.13 ^{mno}	5.46 ± 0.15 ^{opq}	29.76 ± 0.28 ^{pqr}	479,204
	H27	19.95 ± 0.94 ^{qrs}	7.38 ± 0.36 ^{ghi}	27.33 ± 1.31 ^{rs}	439,979
	H28	35.31 ± 0.01 ^{g}	6.09 ± 0.11 ^{mno}	41.40 ± 0.12 ^{hij}	666,512
	H29	36.86 ± 0.15 ^{g}	9.32 ± 0.10 ^{de}	46.18 ± 0.04 ^{ef}	743,407

Note: Each value represents the mean ± standard deviation (SD) of three technical replicates obtained from a single composite biological sample per hybrid. Averages followed by different letters in the same column are significantly different according to Tukey's test ($p < 0.05$) and are intended for comparative purposes under identical analytical conditions. C: Capsaicin; DHC: Dihydrocapsaicin; SHU: Scoville Heat Units.

The major and predominant capsaicinoid in all the Habanero pepper hybrids evaluated was capsaicin, a pattern consistent with previous studies identifying capsaicin as the primary determinant of pungency in highly pungent *C. chinense* genotypes [27,44]. The concentrations detected in the present study ranged from 19.95 to 73.55 mg g^{-1} dry weight (DW) (Table 2 and Figure 3). The lowest capsaicin concentration was observed in hybrid H27 (19.95 mg g^{-1} DW), which is comparable to the capsaicin levels reported for moderately pungent chili peppers. Similar values have been documented in Mexican chili varieties analyzed by HPLC, where capsaicin concentrations of approximately 20–23 mg g^{-1} DW were reported depending on the cultivar and the fruit maturity stage [45]. These results confirm that even the least pungent hybrid evaluated in this study maintains a capsaicin content characteristic of chili peppers with a high pungency value.

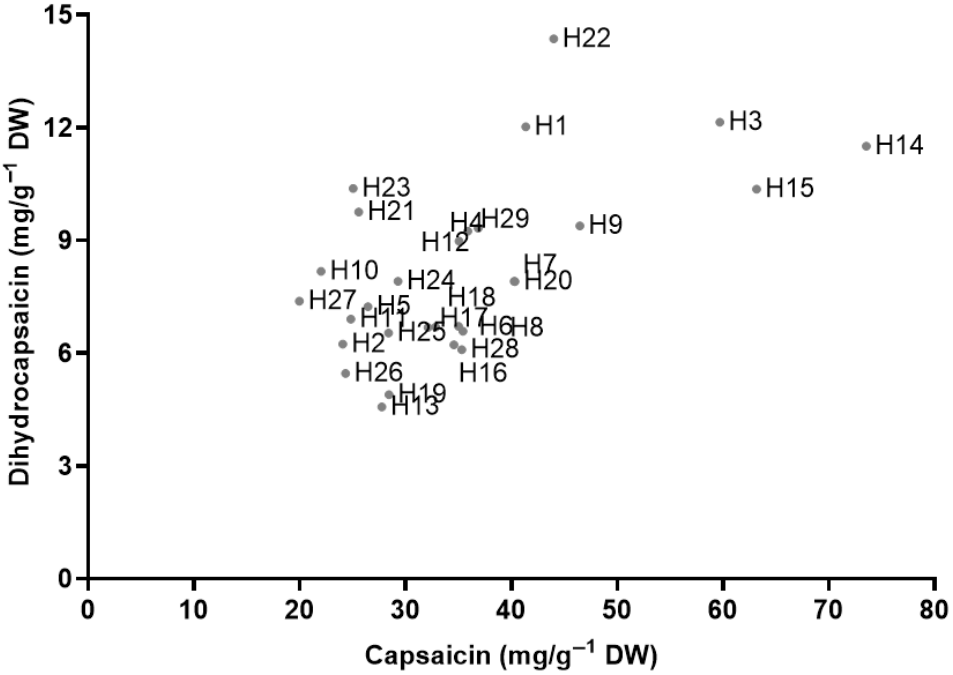


Figure 3. Bivariate relationship between capsaicin and dihydrocapsaicin concentrations in 29 *C. chinense* hybrids grown under the same environmental conditions.

In contrast, hybrids H14, H15, and H3 exhibited markedly higher capsaicin concentrations (73.55, 63.18, and 59.71 mg g^{-1} DW, respectively), positioning them at the upper end of the concentration range reported for *C. chinense*. Previous studies focusing on the placental tissue of red Habanero peppers have reported capsaicin concentrations of approximately 30–35 mg g^{-1} DW [46], which are substantially lower than those observed in these hybrids under the conditions evaluated here. These differences highlight hybrid-specific variation in capsaicin accumulation, although the influence of environmental and developmental factors cannot be excluded.

Dihydrocapsaicin concentrations ranged from 4.57 to 14.36 mg g^{-1} DW (Table 2), with statistically significant differences among hybrids ($p < 0.05$). The highest value recorded in hybrid H22 (14.36 mg g^{-1} DW) is comparable to ranges reported in previous studies. For instance, in different *Capsicum* cultivars, dihydrocapsaicin concentrations have been reported to reach up to approximately 10.14 mg g^{-1} DW in Serrano pepper, with substantial variation among varieties (0.63–10.14 mg g^{-1} DW) [47].

When comparing these results with those reported in the literature for different *Capsicum* species, where dihydrocapsaicin content is often expressed in μg g^{-1}, it is evident that the maximum documented values reach approximately 11.97 mg g^{-1} DW (equivalent to 11,969 μg g^{-1}) in extremely pungent cultivars such as Carolina Reaper (*C. chinense*) [48]. This demonstrates that the values detected in the present study fall within the range previously observed for capsaicinoids in chili fruits, although some of our samples slightly exceed the maximum values previously reported.

The bivariate relationship between capsaicin and dihydrocapsaicin concentrations (Figure 3) illustrates that hybrids with high capsaicin levels generally also exhibit elevated dihydrocapsaicin contents, although the relative contribution of each compound varies among genotypes. This pattern reflects differences in capsaicinoid composition rather than a fixed proportional relationship between the two compounds.

The combined contribution of capsaicin and dihydrocapsaicin resulted in total capsaicinoid concentrations corresponding to pungency levels ranging from 439,979 to 1,369,360 Scoville Heat Units (SHU) (Figure 4). Hybrids H14, H15, and H3 consistently exhibited

SHU values exceeding 1.0×10^6, placing them among the most pungent Habanero pepper hybrids reported to date for materials cultivated in Yucatán. The strong correspondence between total capsaicinoid concentration and SHU values supports the use of HPLC-based quantification as a reliable approach for pungency assessment, in agreement with previous analytical studies [13,49,50].

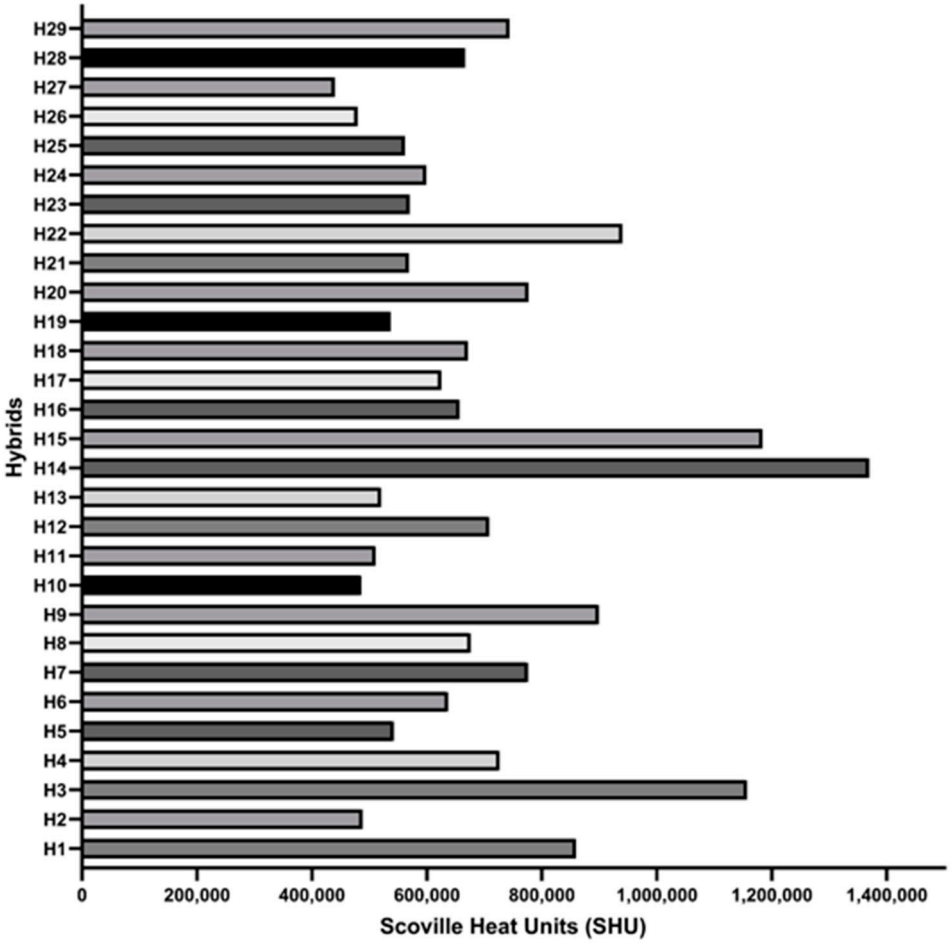


Figure 4. Total capsaicinoid content and corresponding pungency expressed as Scoville Heat Units (SHU) in Habanero pepper (*C. chinense*) hybrids cultivated in Yucatán. Values were calculated from capsaicinoid concentrations determined by HPLC.

For comparison, the natural hybrid Bhut Jolokia (*Capsicum chinense* × *Capsicum frutescens*) has been reported to reach pungency levels of approximately 1,001,304 SHU [40]. In the present study, three hybrids—H14 (1,369,360 SHU), H15 (1,183,915 SHU), and H3 (1,156,762 SHU exhibited pungency values comparable to or exceeding this reference, underscoring the high pungency potential expressed by certain hybrid materials under the evaluated greenhouse conditions. Similar trends have been reported for other hybrid peppers, which often express elevated capsaicinoid levels under controlled agronomic management [39].

Although all hybrids were cultivated under greenhouse conditions to minimize environmental variability, capsaicinoid biosynthesis is known to be influenced by climatic factors, soil characteristics, and their interaction with genetic background [39,51]. In addition, the present study was conducted during a single growing season, and previous research has demonstrated significant interannual variation in capsaicinoid accumulation, including genotype × environment interactions [52]. Consequently, the results presented

here should be interpreted as descriptive of hybrid performance under the specific conditions evaluated rather than as indicators of long-term stability.

Differences between greenhouse and open-field cultivation systems may further influence capsaicinoid accumulation. Studies in *C. annuum* and *C. chinense* have shown that open-field conditions can lead to distinct capsaicinoid profiles compared to greenhouse cultivation, potentially due to increased exposure to abiotic stress factors such as temperature fluctuations and solar radiation [53]. In addition, soil nutrient availability and chemical composition, which were not characterized in detail in the present study, may also contribute to variability in pungency [54].

Despite the limitations associated with evaluation during a single growing season and under greenhouse conditions, the identification of multiple hybrids with capsaicinoid concentrations and pungency values exceeding 1,000,000 SHU constitutes a relevant contribution to the characterization of Habanero pepper germplasm from the Yucatán Peninsula. These results provide high-value preliminary information for agro-industrial applications requiring elevated capsaicinoid contents, including the food, pharmaceutical, and cosmetic industries, where these compounds are valued for their antioxidant, anti-inflammatory, analgesic, and antimicrobial properties [55]. Furthermore, the findings of this study establish a solid foundation for future multi-year and field-based research aimed at evaluating the stability and agronomic performance of high-pungency hybrid materials.

4. Conclusions

The present study provides a comparative evaluation of the capsaicinoid composition of 29 Habanero pepper (*C. chinense*) hybrids cultivated under controlled greenhouse conditions in the Yucatán Peninsula. The results confirmed that capsaicin and dihydrocapsaicin are the predominant compounds contributing to the characteristic pungency of Habanero peppers. A wide range of capsaicinoid contents was observed among the evaluated hybrids, with total capsaicinoid concentrations ranging from 27.33 to 85.05 mg g^{-1} dry weight (DW) and pungency values exceeding 1.3×10^6 SHU in the most pungent materials.

Despite cultivation under homogeneous greenhouse conditions, marked differences in capsaicinoid accumulation were detected among hybrids, indicating consistent genotype-dependent variation under the evaluated conditions. Hybrids H14, H15, and H3 exhibited the highest capsaicinoid levels, highlighting their potential interest for selection and agro-industrial applications requiring elevated pungency. However, because the study was conducted during a single growing season, these results should be interpreted as descriptive of hybrid performance under specific conditions rather than as indicators of long-term stability.

Finally, the satisfactory instrumental precision of the HPLC-FLD method supports its suitability for the comparative quantification of the major capsaicinoids in *C. chinense* fruit extracts. The analytical performance obtained provides confidence in the quantitative results generated under the evaluated conditions, confirming the applicability of the method as a reliable tool for research and quality control purposes in the food and pharmaceutical industries.

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Abbreviations

The following abbreviations are used in this manuscript:

HPLC	High-Performance Liquid Chromatography
SHU	Scoville Heat Units
DW	Dry Weight
C	Capsaicin
DHC	Dihydrocapsaicin
SD	Standard Deviation
ANOVA	Analysis of Variance
LC-MS	Liquid Chromatography–Mass Spectrometry
GC-MS	Gas Chromatography–Mass Spectrometry
UV	Ultraviolet

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