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ORIGINAL PAPER

First comprehensive validation of an HPLC-DAD method for dihydrocapsaicin quantification in *Capsicum chinense* germplasm accessions

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Abstract

Capsaicinoids are alkaloid compounds found exclusively in peppers of the genus *Capsicum*. Among them, capsaicin (CAP) and dihydrocapsaicin (DHC) are primarily responsible for the pungency of the fruit, which is the characteristic burning sensation. The species *Capsicum chinense* produces fruits with the highest pungency levels. The aim of this study was to validate, according to the SANTE/11,312/2021 and ICH Q2(R1) guidelines, an analytical method for quantifying DHC in 53 *C. chinense* accessions from the *Capsicum* Active Germplasm Bank at the Montes Claros State University (UNIMONTES), Minas Gerais, Brazil, to identify high-DHC accessions (high-pungency candidates) for breeding prioritization. Validation included the parameters of selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, matrix effect, precision, and accuracy. Analyses were performed by high-performance liquid chromatography with diode-array detection (HPLC-DAD), a technique whose analytical performance had not yet been fully validated for DHC quantification in *C. chinense*. The validated method showed satisfactory accuracy, with recovery rates ranging from 87.85% to 96.78%, and good precision, with a relative standard deviation (RSD) of 2.33%. To the best of our knowledge, this is the first comprehensive validation of an HPLC-DAD method for DHC determination in *C. chinense* accessions from a germplasm bank. The *C. chinense* accessions were quantified and grouped into 28 clusters according to their mean DHC concentrations, demonstrating high variability of pungency within the germplasm, with six accessions identified as presenting the highest DHC contents. Accessions with high DHC contents represent promising candidates for use in breeding programs aimed at developing cultivars with greater pungency.

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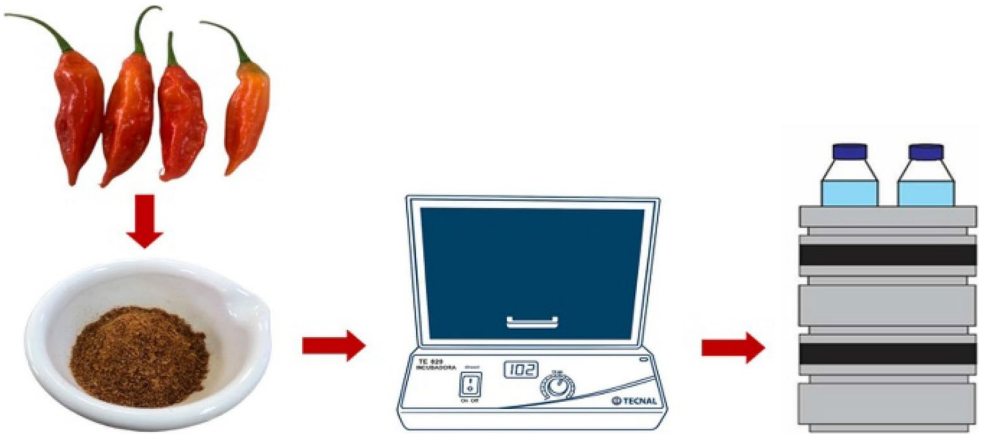
Graphical Abstract

Validated HPLC-DAD method for dihydrocapsaicin (DHC) quantification in *Capsicum chinense*; large-scale analysis reveals high pungency diversity (28 clusters) and identifies six elite high-DHC accessions for breeding applications.



Keywords Capsaicinoids · Pungency Variability · Liquid chromatography · Germplasm Evaluation · Method Validation

Introduction

The genus *Capsicum* comprises approximately 42 recognized species of pepper plants [1, 2]. Despite this wide variety, only five species are domesticated: *Capsicum annuum*, *Capsicum baccatum*, *Capsicum chinense*, *Capsicum frutescens*, and *Capsicum pubescens* [3].

C. chinense peppers are characterized by their pronounced aroma, distinctive fruit morphology, and, in most cases, intense pungency. *C. chinense* is one of the most prevalent *Capsicum* species in Brazil, due to its adaptability to tropical climates and remarkable genetic diversity in the Amazon basin [4]. Several pepper cultivars have been developed within the species *C. chinense*, including "Biquinho", "Pimenta-de-Cheiro", "Bode", "Murupi", "Cumari do Pará", and "Habanero" [5].

Highly pungent peppers, often referred to as "nuclear peppers", are characteristic of *C. chinense*. The Scoville organoleptic test, a sensory evaluation method conducted by trained assessors, is widely used to measure pungency in peppers, with the pungency level expressed as Scoville Heat Units (SHU) [6]. According to Guinness World Records (2023), 'Pepper X' was recognized as the world's most pungent pepper, with an average of 2,693,000 SHU. Peppers of cultivars belonging to the species *C. chinense* are among the world's most pungent, including "Pepper X", "Carolina Reaper", "Trinidad Moruga Scorpion", "Infinity Pepper", "Naga Viper", and "Bhut Jolokia", with pungency ranging from approximately 950,000 to 2,700,000 SHU.

Capsaicinoids are alkaloids responsible for the pungency of *Capsicum* fruits [6]. Capsaicinoids are mainly synthesized and accumulated in the placental tissue of *Capsicum* fruits. However, evidence suggests that capsaicinoids can also be synthesized in the pericarp of highly pungent peppers of the *C. chinense* cultivars, such as "Trinidad Moruga Scorpion Yellow", resulting in greater capsaicinoid accumulation [7–9].

Capsaicin (CAP) and dihydrocapsaicin (DHC) constitute approximately 90% of the total capsaicinoids in *Capsicum* peppers [10, 11]. Other capsaicinoids, including nordihydrocapsaicin, homodihydrocapsaicin, and homocapsaicin, are present in lower concentrations [12, 13].

Additionally, capsaicinoids exhibit significant characteristics for human health, including anti-inflammatory [14, 15], anticancer, analgesic, and antipruritic properties [6], as well as significant antioxidant, antifungal, and antibacterial activities [16, 17], which have been extensively studied. Capsaicinoids have also been used in the production of pepper spray, employed for law enforcement and self-defense purposes [18].

The Scoville organoleptic method has been used for decades to assess pungency; however, it is limited by its reliance on subjective sensory evaluations. Therefore, advanced analytical techniques, such as gas chromatography (GC), high-performance liquid chromatography (HPLC) with various detectors, and ultra-high-performance liquid chromatography (UHPLC), have been employed to detect and quantify capsaicinoids [19, 20]. HPLC is a widely adopted analytical tool in research due to its simpler sample preparation and higher sample throughput [21].

Beyond its culinary and biochemical relevance, the precise quantification of pungency-related traits holds growing relevance within contemporary agricultural systems

increasingly shaped by data-driven decision-making [22, 23]. Recent studies underscore the pivotal role of advanced analytical and phenotyping approaches in germplasm characterization as a foundation for enhancing the strategic use of plant genetic resources and supporting innovation in crop improvement [22–25].

Within this framework, targeted chemical analyses represent an important component of modern phenotyping, as they provide objective and reproducible descriptors of phenotypic variation that are directly applicable to quality-related traits [24, 25]. By enabling the identification and comparison of accessions with differentiated pungency profiles, such analytical tools contribute to value-oriented agricultural systems and breeding initiatives focused on product differentiation [22, 23]. In this context, the validated and accessible HPLC-DAD method presented herein integrates analytical rigor with practical applicability, contributing to broader academic discussions on plant genetic resource characterization and agricultural innovation.

Although several chromatographic methods have been reported for capsaicinoid determination in *Capsicum* matrices, many studies prioritize analytical speed or sensitivity and provide limited statistical validation and matrix-aware calibration, particularly when applied to diverse biological collections.

While several HPLC and UHPLC approaches have been reported for capsaicinoid determination in different *Capsicum* matrices, most studies focus on processed products, single cultivars, or industrial extracts, often prioritizing speed and instrument performance over comprehensive statistical validation. In contrast, the present work establishes a full validation of an accessible HPLC-DAD workflow under internationally recognized guidelines, including matrix-matched calibration with quantified matrix effect and formal regression diagnostics (residual assessment and lack-of-fit testing), and applies it to germplasm-scale screening of a genetically diverse *C. chinense* collection to identify high-DHC accessions (high-pungency candidates) for breeding prioritization. The accessions were obtained from the *Capsicum* Active Germplasm Bank (AGB) at the State University of Montes Claros (UNIMONTES), Minas Gerais, Brazil.

The objectives of this study were: (i) to validate an accessible HPLC-DAD method for DHC quantification in *C. chinense* under internationally recognized guidelines, including matrix-aware calibration and regression diagnostics; and (ii) to apply the validated method to large-scale chemical phenotyping of a genetically diverse germplasm collection ($n = 53$), enabling robust accession ranking and identification of high-DHC genotypes for breeding prioritization. A further aim is to provide a reproducible analytical baseline that supports future multi-environment confirmation and integration with multi-trait breeding and metabolomics frameworks.

By ensuring statistically robust and reproducible quantification of dihydrocapsaicin, the validated method supports reliable germplasm-scale chemical phenotyping. This analytical assurance underpins downstream interpretation and breeding use by enabling consistent accession ranking, comparability across experiments, and future confirmation under multi-environment trials and integration with multi-trait and metabolomics frameworks.

Materials and methods

Plant material

Fifty-three *Capsicum chinense* accessions from the *Capsicum* Active Germplasm Bank (AGB) at the State University of Montes Claros (UNIMONTES) were used. The accessions were cultivated under greenhouse conditions with a 50% shading screen at the experimental area of UNIMONTES, in Janaúba, Minas Gerais (MG), Brazil (15°48’09’’S, 43°18’32’’W, 533 m altitude) from 2023 to 2024. Seeds were sown directly into 3 L plastic pots containing a 1:1:1 mixture of clayey soil, coarse sand, and cattle manure. Pots were labeled and irrigated daily, with adjustments as needed. Cultural management followed recommendations for conventional cultivation [26], adapted for protected cultivation in pots.

Sample preparation

Non-pungent *Capsicum annuum* (bell pepper) fruits were used as a blank matrix for method development and matrix-matched calibration, due to the absence of endogenous capsaicinoids. Both *C. annuum* and *C. chinense* belong to the same genus and share a conserved evolutionary background; differences in pungency among *Capsicum* species are mainly associated with the presence, functionality, and regulation of genes involved in the capsaicinoid biosynthetic pathway, rather than with the absence of a common primary metabolic framework [1, 13, 27]. Thus, bell pepper provides a *Capsicum*-derived plant matrix that preserves general fruit components, such as carbohydrates, organic acids, pigments, and structural elements, while allowing the evaluation of analytical performance parameters without native analyte interference.

Because capsaicinoids are endogenous in pungent *Capsicum* matrices, a truly analyte-free *C. chinense* blank is not practically available for spike-based validation. Therefore, a non-pungent *C. annuum* (bell pepper) matrix was used as a surrogate *Capsicum* blank to enable controlled fortification

experiments (e.g., recovery) and matrix-matched calibration. This surrogate-blank strategy (non-pungent pepper matrices fortified with capsaicinoid standards) has precedent in capsaicinoid analytical validation and performance assessment [28–32].

Bell pepper fruits were acquired from a local supermarket in Janaúba, MG, Brazil. Approximately 100 g of ripe fruits from each accession were harvested at maturity, washed under running water, dried, and stored in labeled Kraft paper bags. Samples were dried in an oven (Cienlab® CE-220) at 65 °C until constant weight, operationally defined as two consecutive weight measurements showing variation below 1%. Drying time varied among accessions depending on fruit size and initial moisture content, typically ranging from approximately 48 to 96 h. After drying, the peduncle was removed, and the fruits were ground in a porcelain mortar (Nalgon®) with a pestle to obtain a homogeneous sample.

For each accession, all fruits harvested at maturity were pooled prior to drying and grinding. After drying, the fruits belonging to the same accession were jointly ground to obtain a single homogeneous powder. The 1 g subsamples used for extraction were taken from this bulk-homogenized material, ensuring that analytical replicates represented the mean chemical composition of each accession rather than individual fruits. The same procedure was applied to the bell pepper fruits. The ground material was stored at −20 °C, protected from light, until extraction. All dihydrocapsaicin (DHC) concentrations are expressed on a dry weight basis ($mg \cdot kg^{-1}$ DW).

Reagents and solutions

All solvents and reagents used were of HPLC grade. Acetonitrile (≥99.8% purity), acetic acid (100%), and absolute ethanol were acquired from Dinâmica® or Supelco® (São Paulo, Brazil). Ultrapure water was obtained using an ELGA PURELAB laboratory water purification system. A dihydrocapsaicin (DHC) reference standard (≥95% purity) was acquired from Sigma-Aldrich®. Stock solutions of the DHC standard were prepared in ethanol at a concentration of 0.20 $mg \cdot mL^{-1}$, with working solutions obtained by appropriate dilution. All solutions were stored at -20 °C until analysis.

Extraction of capsaicinoids

Capsaicinoid extraction was performed according to the method described by Chinn et al. [33], with slight modifications. One gram of dried (DW), ground pepper was homogenized in 25 mL of ethanol, heated at 50 °C, and agitated at 100 rpm for one hour on a light-protected orbital shaker (TE-420 Tecnal). Analyses were conducted in triplicate for each sample. Samples were filtered through a 0.45 µm hydrophilic PTFE syringe filter (Millex®, Merck, Germany), and extracts were stored in Falcon tubes at -20 °C until chromatographic analysis.

Chromatographic analysis

Dihydrocapsaicin was quantified using a Waters Alliance e2695 high-performance liquid chromatograph coupled with a Waters 2998 diode-array detector (DAD). The Empower™ PDA version 2 software (Waters) was used for instrument control and data processing. Chromatographic separation was achieved using a C18 column (250 mm × 4.6 mm, 5 µm; Avantor ACE®, Avantor, UK), maintained at 25 ± 5 °C. The injection volume was 20 µL, and the mobile phase consisted of 40% ultrapure water (pH 3.0, adjusted with 1% acetic acid) and 60% acetonitrile. Elution was performed in isocratic mode at a flow rate of 1.0 $mL \cdot min^{-1}$, with detection at 280 nm and a total run time of 10 min.

Under these conditions, DHC was consistently eluted at a retention time of 7.8 ± 0.1 min, with good chromatographic resolution. Peak identity and selectivity were confirmed by comparison of retention time and UV–Vis spectral profiles obtained by DAD. Spectral overlays acquired across the DHC peak showed consistent absorbance profiles, confirming peak homogeneity and the absence of co-eluting matrix interferences. Isocratic elution was selected as it provided adequate resolution, stable retention time, and a shorter run time for DHC quantification, without evidence of matrix interferences under the validated conditions. A representative chromatogram of a fortified matrix extract and a real sample is shown in Fig. 1.

Methodology validation

The analytical method was validated by assessing the following parameters: selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, matrix effect (ME), precision, and accuracy, according to the SANTE/11,312/2021 [34] and ICH Q2(R1) [35] international guidelines for analytical method validation [34, 36]. All validation steps requiring a blank/surrogate matrix (e.g., fortification-based recovery, matrix-matched calibration, and matrix-effect evaluation) were performed using the non-pungent *C. annuum* matrix described above. Accordingly, these validation results formally apply to this surrogate *Capsicum* matrix, while application to pungent *C. chinense* relies on matrix-matched calibration and dilution-within-range strategy (see Dilution Integrity). Selectivity was evaluated through qualitative analysis, comparing chromatograms from a blank

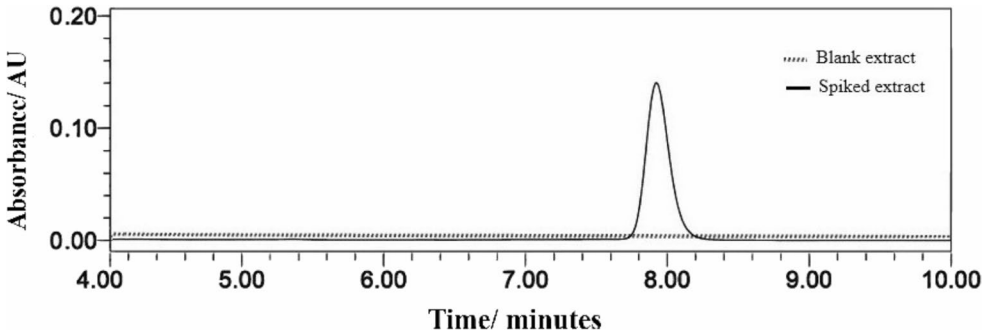


Fig. 1 HPLC–DAD chromatograms of the matrix extract without dihydrocapsaicin (DHC) (dotted line) and the matrix extract fortified with DHC at 0.12 mg·mL⁻¹ (solid line). Chromatographic separation was performed under the conditions described in the *Chromatographic Analysis* section. DHC was eluted at a retention time of 7.8 min. Detection was carried out at 280 nm, with absorbance expressed in arbitrary units (AU)

matrix extract and a matrix extract spiked with DHC (0.12 mg·mL^{-1}), using six independent replicates per sample.

The LOD and LOQ were determined experimentally by analyzing matrix extracts fortified with the DHC standard at varying concentrations, using an instrumental signal-to-noise approach (3:1 and 10:1, respectively), with six independent replicates per sample [35, 37].

Linearity was evaluated using an analytical curve of matrix extracts fortified with the DHC standard at six concentration levels (0.001, 0.04, 0.08, 0.12, 0.16, 0.20 mg·mL⁻¹), with three independent replicates per level. Linear regression parameters were estimated using the ordinary least-squares method (OLSM), after confirming the absence of outliers based on standardized Jackknife residuals [37].

The assumptions of regression were verified by the Ryan-Joiner test for normality, the Brown-Forsythe modified Levene test for homoscedasticity, and the Durbin-Watson test for independence of residuals. Analysis of variance (ANOVA) was conducted to assess the linear regression model's assumptions, significance, and deviation from linearity. All linearity assessments followed procedures described by Souza and Junqueira [38] and were conducted using a tool developed by Bazilio et al. [39].

The matrix effect (ME) was assessed by comparing analytical curves of the matrix extract and solvent. Thus, the same linearity assessment conducted for the matrix extract was performed for the solvent, at the same concentration levels. ME was calculated according to Eq. 1 [40]. Negative ME values indicate suppression, while positive values indicate enhancement of the chromatographic signal [41]. The acceptable ME range is -20% to + 20%) [34, 40].

$$\mathrm{ME}\ (\%) = \left(\frac{\alpha_{matrix}}{\alpha_{solvent}} - 1\right) \times 100 \qquad (2)$$

Where:

a_{matrix}:slope of the matrix-matched calibration curve.

$a_{solvent}$:slope of the solvent calibration curve.

Accuracy and precision were assessed through simultaneous analyte recovery experiments. Accuracy was determined by performing recovery assays on bell pepper matrix extracts fortified with DHC at three concentration levels (0.001, 0.08, and 0.16 mg·mL⁻¹), with three replicates per level. Recovery values within 70–120% were considered acceptable, confirming the trueness of the method. Precision was evaluated by calculating the relative standard deviation (RSD) of seven replicates from a fortified matrix at 0.08 mg·mL⁻¹. An RSD below 20% was adopted as the acceptance criterion for repeatability, according to the same guidelines.

Real samples

The validated HPLC-DAD method was applied to quantify DHC in 53 *C. chinense* accessions. Samples whose concentrations exceeded the upper limit of the calibration range were not quantified in the undiluted form; instead, a minimal two-fold (2×) dilution was applied to bring them within the validated range prior to analysis. Additional dilution factors (4× and 8×) were evaluated exclusively to assess dilution integrity, in accordance with the recommendations of the US Food and Drug Administration [42], and were not used for routine quantification. This strategy avoided potential non-linearity or detector saturation while ensuring that all reported results were obtained within the validated calibration interval.

Experimental data were organized in a completely randomized design with three analytical replicates. Data normality was tested using the Lilliefors test ($p > 0.05$), and the homogeneity of variances was verified through the Bartlett test ($p > 0.05$). Once assumptions were met, an analysis of variance (ANOVA) was conducted at a 5% significance level, followed by the Scott–Knott clustering test (1% significance level) to group accessions based on DHC

concentration. All statistical analyses were performed using the Genes® software package (version 2021.1.0) [43].

Results and discussion

This section presents and discusses the results obtained from the analytical method validation, followed by its application to germplasm-scale screening of DHC content in *Capsicum chinense* accessions, including comparative and practical implications.

Method validation

The method for extracting and determining DHC concentrations in *Capsicum chinense* accession samples was validated for selectivity, LOD, LOQ, ME linearity, precision, and accuracy (Table 1).

Selectivity

In the chromatogram of the blank matrix, no signal appeared at the DHC retention time, confirming the absence of interfering compounds. In contrast, the chromatogram of the fortified extract exhibited a well-defined DHC peak with adequate resolution and no overlap with adjacent peaks, demonstrating excellent selectivity for the analyte (Fig. 1). In the chromatogram of the fortified extract, a well-defined peak was detected at the DHC retention time, exhibiting good resolution without overlap with adjacent signals. These findings confirm that the method demonstrates appropriate selectivity for quantifying DHC in the analyzed matrix, enabling differentiation and quantification of the analyte despite the presence of other matrix components. Selectivity is a critical requirement in analytical methods, ensuring that the measured response is exclusively attributed to the target compound [35].

Table 1 Results of the validation study for dihydrocapsaicin quantification

Parameter	Result	
Linearity range (mg·mL^{-1})	0.001–0.20	
Regression equation	$y = 1.374 \times 10^{7}x - 7.220 \times 10^{3}$	
R^2	0.9998	
LOD (mg·mL^{-1})	0.0005	
LOQ (mg·mL^{-1})	0.001	
Accuracy (Recovery)		
Fortification level (mg·mL^{-1})	Recovery (%)	RSD (%)
0.001 mg·mL^{-1}[a]	87.85	2.19
0.08 mg·mL^{-1}[a]	93.70	2.33
0.16 mg·mL^{-1}[a]	96.78	2.03
Precision (Repeatability, within-day)		
0.08 mg·mL^{-1}[b]	RSD = 2.33%	

LOD = limit of detection; LOQ = limit of quantification; [a] Mean of three replicates; [b] Mean of seven replicates; RSD = relative standard deviation

Limits of detection and quantification

The LOD and LOQ were 0.0005 mg·mL^{-1} and 0.001 mg·mL^{-1}, respectively, representing the lowest detectable and quantifiable concentrations with acceptable precision and accuracy. The sensitivity of the method was comparable to that reported in previous studies for DHC quantification [19, 44–51]. This performance confirms that the optimized HPLC-DAD protocol provides detection limits consistent with, or superior to, earlier HPLC and UHPLC reports.

The method's capacity to detect small variations is critical for ensuring reliable results, particularly in peppers with diverse pungency levels. Based on the established limit of quantification, DHC concentrations can be reliably determined across the investigated samples.

Linearity

No outliers influencing regression estimation were detected in the calibration data, and a strong linear relationship was observed between DHC concentration and peak area (Fig. 2). The regression equation and coefficient of determination (R^2 = 0.9998) confirmed the excellent linearity of the method across the range 0.001–0.200 mg·mL^{-1} (Table 1).

The residuals followed a normal distribution (Fig. 3) and exhibited independence and homoscedasticity (Fig. 4). ANOVA confirmed a highly significant regression with a non-significant lack-of-fit, validating the suitability of the

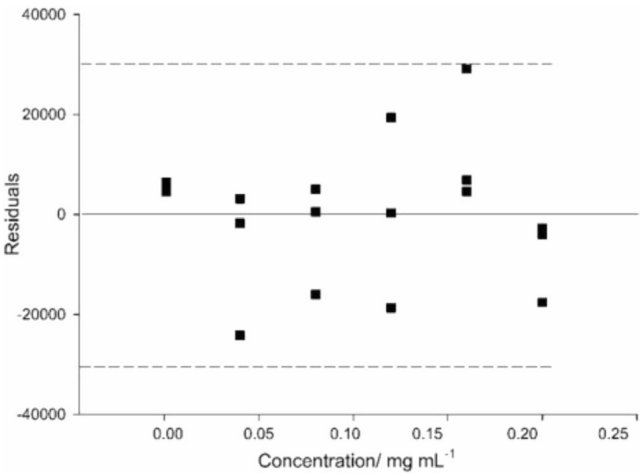


Fig. 2 Regression residuals plot

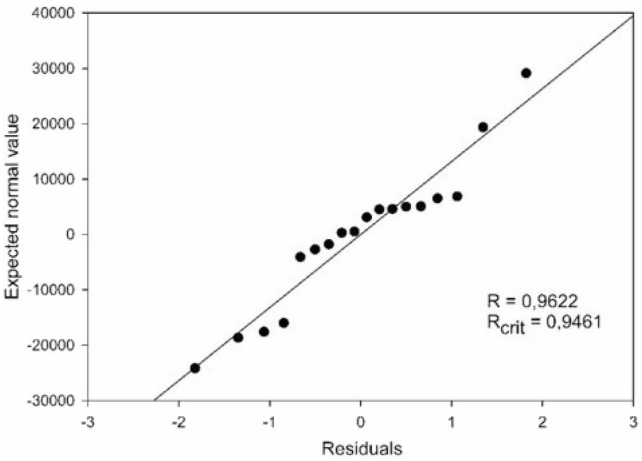


Fig. 3 Normal probability plot of regression residuals

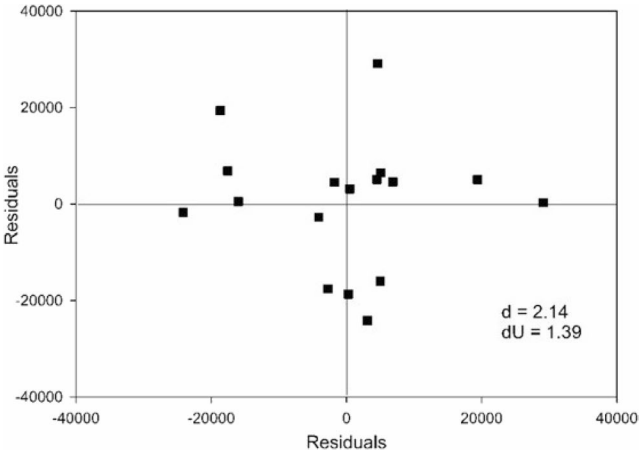


Fig. 4 Durbin-Watson plot for regression residuals

linear model. The residuals exhibited a correlation coefficient of 0.9622 exceeding the critical value of 0.9461, suggesting that deviations from normality were not significant. The homoscedasticity of the regression residuals was confirmed to be uniform.

The random distribution of regression residuals was confirmed (Fig. 4), supported by the Durbin–Watson statistic, in which the calculated value (d = 2.14) exceeded the upper critical limit (dU = 1.39), indicating independence of residuals and absence of autocorrelation.

The regression parameters were estimated using the ordinary least squares method (OLSM), as the regression residual assumptions were validated. The ANOVA confirmed the model's high significance, supporting the suitability of the derived equation to represent the experimental data. Additionally, the non-significant lack of fit indicates that the linear model adequately describes the response variable (chromatographic area) across the range of 0.001–0.200 mg·mL^{-1}, with no need for adjustment by higher-order models (Table 2).

Table 2 Analysis of variance (ANOVA) for the significance of regression and deviation from linearity

Source of variation	DF	MS	F	*P*
Regression	1	1.57×10^{13}	8.57×10^{4}	2.90×10^{-31} **
Residuals	16	1.84×10^{8}		
Lack of fit	4	2.61×10^{8}	1.66	0.224^{ns}
Total	17			

DF = degrees of freedom, MS = mean square, F = F-statistic, P = P-value, ** = highly significant, ^{ns} = not significant

Matrix effect

The matrix effect (ME), determined by comparing the slopes of solvent-based and matrix-matched calibration curves, showed a + 16% signal enhancement, which is within the generally accepted range (−20% to +20%). Based on this result, matrix-matched calibration was adopted for all determinations to ensure reliable quantification (Fig. 5).

The evaluation of the matrix effect was performed using a non-pungent *Capsicum* matrix. Although this approach enables controlled assessment of matrix-related influences on analytical response, it may not fully reproduce the chemical complexity of highly pungent *C. chinense* extracts, which are enriched in secondary metabolites associated with pungency. Therefore, the observed matrix effect should be interpreted as representative of a capsaicinoid-free *Capsicum* surrogate matrix rather than a direct quantification of matrix effects in undiluted high-pungency *C. chinense* extracts. In the present application, extracts exceeding the upper limit of the calibration range were not quantified in undiluted form; instead, they were diluted (minimum 2×) and quantified within the validated interval. Dilution integrity was verified in the four most pungent accessions across 2×, 4×, and 8× dilutions, yielding mean recoveries of 95.56–102.33% with repeatability RSD ≤ 1.1% (Supplementary Table S1), supporting accurate quantification under the applied dilution-within-range workflow. Nevertheless, direct recovery and matrix-effect evaluation in undiluted high-pungency *C. chinense* matrices (e.g., by standard addition) remains an important objective for future work.

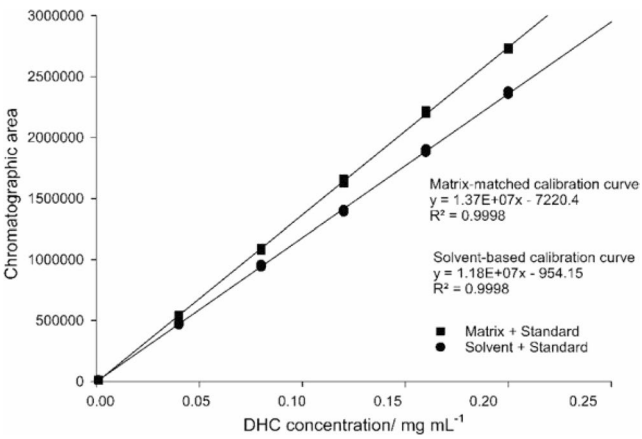


Fig. 5 Matrix-matched and solvent calibration curves

Although *C. annuum* provides a chemically related *Capsicum* matrix suitable for controlled validation, species-specific differences in secondary metabolite composition cannot be entirely excluded and may influence extraction efficiency or detector response in highly pungent *C. chinense* matrices.

Accuracy and precision

Recovery experiments confirmed excellent accuracy and repeatability (Table 1). Recovery values ranged from 87.85% to 96.78%, while the relative standard deviation (RSD) of 2.33% indicated outstanding precision. Both parameters fall within the acceptance limits established by the European Commission [34] and ICH guidelines [35], confirming the reliability of the validated HPLC-DAD protocol.

Real samples and dilution integrity

The validated HPLC-DAD method was applied to the quantification of DHC in 53 *C. chinense* accessions from the UNIMONTES Active Germplasm Bank. Samples whose concentrations exceeded the upper limit of the calibration range were diluted two-fold (2×) to allow quantification within the validated interval. Higher dilution factors (4× and 8×) were evaluated exclusively to assess dilution integrity, as recommended by the US Food and Drug Administration [42].

Thus, the practical 'worst-case' scenario in this dataset was not undiluted injection of highly pungent extracts, but accurate quantification after bringing samples into the validated interval. Across the dilution-integrity tests (4 accessions × 3 dilution levels), recoveries ranged from 95.56% to 102.33% (overall mean≈99.0%) with RSD≤1.03%, supporting accuracy and precision under the applied dilution-within-range workflow.

Dilution integrity was confirmed using the four most pungent accessions, yielding mean recoveries between 95.56% and 102.33% and repeatability (RSD)≤1.1% across all dilution levels. Detailed results are provided in Supplementary Table S1, confirming that dilution did not compromise accuracy or precision.

Data followed a normal distribution and showed homogeneity of variances, as confirmed by the Lilliefors and Bartlett tests ($p > 0.05$). ANOVA detected significant differences among accessions (Table 3), and the Scott–Knott clustering test (1%) grouped them into 28 distinct clusters. The high experimental precision, evidenced by a CV of 2.01%, is classified as excellent according to Pimentel-Gomes [52], reinforcing the statistical reliability of DHC quantification.

The 53 accessions were categorized into 28 Scott–Knott clusters (Fig. 6), with 16 clusters comprising a single accession and the remaining 12 comprising two or more accessions. These groups summarize the variability in DHC concentration and support identification of accessions with comparatively higher or lower DHC levels for germplasm screening and breeding prioritization. To facilitate interpretation and data reuse, a tabular summary of the Scott–Knott clusters, including cluster size (n), mean DHC concentration (mg/kg DW), and accession assignments (UNI IDs), is provided in Supplementary Table S2.

The a–f groups, each consisting of a single accession, exhibited the highest DHC concentrations: UNI 95 (9222.86 $mg \cdot kg^{-1}$ DW), UNI 96 (7684.63 $mg \cdot kg^{-1}$ DW), UNI 151 (7424.20 $mg \cdot kg^{-1}$ DW), UNI 146 (6449.75 $mg \cdot kg^{-1}$ DW), UNI 93 (4136.11 $mg \cdot kg^{-1}$ DW), and UNI 158 (3644.12 $mg \cdot kg^{-1}$ DW), respectively. The DHC concentration values

Table 3 Analysis of variance (ANOVA)

Source of variation	Degrees of freedom	Mean square
Accessions	52	$1.28 \times 10^{7**}$
Residuals	106	6.84×10^{2}
Total	158	
Mean DHC concentration ($mg \cdot kg^{-1}$ DW)		1304.61
CV of DHC concentration (%)		2.01

CV coefficient of variation

** = highly significant. Normality and homoscedasticity assumptions were verified using the Lilliefors and Bartlett tests, respectively ($p > 0.05$)

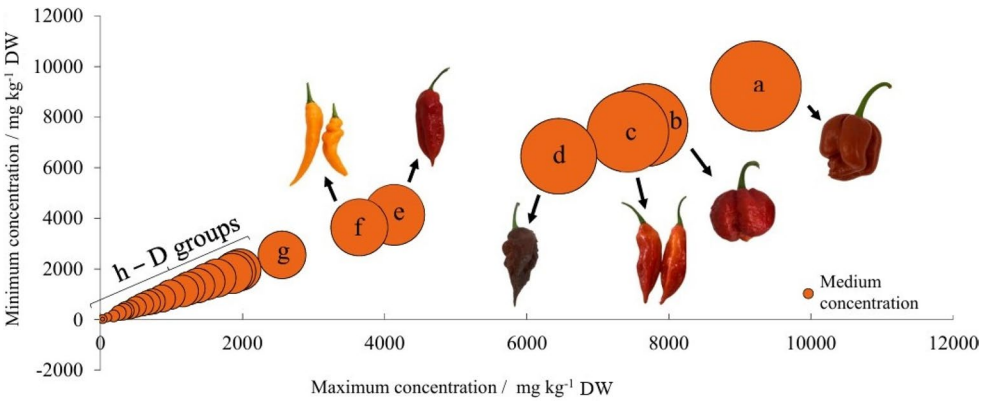


Fig. 6 Clustering of *Capsicum chinense* accessions based on mean dihydrocapsaicin (DHC) concentration (mg DHC kg^{-1} dry weight). Circles represent the mean DHC concentration of each cluster, positioned according to the minimum (y-axis) and maximum (x-axis) DHC concentrations observed within the cluster. Clusters are ranked alphabetically according to decreasing mean DHC concentration, with cluster 'a' comprising accessions with the highest mean DHC levels

of these accessions fall within the range reported by Sarpras et al. [53] for 63 samples of the same species (2600–26500 $mg \cdot kg^{-1}$ DW), exceed those obtained by Schmidt et al. [21] using a UHPLC (1910 $mg \cdot kg^{-1}$ DW), and are lower than those observed by Duelund and Mouritsen [54] (18100 $mg \cdot kg^{-1}$ DW) and Hudakova et al. [55] (42400 $mg \cdot kg^{-1}$ DW) using HPLC methods, all analyzing 'Bhut Jolokia' (*C. chinense*), one of the world's most pungent peppers. These findings suggest that certain accessions in the *Capsicum* Active Germplasm Bank (AGB) hold potential for selection in breeding programs aimed at developing cultivars with high pungency.

Numerous studies have shown that *Capsicum chinense* accessions generally exhibit higher capsaicinoid contents than other *Capsicum* species [56]. Capsaicin (CAP) and dihydrocapsaicin (DHC) are the predominant capsaicinoids and together account for most of the pungency perceived in highly pungent cultivars. In *C. chinense*, these compounds are consistently reported to be strongly correlated, with coefficients ranging from 0.73 to 0.90 in cultivars such as 'Bhut Jolokia', 'Carolina Reaper', 'Trinidad Moruga Scorpion', and 'Habanero' [57–59]. This relationship is biologically expected, as CAP and DHC share the same biosynthetic pathway and are jointly influenced by genetic and environmental factors [27, 60].

In the present study, only DHC was quantified; therefore, it is interpreted as a literature-supported indicator of relative pungency rather than a direct measure of total capsaicinoid content. This analytical choice was made to enable a fully validated and accessible HPLC-DAD workflow for large-scale comparative screening across a genetically diverse germplasm set. Consequently, the results are intended primarily for relative ranking of accessions under the tested conditions; breeding decisions requiring absolute pungency estimates (e.g., SHU) should incorporate CAP + DHC (or total capsaicinoids). Although CAP: DHC ratios may vary among genotypes and environments, CAP is consistently reported as the predominant capsaicinoid in *C. chinense*, with DHC occurring at lower but strongly correlated levels [61–63]. Reported CAP-to-DHC ratios for *C. chinense* cultivars typically range from 1.78:1 to 2.4:1 [59, 64], supporting the use of DHC as a comparative chemical marker for ranking accessions according to relative pungency within this species.

Scoville Heat Units (SHU), calculated from the combined concentrations of CAP and DHC, were not directly estimated in this study. Although approximate conversions between capsaicinoid concentration and SHU are commonly used, indirect estimation based solely on DHC would require assumptions regarding the CAP: DHC ratio that may not be uniformly applicable across all genotypes within this germplasm set. Consequently, pungency comparisons were restricted to relative rankings based on experimentally determined DHC concentrations.

For practical interpretation, an approximate SHU equivalent may be inferred using commonly reported conversion factors for capsaicinoids (≈16 SHU per mg of capsaicinoids per kg dry weight). Under this approximation, the highest dihydrocapsaicin concentration observed in this study (9222.86 $mg \cdot kg^{-1}$ DW) would correspond to approximately 147,000 SHU derived from DHC alone, acknowledging that total pungency would be higher when capsaicin is considered. This estimation is provided solely for contextual comparison and does not replace direct SHU determination based on total capsaicinoid content.

All accessions were cultivated under a single controlled greenhouse environment. While this design minimizes environmental variability and strengthens genotype comparisons under uniform conditions, pungency is a quantitative trait influenced by factors such as temperature, water availability, and crop management. Therefore, the DHC concentrations and accession rankings reported here reflect genotype performance under the specific conditions evaluated, and shifts may occur under different environments. Future multi-environment trials and direct quantification of total capsaicinoids are recommended to further assess genotype × environment interactions and pungency stability. Thus, accession prioritization based on the present ranking should be viewed as a first-pass selection under controlled conditions and confirmed under multi-environment trials before final breeding decisions.

Accordingly, the groups identified with higher DHC concentrations in the present study may represent the most pungent accessions within the *Capsicum* Active Germplasm Bank (AGB) at the State University of Montes Claros (UNIMONTES), supporting their prioritization in breeding and selection programs.

It should be noted that the present study emphasizes analytical reproducibility and accession-level chemical differentiation. Within-accession biological variability among individual fruits was not explicitly quantified and may contribute additional variance under field or multi-environment conditions. Therefore, the clustering results reflect accession means obtained from homogenized samples rather than fruit-level biological variation.

Comparative analytical framing

Extensive studies have quantified capsaicinoids using chromatographic techniques in various *Capsicum* matrices. Betts [65] introduced educational quantifications linking chemical data to SHU, while Barbero et al. [66] developed a reliable HPLC-FLD method for the accurate quantification of the major capsaicinoids in hot peppers. Ha et al. [44] developed

an ultra-HPLC/FLD method for gochujang, emphasizing speed and sensitivity, and Othman et al. [45] examined market peppers via HPLC-DAD, establishing LOQ in $\mu g \cdot g^{-1}$ and correlating DHC to SHU and intake levels.

González-Zamora et al. [46] investigated the effect of high temperatures on capsaicinoids in *C. annuum*, whereas Kuzma et al. [47] validated a pharmacopoeial HPLC–DAD method (LOQ ≈ 0.5 $\mu g \cdot mL^{-1}$) for the quantitative determination of major capsaicinoids in *Capsicum* extracts according to USP criteria. In parallel, Sganzerla et al. [19] quantified capsaicinoid levels among *C. chinense* cultivars, providing one of the first matrix benchmarks for the species. Usman et al. [43] applied UFLC-DAD to 21 genotypes with SHU correlation and extremely low LOQ (0.11 $\mu g \cdot kg^{-1}$ for CAP and 0.368 $\mu g \cdot kg^{-1}$ for DHC.). Later, Duelund and Mouritsen [54] reported a HPLC-MS method for four capsaicinoids with high robustness, and Vázquez-Espinosa et al. [50] introduced a UHPLC-PDA method capable of simultaneously determining different compounds (< 2 min runtime, high throughput). Most recently, Lalić et al. [51] performed a HPLC method validation in accordance with ICH guidelines for a complex pharmaceutical matrix.

Although these studies greatly advanced chromatographic determination of capsaicinoids, they generally targeted processed foods, single cultivars, or industrial extracts, often prioritizing speed over statistical rigor. None performed a comprehensive HPLC-DAD validation with full statistical diagnostics, including matrix-matched calibration with quantified ME, formal residual testing, and lack-of-fit analysis, applied to a large population dataset ($n = 53$) of *C. chinense* accessions.

This work fills that gap by demonstrating a full-stack validation of an accessible HPLC-DAD platform and its practical application to germplasm-scale screening. In practical terms, the present method provides mid-range throughput (~ 6 analyses$\cdot h^{-1}$) with solvent consumption < 10 mL per analysis, while UHPLC-PDA approaches can reduce runtime to < 2 min per run. We acknowledge that UHPLC platforms typically provide higher throughput and may achieve lower LOD/LOQ for capsaicinoids, at the expense of higher acquisition and maintenance costs and more specialized infrastructure [50]. Thus, the proposed HPLC-DAD workflow offers a cost-effective and scalable compromise for routine phenotyping and breeding pipelines operating under standard HPLC infrastructure, whereas UHPLC is advantageous when maximum throughput and sensitivity are prioritized.

From a sustainability-oriented perspective, the analytical workflow adopted in this study aligns with environmentally conscious and transferable practices by combining conventional extraction conditions with the use of ethanol as solvent and standard HPLC-DAD instrumentation. While the method was not designed as a green extraction approach per se, its reliance on low-toxicity solvents, moderate temperatures, and widely available chromatographic infrastructure supports reproducibility, accessibility, and reduced operational burden. In this sense, robust chromatographic validation complements sustainability-driven extraction philosophies by enabling reliable chemical phenotyping without dependence on high-energy or specialized extraction-assisted technologies. Accordingly, extraction-assisted workflows (e.g., microwave- or ultrasound-enabled extraction) were intentionally not adopted here, as the study prioritizes robustness, reproducibility, and transferability using conventional extraction conditions and standard HPLC-DAD infrastructure.

Robust chromatographic validation is a prerequisite for meaningful sustainability claims in extraction workflows, because greener solvent choices or reduced solvent volumes must still deliver reliable quantification. In this sense, the present validation framework (matrix-matched calibration, quantified matrix effect, and regression diagnostics) provides analytical assurance that complements sustainability-driven extraction philosophies and green recovery strategies in modern food research [67].

Differences in reported DHC concentrations across studies should be interpreted in light of methodological and biological factors that strongly influence measured values. Apparent discrepancies may arise from the analyzed tissue fraction (placenta-enriched material versus whole fruit), the reporting basis (fresh weight versus dry weight), and extraction parameters (solvent composition, solid-to-solvent ratio, temperature, time, and cleanup). Therefore, comparisons of absolute DHC levels across reports should consider these variables, even when similar chromatographic platforms are used.

Beyond these comparative performance considerations, the selection of an HPLC-DAD platform in this study reflects a strategic methodological positioning aligned with practical laboratory realities. While UHPLC-based approaches emphasize analytical speed and ultra-high sensitivity, their routine implementation is often constrained by higher acquisition costs, maintenance demands, and reliance on specialized infrastructure. In contrast, the validated HPLC-DAD method demonstrated here enables statistically robust quantification of dihydrocapsaicin using widely available instrumentation, supporting its routine application in agricultural and food research laboratories.

This balance between analytical rigor and operational feasibility defines a clear analytical niche for the method within the broader chromatographic landscape. In contrast to simpler approaches such as UV–Vis spectrophotometry, which are often limited by low selectivity and pronounced matrix interference in complex plant extracts [68], the

validated HPLC-DAD method ensures compound-specific quantification while remaining compatible with standard laboratory infrastructure. By enabling large-scale germplasm screening under standard chromatographic conditions, the approach supports the independent generation of reproducible chemical phenotyping data. Consequently, the methodological framework facilitates the autonomous characterization of *C. chinense* genetic resources and reinforces the relevance of accessible, well-validated HPLC-DAD methods for sustainable breeding programs and inclusive agricultural research.

Biological and breeding implications

Among the 53 accessions evaluated, six (UNI 95, UNI 96, UNI 151, UNI 146, UNI 93, and UNI 158) exhibited the highest DHC concentrations (3.6–9.2 × 10^3 mg·kg^{-1} DW). These values are consistent with those reported by Sarpras et al. [53] for *Capsicum* chinense (2,600–26,500 mg·kg^{-1} DW) and exceed the concentrations reported by Schmidt et al. [21] using UHPLC analysis (1,910 mg·kg^{-1} DW), confirming the suitability of the proposed method for the detection of highly pungent genotypes.

The strong correlation between capsaicin and DHC concentrations, previously described for *C. chinense* [57, 59], supports the use of DHC as a reliable proxy for pungency in breeding programs. In this context, the elite accessions identified in the present study represent valuable genetic resources for the development of cultivars with enhanced pungency and stable capsaicinoid profiles.

Beyond pungency improvement, high-DHC accessions offer opportunities for the development of cultivars targeted to specific market demands. In the culinary sector, differentiated pungency levels can support niche and gourmet markets, while in pharmaceutical, nutraceutical, and agro-industrial applications, standardized capsaicinoid composition is essential due to the biological activities of these compounds, including analgesic, anti-inflammatory, antimicrobial, and insect-repellent effects.

From an agronomic and socio-economic perspective, the incorporation of high-DHC genotypes into breeding programs may contribute to value-added agricultural systems, particularly in countries such as Brazil, where *Capsicum* species are predominantly cultivated by smallholder farmers. Cultivars with elevated capsaicinoid content are especially suitable for industrial extraction processes, enabling the valorization of processing by-products such as placental tissues, seeds, and non-commercial fruits.

This approach aligns with the principles of the circular bioeconomy, in which agricultural residues are reintegrated into the production chain as secondary resources rather than discarded as waste. The recovery of bioactive compounds from agricultural by-products to produce bioactive extracts, natural pesticides, or pharmaceutical ingredients may enhance resource efficiency, reduce waste, and generate additional income streams [69]. Therefore, the selection and utilization of high-DHC accessions may play a strategic role in sustainable pepper breeding programs that integrate genetic improvement, market diversification, and socio-economic development.

Beyond breeding-oriented screening, quantitative capsaicinoid profiling can also inform biofunctional perspectives, as capsaicinoids have been associated with physiological and sensory mechanisms extending beyond pungency. Although the present work does not evaluate bioactivity, the validated quantification framework enables reproducible chemical phenotyping that can support future studies linking capsaicinoid composition to functional outcomes in food and health contexts [70].

Beyond breeding-oriented selection, capsaicinoid quantification can also support broader valorization routes in which pungency-related compounds are treated as high-value ingredients. In functional, cosmetic, and industrial contexts, decisions often depend not only on concentration but also on compositional consistency and stability across processing and storage. Although such applications are not evaluated here, the validated HPLC-DAD framework provides a reproducible chemical phenotyping baseline that can support downstream screening of accessions for ingredient-oriented pipelines where compound reliability is critical.

Emerging nonthermal processing technologies have been reported as promising strategies for preserving bioactive compounds, including capsaicinoids, during food and ingredient processing [71]. Although processing effects were not evaluated in the present study, the robust chemical characterization enabled by validated analytical methods is essential for future investigations assessing how such technologies may influence capsaicinoid stability and functional performance. In particular, matrix effects and extraction efficiency can shift after processing, making matrix-matched calibration and validated performance criteria critical for reliable quantification.

Further expansion of post-quantification stabilization or formulation considerations would strengthen applicability, particularly for preserving high-pungency profiles through encapsulation and drying technologies. Such strategies can improve capsaicinoid stability and usability in downstream applications (e.g., spray-dried capsaicin microcapsules) [72].

Concluding comparative remarks

While UHPLC platforms can deliver higher analytical speed and sensitivity [44, 50], their adoption in developing

regions is limited by cost and maintenance. The validated HPLC-DAD method presented here achieves a balanced compromise between analytical performance and operational accessibility, enabling statistically robust, reproducible, and scalable quantification of dihydrocapsaicin in a genetically diverse *C. chinense* population.

Limitations of the study

A limitation of this study is that recovery and matrix effects were validated using a non-pungent *C. annuum* surrogate blank matrix, which may not fully reproduce the chemical background of undiluted high-pungency *C. chinense* extracts. This limitation is partially addressed by the fact that, under the present screening workflow, highly concentrated extracts were quantified after dilution into the validated range, supported by dilution-integrity results in the four most pungent accessions. However, matrix effects and recovery were not directly evaluated in undiluted high-pungency *C. chinense* matrices, and standard-addition experiments in representative pungent matrices are recommended as future work.

Conclusion

This study established a validated, statistically robust, and reproducible HPLC-DAD workflow for DHC quantification in *C. chinense* germplasm accessions, representing the first fully validated protocol specifically applied to this species. The method demonstrated consistent analytical performance and applicability to large population datasets, providing a reliable foundation for chemical phenotyping in genetic improvement programs.

Beyond its analytical rigor, the approach demonstrates that DHC quantification can serve as an efficient biochemical marker for assessing pungency variability in *C. chinense*, enabling the identification of accessions with contrasting and superior DHC levels. By integrating statistical validation with large-scale germplasm evaluation, the study strengthens the connection between analytical chemistry and plant breeding, offering a practical, accessible, and transferable methodology for capsaicinoid research and cultivar development.

Future research may expand the biological interpretation of the validated approach by integrating DHC profiles with sensory pungency data and broader capsaicinoid composition, thereby enhancing phenotypic characterization without affecting the robustness of the analytical method itself. Additionally, the high-DHC accessions identified here represent valuable parental material for crossing programs aimed at developing novel pungent cultivars.

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Data Availability The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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