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Research Article

The pungency of Malaysian peppers: Quantification of capsaicin using high-performance liquid chromatography

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Abstract
Capsaicin, a pungent alkaloid primarily found in chili peppers (*Capsicum* spp.), has broad applications in food, medicine, and agriculture due to its bioactive properties. This study aimed to rapidly quantify capsaicin content in ten cultivars of Malaysian chili peppers using a validated high-performance liquid chromatography (HPLC) method. Samples were categorized into pericarp, placenta, and seeds to assess tissue-specific distribution. The analysis revealed significant variability in capsaicin concentrations among cultivars and tissues, with the highest levels observed in *Capsicum chinense* (habanero) and *C. frutescens* (bird's eye chili), and the lowest in *C. annuum* bell peppers. The placenta consistently exhibited the highest capsaicin content, followed by the pericarp and seeds. These findings highlight the critical role of genetic and anatomical factors in determining pungency and confirming the utility of HPLC for accurate and efficient quantification of capsaicin. This research contributes to understanding pungency in Malaysian peppers and supports their potential applications in functional foods, nutraceuticals, and crop breeding programs.

Keywords: *Capsicum*, capsaicin, pungency, high-performance liquid chromatography, Malaysian peppers

Introduction

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide, $C_{18}H_{27}NO_3$) has long been recognized as the primary pungent component in peppers. It is a naturally occurring volatile compound responsible for the characteristic heat and irritation experienced when consuming chili peppers [1, 2]. While most associated with chili peppers, capsaicin has also been identified in other plant species. Structurally, capsaicin consists of a substituted benzene ring attached to a long hydrophobic alkyl chain via a polar amide linkage [3]. Among the group of capsaicinoids, capsaicin is the most prevalent, typically followed by dihydrocapsaicin and nordihydrocapsaicin, which make up around 66%, 22%, and 7%, respectively; the remaining 5% consists of minor capsaicinoids [4, 5]. These compounds share a conserved chemical backbone but vary in their hydrophobic side chains [6], as illustrated in **Figure 1**.

The concentration of capsaicinoids in chili peppers is closely linked to the stage of fruit ripeness [7]. As the peppers mature and change in colour from green to red, there is a progressive increase in capsaicinoid accumulation. Research has shown that both genetic factors and environmental conditions significantly influence the pungency and capsaicinoid levels in chili peppers. Other variables, such as the ripening stage, species, cultivar, and climate, also contribute to these differences [8, 9]. For instance, dry environments tend to reduce the levels of capsanthin, a major pigment in peppers [10]. Furthermore, the method used for drying chili peppers affects their capsaicinoid profiles, with drying generally leading to an increase in capsaicinoids over storage time [11]. Capsaicinoids are primarily synthesized and stored in the placental epidermis as the fruit matures, but they are also found in the pericarp and seeds [12].

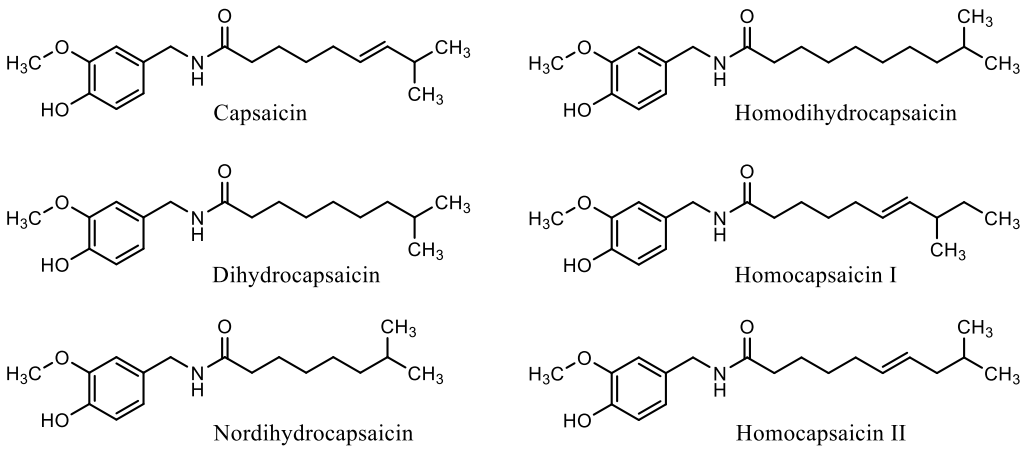

Figure 1. Structures of capsaicin and its analogs

Capsaicin's pungency arises from its capacity to activate sensory neurons in the oral mucosa, producing the characteristic burning sensation associated with chili consumption [13]. The degree of heat is quantified in Scoville Heat Units (SHU), where a higher SHU denotes a more intense spiciness [14]. It is estimated that 1 µg of capsaicin corresponds to approximately 16 SHU [15, 16]. Both the intensity of red coloration and the level of pungency are widely recognized as key indicators of chili quality [17]. A study on capsaicinoid content in foods consumed in Korea showed that capsaicin concentrations vary significantly among pepper cultivars [18]. Based on SHU, chili peppers can be classified into three categories: mildly spicy (SHU < 2,500), moderately spicy (2,500 < SHU < 30,000), and extremely spicy (SHU > 30,000) [19]. In comparison, pure capsaicin registers between 15,000,000 and 16,000,000 SHU [20]. Notably, capsaicin is synthesized mainly in the vacuoles of epidermal cells within the placenta of the fruit. It is then translocated through the ovary septum to other tissues, making the upper interior region of the fruit -often called the "pepper roof"- the hottest part [21].

The biosynthetic pathway of capsaicin involves a complex network of enzymatic reactions, many of which are still not fully elucidated, making the regulation of capsaicin synthesis challenging. Four major enzymes play critical roles in this process. Phenylalanine ammonia-lyase (PAL) initiates the phenylpropanoid pathway by converting phenylalanine into cinnamic acid [22]. Additional enzymes, including cinnamic acid-4-hydroxylase (C4H), *p*-coumaric acid-3-hydroxylase (C3H), and caffeic acid *O*-methyltransferase (CAOMT) contribute to the upregulation of capsaicin levels by catalysing key steps in this pathway [23]. Simultaneously, the fatty acid biosynthesis route generates 8-methyl-6-CoA, a crucial precursor for the branched-chain fatty acids involved in capsaicin formation. Ultimately, the condensation of vanillylamine and 8-methyl-6-nonenoyl-CoA, facilitated by the enzyme acyltransferase (AT), results in the final production of capsaicin (**Figure 2**) [24].

High-performance liquid chromatography (HPLC) is widely recognized as a reliable technique for the rapid and accurate quantification of capsaicin in plant matrices [25]. Previous studies have reported variations in capsaicin content across different *Capsicum* species, with concentration differences observed between different fruit tissues pericarp, seeds, and placenta [26]. However, limited studies have specifically examined Malaysian peppers, which are known for their unique pungency profiles.

This study aims to rapidly determine and quantify the capsaicin content in ten cultivars of Malaysian peppers (*Capsicum* spp.) using HPLC. The research sought to investigate the distribution of capsaicin across different fruit tissues, pericarp, seeds, and placenta, to better understand the variations in pungency within and between species. By developing a reliable and efficient HPLC method for capsaicin analysis, this study provides valuable insights into the bioactive composition of Malaysian peppers, supporting their potential applications in the food industry, pharmacological research, and nutraceutical development.

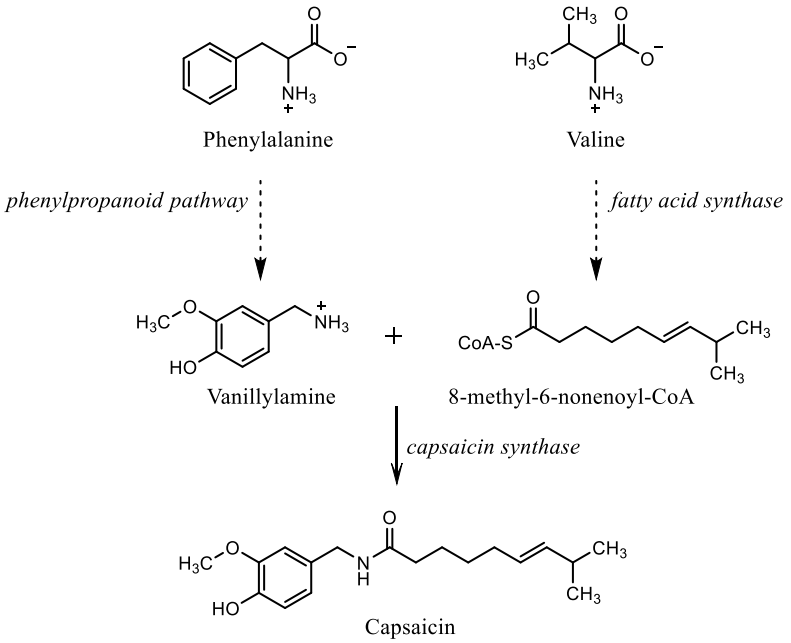

Figure 2. Biosynthesis of capsaicin

Materials and Methods

Plant material

The capsaicin content was analysed in 10 chili pepper cultivars of the following species: *C. annuum* (8 cultivars; dry chili, green chili, red chili, bell peppers of different colors, lada solok, and friggitello), *C. chinense* (1 cultivar; habanero), and *C. frutescens* (1 cultivar; bird-eye chili). The samples (**Figure 3**) were purchased from local supermarkets.

Chemicals and reagents

The reference standard of capsaicin (≥ 98%) from Capsicum spp. was purchased from Henan Qiambaiguan Biotechnology Co., Ltd (Mengzhou, Henan). The solvents (methanol, hexane, and acetone) for optimising the capsaicinoid extraction process were of analytical grade. The acetonitrile for chromatographic separation was from Fischer Scientific, Waltham, (MA, USA). Tap water was purified by ELGA PURELAB® Option water purification system with a resistivity of 18 MΩ.cm^{-1} at 25°C (Veolia Water Technologies, Paris, France) to produce ultrapure water.

Preparation of reference standard solution

A capsaicin stock solution was prepared by accurately weighing 50 mg of capsaicin reference standard and dissolving it in 25 mL of methanol (99.8%, Fischer Scientific, Waltham, MA, USA), yielding a final concentration of 2000 µg/mL. The solution was sonicated for 30 minutes to ensure complete dissolution. This stock solution was then diluted with methanol to prepare a working standard solution of 100 µg/mL. A series of standard solutions was prepared via two-fold serial dilution, resulting in final concentrations of 100, 50, 25, 12.5, and 6.25 µg/mL. All standard solutions were stored at 4 °C for short-term use, and at –18 °C for long-term preservation.

Preparation of sample solution

The pepper fruits were initially washed and air-dried before being manually separated into three distinct tissue parts: pericarp, seeds, and placenta (**Figure 4**). Each tissue type was then oven-dried and finely ground into a uniform powder. For extraction, approximately 10.0 ± 0.5 g of each dried sample was accurately weighed and subjected to sonication in 99.8% methanol for 1 hour to facilitate the extraction of capsaicin. The resulting mixture was filtered through Whatman filter paper to obtain a clear extract. The extraction was repeated until capsaicin was no longer detected in the sample by TLC analysis with a capsaicin standard. The methanol was then evaporated under reduced pressure using a rotary evaporator set at 60 rpm to concentrate the extract. The residue was weighed and reconstituted in 10 mL of 99.8% methanol to achieve a final concentration of 1 mg/mL extracts for subsequent HPLC analysis.

HPLC analysis and quantification of capsaicinrea

Quantification of capsaicin was carried out using an HPLC system equipped with a UV detector. Separation was achieved using a pH resistant Inertsil C18 column (150 × 4.6 mm, 5 µm). The mobile phase consisted of a gradient elution of acetonitrile and water (ACN:H_2O), starting from 30:70 and increasing

to 60:40 over a 20-minute run. The flow rate was maintained at 1.0 mL/min. The injection volume for each sample was 20 µL, and the column temperature was set at 35 °C. Detection was carried out at a wavelength of 280 nm, and chromatographic data were processed and recorded using a compatible integrator.

The injection sequence included a blank (diluent), three replicates of the capsaicin reference standard, the test solution, and a final blank for rinsing. Capsaicin concentrations in the samples were determined based on a calibration curve constructed from standard solutions with concentrations ranging from 10 to 100 µg/mL. Each standard was injected in triplicate, and the calibration curve was generated by plotting the mean peak area against the corresponding capsaicin concentration. Linear regression analysis was performed to determine the slope, intercept, and correlation coefficient (r) of the curve. Capsaicin levels in the test extracts were quantified by interpolating their peak areas onto the standard calibration curve.

Results and Discussion

A series of standard reference solutions of capsaicin with concentrations of 100, 50, 25, 12.5, and 6.25 µg/mL were prepared and analysed using high-performance liquid chromatography (HPLC). Each standard solution was injected into the HPLC column in triplicate to ensure analytical reproducibility and precision. The capsaicin peak was consistently observed at a retention time of approximately 8.531 minutes (**Figure 5**), confirming the specificity of the method.

The calibration curve for capsaicin quantification was constructed using five standard solutions prepared in methanol at concentrations of 100, 50, 25, 12.5, and 6.25 µg/mL. The mean peak areas obtained from HPLC analysis were plotted against the corresponding concentrations to generate the curve (**Figure 6**). The resulting regression equation, $y = 60.191x - 2.0417$, demonstrated excellent linearity, with a correlation coefficient of $R^2 = 0.9999$, confirming a strong linear relationship between peak area and capsaicin concentration. The method's sensitivity was further validated by determining a limit of detection (LOD) of 0.145 µg/mL and a limit of quantification (LOQ) of 0.480 µg/mL.

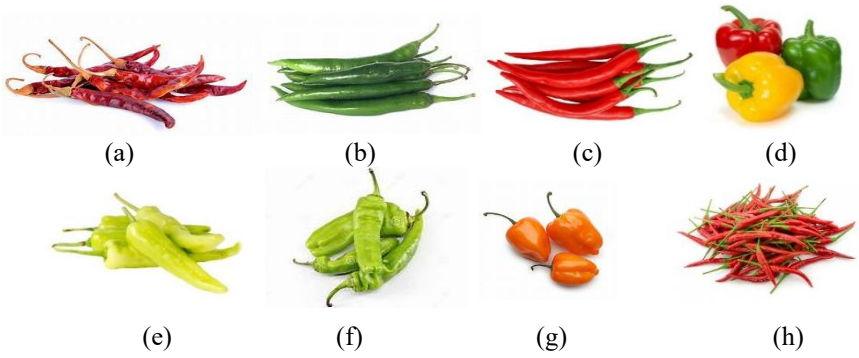

Figure 3. Cultivars of Malaysian chili peppers; (a) dry chili, (b) green chili, (c) red chili, (d) bell peppers, (e) lada solok, (f) friggitello, (g) habanero, and (h) bird-eye chili.

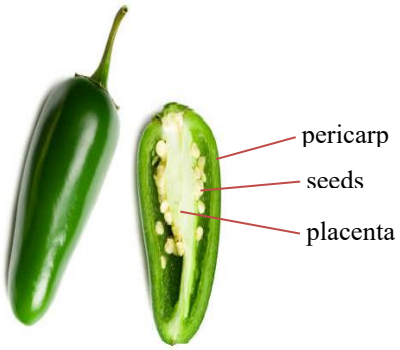

Figure 4. The cross-section of a chili pepper showing the pericarp, seeds, and placenta of the fruit

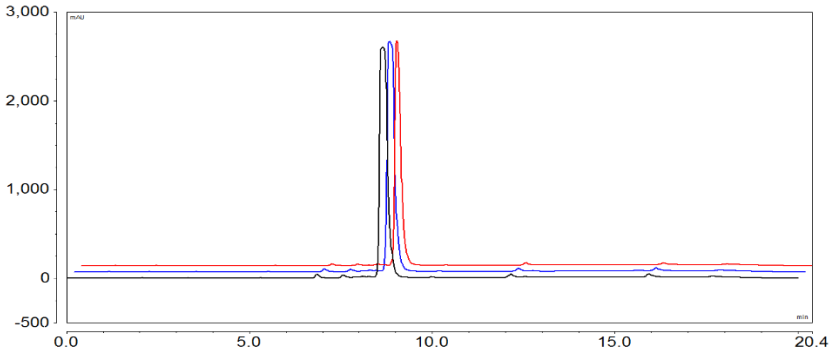

Figure 5. Overlayed chromatograms of capsaicin standard, analysed in triplicate

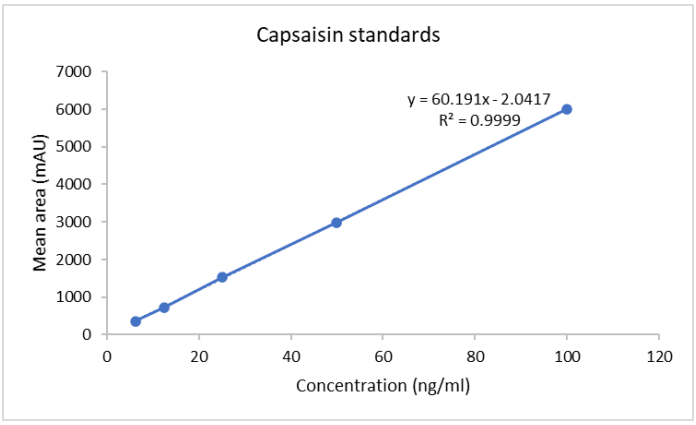

Figure 6. Calibration curve of capsaicin standards

This calibration model was subsequently used to quantify capsaicin content in chili pepper extract samples by interpolating their peak areas onto the calibration curve. This approach enabled the accurate and reliable determination of capsaicin concentrations in the various tissue samples examined in the study.

The identification of capsaicin in chili pepper extracts was carried out using the same chromatographic conditions that were optimized for the capsaicin reference standard. This ensured consistency and reliability in compound detection across all samples. Capsaicin in each extract was identified by comparing the retention time (Rt) of sample peaks with that of the authentic capsaicin standard. The standard exhibited a well-defined peak at 8.531 minutes. In all sample chromatograms, a corresponding peak was observed consistently within a narrow retention time window of 8.511 to 8.532 minutes, indicating the presence of capsaicin. The close agreement in retention times confirmed the identity of the compound in the extracts as capsaicin.

Each chili pepper cultivar was dissected into three distinct fruit tissues: pericarp, placenta, and seeds, to examine the tissue-specific distribution of capsaicin. These individual tissues were analysed independently under the same HPLC conditions. The chromatographic data for all tissues across the ten cultivars revealed that capsaicin was present in all tissue types, though with marked differences in concentration, which are discussed in the subsequent section. A representative chromatogram highlighting the capsaicin peak is shown in **Figure 7**.

Quantification of capsaicin was carried out by comparing the peak areas of the sample extracts with a previously established capsaicin calibration curve. The calibration curve was generated using capsaicin standard solutions at concentrations ranging from 6.25 to 100 ng/mL. For analysis, chili pepper extracts were prepared at a concentration of 1 mg/mL. From this extract, 20 nL was injected into the HPLC, corresponding to 20 ng of sample introduced per injection.

Although quantification was based on comparison of peak areas between the standards and samples, it was essential to account for the precise amount of sample injected. Therefore, the capsaicin concentrations obtained from the calibration curve were normalized to the amount of extract injected. Final capsaicin

concentrations in the chili pepper extracts were thus calculated and expressed as ng of capsaicin per mg of extract, allowing for accurate and comparable quantification across all samples. The capsaicin content in each sample was calculated and expressed in ng/mg of extract. Chromatographic retention times, peak areas, and calculated capsaicin concentrations for all samples are summarized in **Table 1**.

All capsaicin concentrations measured in the pepper extracts were well above both the LOD and LOQ, affirming the method's robustness and reliability. The precision and accuracy of the quantification were within acceptable analytical limits. The method's precision deviation did not exceed 2.09%, indicating a high level of reproducibility and analytical confidence.

The analysis demonstrated significant differences in capsaicin content across the ten *Capsicum* cultivars studied. Among them, the highest concentrations of capsaicin were observed in *Capsicum chinense* (habanero) and *Capsicum frutescens* (bird's eye chili), followed by *Capsicum annuum* cultivars such as friggitello, dry chili, red chili, green chili, and lada solok. In contrast, bell peppers—specifically the red, yellow, and green varieties—exhibited the lowest levels of capsaicin. This pattern suggests a clear trend where smaller, hotter pepper varieties contain substantially higher amounts of capsaicin compared to larger, milder ones. These findings are consistent with previous research, which has highlighted the influence of genetic factors on capsaicin biosynthesis and accumulation [27]. Such variations are likely attributable to differences in the expression of biosynthetic pathway enzymes among species and cultivars.

Among the different fruit tissues analysed, the placenta exhibited the highest concentration of capsaicin, followed by the pericarp and, lastly, the seeds. This distribution pattern supports previous findings that capsaicin biosynthesis primarily takes place in the placental tissue of chili peppers, where specialized epidermal cells are responsible for its production [5]. The elevated capsaicin content in the placenta is attributed to the presence of capsaicinoid-synthesizing enzymes and active metabolic pathways concentrated in this tissue. In contrast, the pericarp and seeds contain comparatively lower levels, likely due to limited direct biosynthetic activity and passive diffusion from adjacent tissues. These findings suggest that the pungency level of Malaysian peppers is strongly influenced by the capsaicin concentration within the placenta, making it the key determinant of overall heat intensity in the fruit. Understanding this tissue-specific distribution of capsaicin is crucial for both breeding programs targeting pungency traits and for optimizing the use of chili peppers in functional food applications.

The HPLC method employed in this study proved to be efficient, producing well-resolved capsaicin peaks with minimal interference. The method demonstrated high sensitivity and reproducibility, making it suitable for routine capsaicin quantification in food and pharmaceutical applications. The use of methanol-based extraction provided optimal capsaicin recovery, like previous extraction protocols [25].

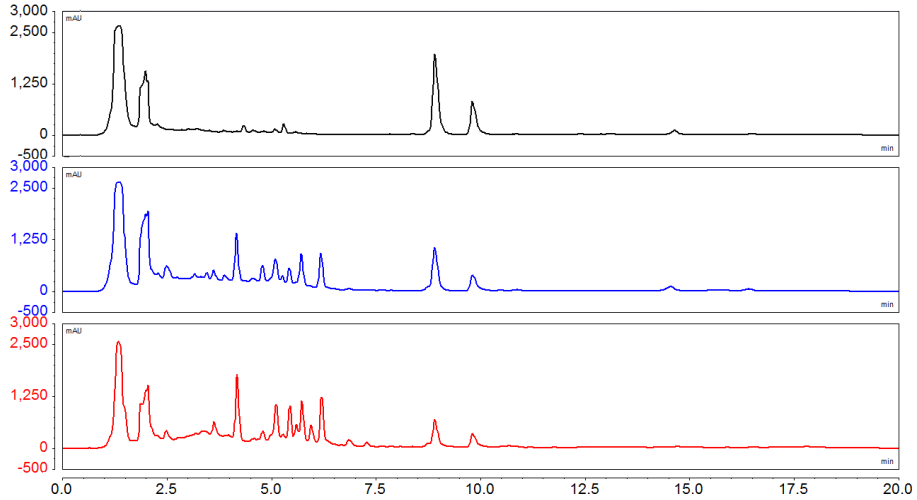

Figure 7. Chromatograms of pepper tissue extracts; placenta (top), pericarp (middle), and seeds (bottom). The peak corresponding to capsaicin is at Rt 8.530 min

Table 1. Capsaicin concentration in chili pepper extracts

Cultivar	Tissue	Retention Time (min)	Peak Area (mAU·s)	Capsaicin Concentration (ng/mg extract) (Mean ± SD)
Dry chili	Placenta	8.531	1491.010	1238.6 ± 4.2
	Pericarp	8.532	1036.448	861.0 ± 3.1
	Seed	8.532	325.712	270.6 ± 2.3
Green chili	Placenta	8.515	574.181	477.0 ± 1.4
	Pericarp	8.511	553.957	460.2 ± 1.2
	Seed	8.512	338.232	281.0 ± 3.3
Red chili	Placenta	8.522	985.406	818.6 ± 0.4
	Pericarp	8.522	1055.709	877.0 ± 1.2
	Seed	8.523	294.413	244.6 ± 4.6
Bell pepper (green)	Placenta	8.515	325.712	270.6 ± 3.2
	Pericarp	8.512	314.156	261.0 ± 3.6
	Seed	8.511	-	ND
Bell pepper (yellow)	Placenta	8.521	317.045	263.4 ± 4.7
	Pericarp	8.512	309.340	257.0 ± 3.2
	Seed	8.522	-	ND
Bell pepper (red)	Placenta	8.517	385.903	320.6 ± 4.8
	Pericarp	8.515	333.417	277.0 ± 2.6
	Seed	8.511	-	ND
Lada solok	Placenta	8.530	535.177	444.6 ± 4.6
	Pericarp	8.529	526.028	437.0 ± 1.2
	Seed	8.532	316.082	262.6 ± 2.6
Friggitello	Placenta	8.514	2059.213	1710.6 ± 1.2
	Pericarp	8.520	1778.001	1477.0 ± 5.1
	Seed	8.518	338.232	281.0 ± 4.3
Habanero	Placenta	8.531	6312.791	5244.0 ± 3.2
	Pericarp	8.531	6161.711	5118.5 ± 5.5
	Seed	8.532	318.489	264.6 ± 1.1
Bird-eye chili	Placenta	8.512	5114.990	4249.0 ± 5.2
	Pericarp	8.516	3778.148	3138.5 ± 4.3
	Seed	8.518	294.413	244.6 ± 2.2

ND = not detected

Conclusion

This study successfully quantified capsaicin content in ten Malaysian chili pepper cultivars using a reliable and efficient HPLC method, providing new insights into the variation in pungency across different species and fruit tissues. The findings clearly indicate that both the cultivar and the anatomical part of the fruit significantly influence capsaicin levels. The highest concentrations were consistently found in the placenta, reaffirming its role as the primary site of capsaicin biosynthesis. Among the cultivars analysed, *Capsicum chinense* and *C. frutescens* exhibited markedly higher capsaicin content compared to *C. annuum*, underscoring the importance of genetic factors in determining pungency levels.

The results not only confirm patterns observed in previous studies but also expand current understanding by characterizing locally available Malaysian varieties, which are often underrepresented in global datasets. The use of HPLC in this context proved to be a robust and reproducible approach for capsaicin quantification, making it suitable for application in both research and industry settings.

From an applied perspective, this study has implications for chili pepper breeding, quality control in food production, and the development of functional foods or therapeutic agents containing capsaicin. Future research should explore the influence of environmental factors, post-harvest processing, and storage conditions on capsaicin stability and investigate the full spectrum of capsaicinoids and related bioactive present in these peppers. Additionally, integrating molecular approaches to study gene expression related to capsaicin biosynthesis may further unravel the genetic mechanisms behind pungency traits in *Capsicum*

species.

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