Research Article

Measurement of Capsaicinoids in Chiltepin Hot Pepper: A Comparison Study between Spectrophotometric Method and High Performance Liquid Chromatography Analysis

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Direct spectrophotometric determination of capsaicinoids content in Chiltepin pepper was investigated as a possible alternative to HPLC analysis. Capsaicinoids were extracted from Chiltepin in red ripe and green fruit with acetonitrile and evaluated quantitatively using the HPLC method with capsaicin and dihydrocapsaicin standards. Three samples of different treatment were analyzed for their capsaicinoids content successfully by these methods. HPLC-DAD revealed that capsaicin, dihydrocapsaicin, and nordihydrocapsaicin comprised up to 98% of total capsaicinoids detected. The absorbance of the diluted samples was read on a spectrophotometer at 215–300 nm and monitored at 280 nm. We report herein the comparison between traditional UV assays and HPLC-DAD methods for the determination of the molar absorptivity coefficient of capsaicin ($\varepsilon_{280} = 3,410$ and $\varepsilon_{280} = 3,720\,\mathrm{M^{-1}\,cm^{-1}}$) and dihydrocapsaicin ($\varepsilon_{280} = 4,175$ and $\varepsilon_{280} = 4,350\,\mathrm{M^{-1}\,cm^{-1}}$), respectively. Statistical comparisons were performed using the regression analyses (ordinary linear regression and Deming regression) and Bland-Altman analysis. Comparative data for pungency was determined spectrophotometrically and by HPLC on samples ranging from 29.55 to 129 mg/g with a correlation of 0.91. These results indicate that the two methods significantly agree. The described spectrophotometric method can be routinely used for total capsaicinoids analysis and quality control in agricultural and pharmaceutical analysis.

1. Introduction

Pepper (*Capsicum annum L.*) is an important horticultural crop of major economic significance globally, imparting flavor, aroma, and color to foods [1]. The demand for new cultivars with high levels of phytochemicals has received increasing attention for biochemists, pharmaceutical companies, plant breeders, and general public due to their health benefits.

Chiltepines (*Capsicum annuum* var. *glabriusculum*) are a wild relative of domesticated peppers that grow throughout Mexico in undisturbed sites of tropical deciduous forests and orchards, in pasture grasslands, and along roads [2, 3]. Chiltepin production in Mexico has been estimated at 50 tons/year, having great importance for subsistence farmers of the central and northern regions of the country [3, 4]. The area where Chiltepin peppers are commercially harvested and

recognized for the quality flavor is in the mountains in the state of Sonora, Mexico, which rarely exceed 1000 m altitude [5].

Montoya-Ballesteros et al. [6] observed that Chiltepin fruits in green and red stage of maturity showed differences in color, pungency, and capsaicin and dihydrocapsaicin content. These results suggest that, during fruit ripening, several biochemical, physiological, and structural modifications occur and these changes determine the attributes of fruit quality.

In relation to the importance of phytochemicals for functional aspects of pepper, the capsaicinoids are a specific class of compounds that causes the spicy sensation (pungency) of chili pepper fruit. The main capsaicinoid is capsaicin, followed by dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin, and homocapsaicin. Of these compounds, capsaicin and dihydrocapsaicin account for approximately 90% of capsaicinoids in chili pepper fruit. These compounds are the two most potent capsaicinoids; their molecules differ only in the saturation of an acyl group [7] and their amount represents a quality parameter for different kind of products. In addition, capsaicin has been used traditionally for muscular pain and headache and to improve circulation and also commonly added to herbal formulations because it acts as a catalyst for other herbs and aids in their absorption. Moreover, capsaicin and other capsaicinoids have shown strong evidence that have promising potential in the fight against many types of cancer [8]. High concentrations of capsaicin occur in Chiltepin (*C. annuum* L. var. *glabriusculum* (Dunal) Heiser & Pickersgill) [9], Bhut Jolokia (*C. chinense* Jacq.) [10]; and the amounts vary with genotype, fruit maturity, and conditions of cultivation [11, 12].

The fertilization regime also affects the concentrations of many secondary metabolites such as capsaicinoids and phenols [13, 14]. Golcz et al. [15] stated that chili pepper has the greatest requirement for potassium (40%) and nitrogen (31%) in relation to the total amount of absorbed nutrients. Little is currently known about the effect of soil nutrients on pepper fruit pungency. Nitrogen availability may have a more direct effect on capsaicin accumulation since the synthesis of a single capsaicin molecule requires 3 mol of nitrogen to be formed [16]. Hence nitrogen availability may affect pepper pungency through its content in the fruit tissues. On the other hand, potassium may also affect pepper pungency given its positive effect on fruit development [17]. Previously, some studies have documented N and K effect on hot pepper [18, 19]. However, the results obtained by researchers suggest that K apparently does not notably interfere in capsaicin metabolization.

In the past, organoleptic tests, such as the Scoville heat test, were used to determine the pungency in peppers [20]. These methods have been replaced by analytical methods as colorimetry [21], gas chromatography [22], liquid chromatography, GC/LC-MS [23], and NMR-flow probe analysis [24]. Quantification of capsaicinoids often employs separation methods combined with ultraviolet (UV) detection [25]. Among these, the most widely used is high performance liquid chromatography (HPLC), which has become one of the most important tools in the separation and identification of capsaicinoids from raw pepper fruit extracts. In this method,

a 4.6 mm × 250 mm C-18 column (10 μm packing) is used with mobile phase consisting of a mixture of acetonitrile, dioxane, water, methanol, and perchloric acid depending on the sample concentration. For samples with more than 700 ppm, absorption UV detection at 280 nm is used. For samples with less than 700 ppm, fluorescence detection is employed with excitation at 288 nm and emission monitored at 320 nm [26].

At present, evaluation of capsaicinoids content in chili peppers is of great interest to plant breeders, food technologists, and nutritionists and also to assess breeding lines for genetic selection purposes or monitor levels during storage or processing. This interest has underscored the need for rapid and efficient quantification methods. HPLC methods, although more precise, are costly and require a trained operator. Simple spectrophotometric assays allow more rapid analysis [27, 28]. However, ordinary UV-visible spectrophotometry at a single wavelength generally requires that the analyte be separated from the other absorbing constituents in the complex sample matrix before the absorbance measurements are made. Davis et al. [29] proposed a novel spectrophotometric method for the determination of capsaicinoids in habanero pepper extracts that does not require prior analyte separation. We therefore took advantage of the enhanced sensitivity of currently available spectrophotometer (absorbance measured down to a sensitivity of 0.0001) to develop an assay that uses capsaicinoids. The theory of the calculation of the molar absorptivity is briefly illustrated.

We address here the precision of spectrophotometer values as a reflection of total amount of capsaicin and dihydrocapsaicin, which represents the majority of capsaicinoids content in chili peppers, and we compared values obtained with simple spectrophotometer readings with the cumulative amounts of individual capsaicinoids identified through HPLC analysis. The presence of correlation between the two methods is disclosed and discussed.

2. Experimental

2.1. Reagents. All the reagents, standards (capsaicin >95%; dihydrocapsaicin about 90% from *Capsicum* sp.), and solvents used were of high degree of purity or HPLC grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant Material and Growth Conditions. A sample of seeds of three different semidomesticated cultivars (named here Ch1, Ch2, and Ch3) was obtained from the semiarid regions of the state of Sonora, in Northwest Mexico. These three cultivars are not significantly different morphologically and grow in different regions of the Sonoran Desert where people harvest the fruits and aid in seed dispersal. The three cultivars were cultivated in an experimental area within a rural land of the municipality of Francisco I. Madero, Coahuila, Mexico ($25°$ $46'$ $31''$ N; $103°$ $16'$ $23''$ W). In this region, the average altitude is 1105 m, the climate is predominantly semidry, the average annual temperature is $22°$C, with maximum temperatures of $45°$C in summer and minimum of $4°$C in winter, and the average annual rainfall is less than 400 mm [30].

According to technical recommendations by Molina et al. [31], seeds were treated before germination as follows: they were soaked in water without chlorine for about 72 h and afterward they were germinated in greenhouse conditions at 25°C in Jan. 2013. Seeds were placed within polystyrene trays of 200 cavities filled with a mixture of peat moss and vermiculite as substrate; one seed per cavity was placed at a depth of 5 mm from the base and sprayed with water. The trays were placed under shade at 22°C and covered with black plastic. Irrigation was performed once a week until the day of transplant, which was performed 82 days after the date of germination. The transplant was directly from the trays to the field, when seedlings had an approximate minimum height of 20 cm, in Apr. 2013. Distance between plants and rows was 1 m. Vermicompost was used as fertilizer in an amount of 4.5 t ha^{-1}. The irrigation drip was provided every 20 days at field capacity. The fruit was harvested GM in July 2013, 77 days after to transplant and then RR 87 days after transplant.

2.3. Collection of Fruits from Plant Materials. Samples were collected at two stages of maturity, green mature (fresh) and red ripening, and then dried using a cabinet-type convective dryer at 65°C by 24 h and triturated.

2.4. Capsaicinoids Extraction. The capsaicinoids were extracted from the dried fruits following the methods proposed by Al Othman et al. [32] and Parrish [33] slightly modified as follows: briefly, the dried fruits were powdered and 1 g was treated with 10 mL of acetonitrile at 65°C along 20 min under sonication, with a working frequency of 35 kHz. The extracts were evaporated to dryness at 60°C, resuspended in 0.5 mL of acetonitrile, and filtrated through 0.45 µm cellulose acetate membrane filter (GVS Filter Technology, Indianapolis, IN, USA). Samples were stored at −20°C until they were analyzed.

2.5. Capsaicinoids Analysis by HPLC-DAD. Chromatography conditions were based on the validated method reported previously [9]. The analyses of capsaicinoids were performed by HPLC-DAD (Agilent 1200, Agilent Technologies Palo Alto, CA, USA) employing a reversed phase column Kromasil Eternity-5-C18 (4.6 × 150 mm) with Precolumn (SUPELCO Analytical, Sigma-Aldrich) at 25°C. Elution was performed with an isocratic mixture of water : acetonitrile 50 : 50. Detection was set at 202, 222, and 280 nm. Injection volume was 20 µL. All peaks related to capsaicinoids were eluted in about 15 min. Quantitative analysis was performed following the external standard method. Calibration curves were built by injecting by triplicate of ten increasing concentrations of standard. Nordihydrocapsaicin was quantified using the calibration curve of dihydrocapsaicin, since it was not commercially available.

2.6. Spectrophotometric Method. The absorbance of the unknown samples solutions was read using one cm quartz cell in a UV-Vis spectrophotometer (HACH DR-5000).

Preparation of Standard Stock Solution. Pure capsaicin or dihydrocapsaicin was weighed and dissolved in acetonitrile up to 2.0 mL to get the stock solution of 2 mg/mL. The solution was then filtered with 0.45 µm syringe filter. The stock solution of capsaicin or dihydrocapsaicin was diluted as required. Calibrate daily with at least six working standards over the range of 10 to 200 mg/L for each standard. These dilutions were scanned in the UV-Vis spectrometer and $\lambda_{\max}$ of capsaicin was selected as wavelength of detection. The instrument was set at 280 nm. The capsaicinoids content in the unknown extract solutions was calculated based on the absorbance values of known standard solutions.

2.7. Pungency. Capsaicinoid content was converted to Scoville heat unit (SHU) by multiplying the concentration of capsaicinoid in dry weight of pepper in parts per million (ppm) by the coefficient of the heat value for each compound, 9.3 for nordihydrocapsaicin and 16.1 for both capsaicin and dihydrocapsaicin for HPLC [22]:

$$\begin{aligned}\text{Total SHU} \\ = \left[\text{C (ppm)} + \text{DHC (ppm)} \right] \times 16.1 \qquad (1) \\ + \left[\text{n-DHC (ppm)} \times 9.3 \right]. \end{aligned}$$

Then the conversion to SHU from total capsaicinoids content obtained by spectrometric method in dry weight of pepper was done by multiplying the coefficient corresponding to the heat value for capsaicin, which is 1.6×10^7 [34].

2.8. Statistical Analysis. Experimental data are shown as the mean ± standard error of assay run in triplicate for capsaicinoids content. The statistical test was an ANOVA, for which we used SPSS version 15.0 for Windows. The means of treatments were compared with Tukey's multiple range test ($P \leq 0.05$). Linear correlation analysis for total capsaicinoids using HPLC and spectrophotometric method was conducted using Deming regression analysis with Bland-Altman plots. All statistical calculations of correlation were performed using Medcalc version 12.3 (Mariakerke, Belgium).

3. Results and Discussion

3.1. Calculation of the Molar Absorptivity Coefficient by HPLC and Spectrometric Method. Few attempts have been made to calculate absorption coefficient for capsaicinoids. The majority of capsaicinoids exhibit absorption in the UV region of the spectrum, between 200 and 350 nm. Because these obey the Beer-Lambert law (absorbance is linearly proportional to the concentration), absorbance measurement can be used to quantify the concentration of a pure (standard) capsaicinoid or to estimate the total capsaicinoid concentration in a mixture or extract of capsaicinoids in a sample. Standards of capsaicinoids (capsaicin and dihydrocapsaicin) commercially purchased were dissolved in acetonitrile and the spectrum was scanned in order to evaluate their fine structure (Figures 1(a) and 1(b)). Using the absorbance measured at its maximum wavelength (280 nm) and taking into consideration the dilution factor, purity degree, and molecular mass (capsaicin 305.41 g/mol; dihydrocapsaicin 307.43 g/mol), the
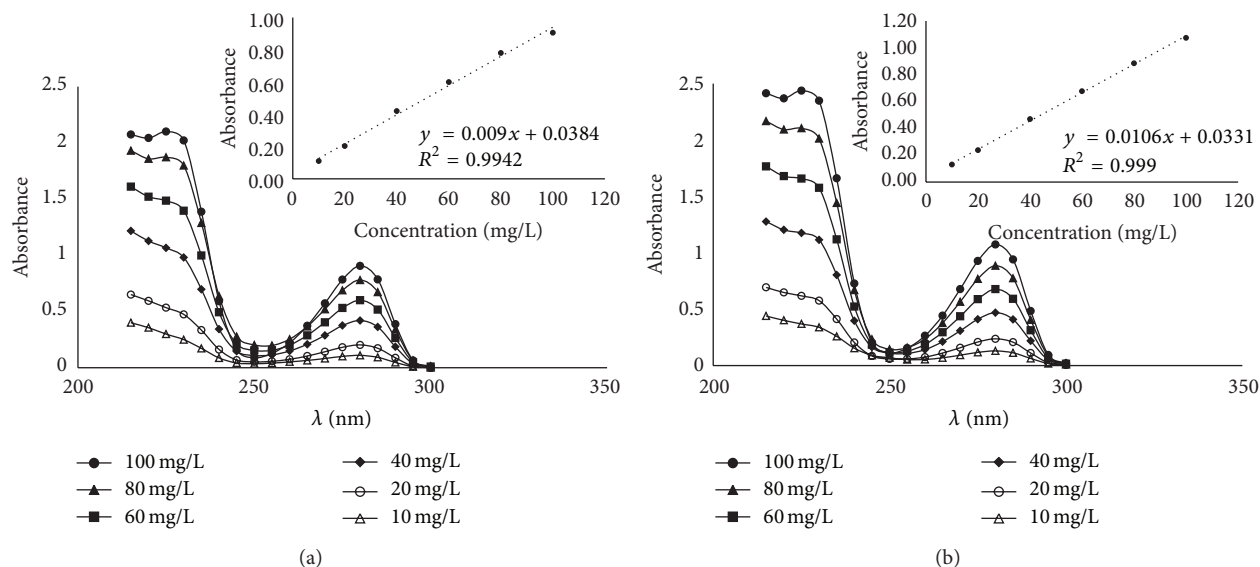
FIGURE 1: UV-Vis spectra of different concentrations of capsaicin (a) and dihydrocapsaicin (b). Insert, calibration curve of each capsaicinoid. The regression line equations for capsaicin and dihydrocapsaicin were ($y = 0.0097x + 0.03$) and ($y = 0.0106x + 0.031$), respectively.

molar absorption of each standard capsaicinoid was calculated, using the following well-known equation:

$$A = \varepsilon \cdot b \cdot c, \tag{2}$$

where A is absorbance (dimensionless), b is the path length through the sample in cm, ε is the molar absorbance coefficient in liter mol^{-1}, and c is the molar concentration in mol liter^{-1}.

Regarding linearity, spectrophotometric test exhibited a linear response between 10 μg/mL and 100 μg/mL and the representative equation of analysis was $y = 0.009x + 0.038$; a collection of 3 calibration curves presented a correlation coefficient 0.994 for capsaicin and $y = 0.01x + 0.033$, with a correlation coefficient of 0.99 for dihydrocapsaicin (inset, Figures 1(a) and 1(b)). The molar absorptivity coefficient of capsaicin is ($\varepsilon_{280} = 3410$ M^{-1} cm^{-1}) and of dihydrocapsaicin is ($\varepsilon_{280} = 4175$ M^{-1} cm^{-1}). LOD and LOQ were found to be 0.070 and 0.213 μg·mL for capsaicin, while LOD and LOQ for dihydrocapsaicin were 0.161 and 0.49 μg·mL, respectively. The results indicated excellent recoveries ranging from 98.5 to 99.3% and 99 to 101.6% for capsaicin and dihydrocapsaicin, respectively, and reliability of the method.

The same experimental conditions were applied to all capsaicinoids obtained by the HPLC-DAD method. Lambert-Beer's law is valid for a DAD detector, which is a spectrophotometer submitted to a dynamic and continuous HPLC flow, operating on chromatographically separated molecules. As proposed by Locatelli et al. [35], (3) can be used when ε is calculated as the slope of a straight-line regression built using the injected mass and the corresponding areas recorded by the DAD detector as follows:

$$\text{Area} = \varepsilon \cdot \frac{0.06 \cdot b \cdot m_0}{F \cdot \text{MW}}. \tag{3}$$

Area is expressed in (μAUs), MW (g mol^{-1}) is the molecular weight, and m_0 (ng) is the injected mass. The application of (3) at an HPLC flow of 1.0 mL min^{-1} yielded for capsaicin (C) $\varepsilon_{280} = 3,720 \pm 36$ M^{-1} cm^{-1} and for dihydrocapsaicin (DHC) $\varepsilon_{280} = 4,350 \pm 30$ M^{-1} cm^{-1}. The ratio between C and DHC ε values, at 280 nm, was εC, 280/εDHC, 280 = 0.855. Similarly, the lower limit of quantitation (LOQ) was determined by injecting a standard at a concentration that resulted in an S/N ratio of 10. The determination of the limit of detection (LOD) was analysed on the basis of S/N ratio of 3. The LOD and LOQ for this method were 0.0038 μg·mL and 0.0116 μg·mL for capsaicin, respectively. Relative standard deviation (RSD) of the within-day and day to day was 0.02–0.05% and 0.08–0.1% for capsaicin and dihydrocapsaicin, respectively.

3.2. Assessment of the Accumulation of Capsaicinoids at Two Ripening Stages in Chiltepin Fruits.

The pungent metabolites in the fruits of *Capsicum* species are called capsaicinoids, which are a group of 12 or more alkaloids with a structure of vanillylamide of branched fatty acids with 9–11 carbons [36], but capsaicin and dihydrocapsaicin are responsible for more than 90% of the pungency [37]. The capsaicin content in chili peppers is variable and ranges from 0.1 to 1% of the fruit weight approximately, but the amount varies depending on the temperature at which the plant is grown, the age of the fruit, the light, and soil composition [32]. The addition of mineral supplements to the pepper crops causes an increase in the capsaicinoid content of the fruit [38] and this nitrogen supply is essential for the synthesis of such compound [39]. However, due to adverse effects of chemical fertilizers, interest has been stimulated for the use of organic manures. Vermicompost is produced by biodegradation of organic material through interactions between earthworms and microorganisms [40].

Chiltepin has high phenotypic plasticity, shown by the variation of traits such as leaf morphology, fruit shape, seed germination pattern, or resistance to pathogens [2]. In Mexico, it can be found from the Yucatan peninsula and the Gulf of Mexico, where it grows in deep soils with dense evergreen vegetation, to xeric regions in the Sonoran desert or the central plateau, where it is commonly associated with nurse trees [41]. Harvesting the fruits of Chiltepin is still a common practice in central and northern Mexico and the total harvest has been estimated to be 50 metric tons per year [3]. However, environmental degradation, grazing, and harvesting have severely impacted many native populations of this species [41].

In the present work we have determined the content of capsaicin, dihydrocapsaicin, and nordihydrocapsaicin by HPLC-DAD to compare the total capsaicinoids level obtained by spectrometric method to study the influence of maturity stage and vermicompost on quality in capsaicinoids composition in Chiltepin fruits.

Chiltepin chili samples showed a high concentration of capsaicinoids in the two stages of maturity GM and RR (Table 1). Nordihydrocapsaicin, capsaicin, and dihydrocapsaicin were observed in the fruit of all samples. However, the amount of nordihydrocapsaicin was lower (5 to 9%) than capsaicin (30 to 59%) and dihydrocapsaicin (32 to 63%) relative to the total of capsaicinoids. Homocapsaicin and homodihydrocapsaicin were only present in some samples and in small percentages (from less than 1% to 4%) compared to total capsaicinoids content. HPLC revealed that capsaicin and dihydrocapsaicin comprised up to 93% of total capsaicinoids content detected in the studied samples. Capsaicin content of the control samples without vermicompost ranged from 1.63 ± 0.04 to 5.41 ± 0.29 mg/g dry weight in red ripe fruit. Levels of capsaicin assessed of samples with vermicompost varied from 3.23 ± 0.18 to 6.43 ± 0.22 mg/g dry weight in red ripe fruit. Capsaicin content was higher in green mature fruit than red ripe fruit (see Table 1). The content ranged from 23.78 ± 0.87 to 40.14 ± 0.25 mg/g dry weight in green mature samples without vermicompost (control) and 42.02 ± 1.93 to 56.68 ± 1.49 mg/g dry weight in green mature samples treated with vermicompost. In most cases, capsaicin levels were greater than dihydrocapsaicin, except for Ch1 RR-Cr, Ch1 Gm-Cr, and Ch2 Gm-Cr. Ch2 GM-Vc was the cultivar showing the highest values of total capsaicinoids ($107.01 \pm 1.55 \, \mathrm{mg \, g^{-1}}$) and therefore the highest value of pungency. Lowest values of capsaicinoids were observed in Ch1 to GM (71.12 ± 2.51 mg $\mathrm{g^{-1}}$) and Ch3 to RR ($30.70 \pm 1.07 \, \mathrm{mg \, g^{-1}}$), except for the case of dihydrocapsaicin where Ch1 to GM (29.49 ± 1.32 mg $\mathrm{g^{-1}}$) and Ch3 to RR ($11.96 \pm 0.26 \, \mathrm{mg \, g^{-1}}$) showed the lowest values. The difference between the lowest and highest value is 3.5-fold.

The direct-absorbance measurement of total capsaicinoids levels could have interferences from other compounds present in the pepper fruit extracts. Therefore, after the validation by HPLC, we evaluated the spectrophotometric method applied for total capsaicinoids in Chiltepin fruit samples. Figure 2 shows the typical spectrum of Ch1 RR-Cr and Ch1 RR-Cr-C+DHC complex overlain spectra and the results
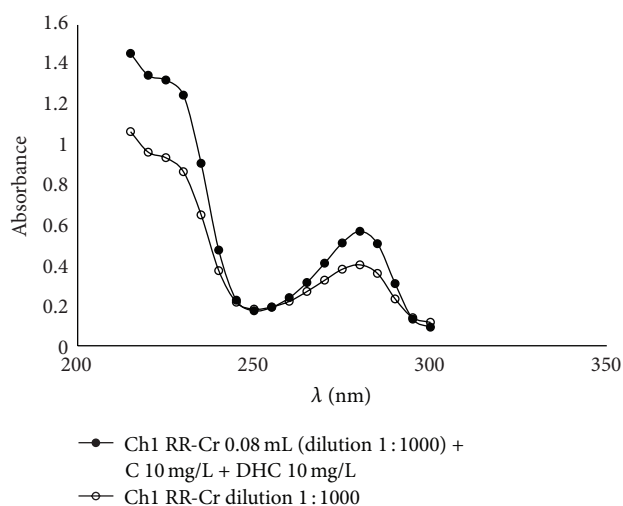

FIGURE 2: UV absorption spectrum of dry Chiltepin fruit extract without standards (white circle) and with standards (capsaicin "C" and dihydrocapsaicin "DHC"; black circle).

obtained are shown in Table 1 in accordance with the HPLC-DAD analysis. Recoveries obtained for the standards did not differ significantly from 100% showing that there was no interference from other common compounds present in extract and thus indicating accuracy and reliability of the method.

The capsaicinoids content, as determined by the spectrophotometric method, was generally higher in GM ripe fruit than red fruit (see Table 1). The content varied from 20.18–97.73 mg/g for RR samples to 59.47–118.93 mg/g for GM stage. Samples showed a considerable percentage of decrease in capsaicinoids levels depending on the stage of maturation from GM to RR. Ch3 was the cultivar that showed the greatest variation in the capsaicinoids content. The values of these metabolites in RR stage decreased by 62.8%, 51.2%, and 45.6% for nordihydrocapsaicin, capsaicin, and dihydrocapsaicin, respectively. This produces fruits with an average 50% less pungency in the RR stage regarding GM. For Ch1 and Ch2 pungency levels are 20% lower in RR than in GM.

3.3. Effects of Vermicompost Fertilization on Capsaicinoids Content in Chiltepin Pepper Fruit.

Vermicompost and compost can meet the nutrient demand of crop and significantly reduce the excessive use of chemical fertilizers in agriculture [42], and, for vermicompost in particular, increase soil fertility without polluting the soil, as well as the quantity and quality of harvested product [43]. According to Ramesh et al. [44], organic production is an alternative for consumers who prefer food free of pesticides and synthetic risk-free fertilizers, with high nutritional value, and growing media promotes the development and productivity of pepper [45, 46].

Under the studied conditions, the fruit in the vermicompost treatment experienced very high capsaicinoids accumulation in different samples. The results presented in Table 1 clearly demonstrated that vermicompost had a significant effect on the capsaicin content of Chiltepin peppers; this

TABLE 1: Content of capsaicinoids and pungency levels in Chiltepin chili samples cultivated in Lagunera Region, Mexico.

Sample (maturation stage-fertilization)	HPLC									Spectrophotometer	
	n-DHC mg g^{-1} dry weight	%	C mg g^{-1} dry weight	%	DHC mg g^{-1} dry weight	%	C:DHC ratio	Total capsaicinoids mg g^{-1} dry weight	SHU	Total capsaicinoids mg g^{-1} dry weight	SHU*
Ch1 RR-Cr	3.96 ± 0.25[bc]	7	17.79 ± 1.04[a]	30	37.21 ± 2.19[e]	63	1:2.1	58.95 ± 3.46[b]	922,233 ± 54,074[bc]	53.38 ± 2.00[b]	859,404 ± 32,248[b]
Ch1 GM-Cr	4.60 ± 0.19[cd]	6	23.78 ± 0.87[b]	33	41.67 ± 1.48[f]	59	1:1.8	70.04 ± 2.54[c]	1,096,393 ± 39,631[cd]	59.47 ± 2.11[b]	957,517 ± 34,022[b]
Ch1 RR-Vc	4.63 ± 0.36[cd]	8	31.24 ± 2.12[c]	52	24.08 ± 1.61[c]	40	1:0.8	59.95 ± 4.09[b]	933,736 ± 63,333[bc]	ND	ND
Ch1 GM-Vc	6.11 ± 0.33[ef]	8	42.02 ± 1.93[d]	54	29.49 ± 1.32[d]	38	1:0.7	77.62 ± 3.58[cd]	1,208,086 ± 55,466[de]	79.95 ± 0.99[c]	1,287,262 ± 16,010[c]
Ch2 RR-Cr	5.41 ± 0.29[de]	7	43.08 ± 1.26[de]	59	23.37 ± 0.50[bc]	32	1:0.5	71.87 ± 2.04[c]	1,120,242 ± 30,824[cd]	81.83 ± 2.54[c]	1,317,507 ± 40,947[c]
Ch2 GM-Cr	8.55 ± 0.33[h]	9	40.14 ± 0.25[d]	43	43.77 ± 0.24[f]	47	1:1.1	92.45 ± 0.61[e]	1,430,315 ± 8,629[fg]	109.64 ± 1.59[e]	1,765,283 ± 25,650[e]
Ch2 RR-Vc	6.43 ± 0.22[fg]	7	43.06 ± 0.72[de]	48	37.39 ± 0.67[e]	42	1:0.9	86.88 ± 1.45[e]	1,355,038 ± 22,338[ef]	97.73 ± 4.37[d]	1,573,485 ± 70,367[d]
Ch2 GM-Vc	8.38 ± 0.27[h]	8	56.68 ± 1.49[f]	53	39.63 ± 1.03[ef]	37	1:0.7	104.69 ± 2.77[f]	1,628,524 ± 42,886[g]	118.93 ± 3.47[f]	2,468,807 ± 55,855[f]
Ch3 RR-Cr	1.63 ± 0.04[a]	5	15.96 ± 0.40[a]	52	11.96 ± 0.26[a]	39	1:0.8	29.55 ± 0.7[a]	464,591 ± 11,055[a]	20.18 ± 0.21[a]	324,924 ± 3,381[a]
Ch3 GM-Cr	5.97 ± 0.14[ef]	8	39.92 ± 0.66[d]	55	25.24 ± 0.39[c]	35	1:0.6	71.13 ± 1.16[c]	1,104,602 ± 17,743[cd]	76.79 ± 0.52[c]	1,236,362 ± 8,379[c]
Ch3 RR-Vc	3.23 ± 0.18[b]	6	26.75 ± 1.11[bc]	54	19.15 ± 1.88[b]	39	1:0.7	49.12 ± 2.69[b]	768,946 ± 42,380[b]	62.18 ± 2.19[b]	1,001,040 ± 35,293[b]
Ch3 GM-Vc	7.08 ± 0.70[g]	8	47.53 ± 3.54[e]	54	31.93 ± 2.83[d]	37	1:0.7	86.53 ± 7.02[de]	1,345,094 ± 108,385[ef]	89.77 ± 2.57[c]	1,445,312 ± 41,422[c]

Red ripening (RR), green mature (GM), control (Cr), and vermicompost supplement (Vc); nordihydrocapsaicin (n-DHC), capsaicin (C), and dihydrocapsaicin (DHC); Scoville heat units = SHU. Content of n-DHC was calculated as equivalents of DHC. Means in the same column followed by different letters (a–h) were significantly different by Tukey's test at $P \leq 0.05$. ND = not detected. *Capsaicin content converted to SHU using equation from [21].
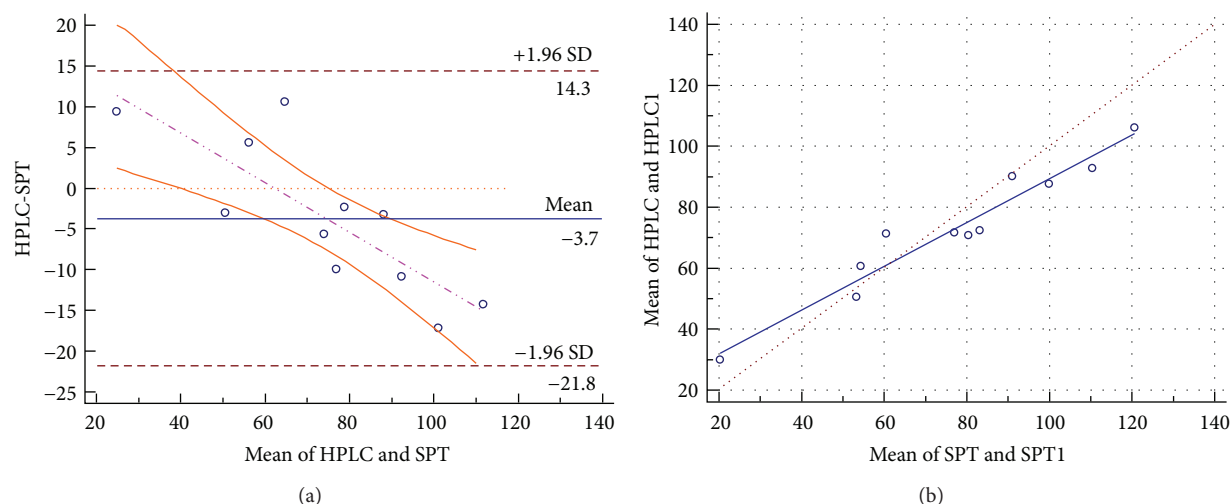
(a)

(b)

FIGURE 3: Bland-Altman plot for total capsaicinoids in Chiltepin fruit extracts. (a) The solid line represents the mean difference in measured capsaicinoids concentrations between methods, and the dashed lines are ±2 SD. Mean difference: −3.7, standard deviation: 1.96, and 95% limit of agreement: −21.8 to 14.3. (b) By Deming regression of the scatter plot, the slope is 0.716; the intercept, 17.59; and the correlation coefficient (r) = 0.88. These results indicate a fair degree of correlation between HPLC and spectrometric (SPT) values.

treatment showed about two times more capsaicin than control treatment. Also, dihydrocapsaicin concentration was affected significantly by the vermicompost treatment, but considerable variability was observed for this capsaicinoid in the different samples. The accumulation of capsaicinoids depends on synthesis and degradation. Capsaicinoids synthesis generally begins with one week after fruit set and continues as placental tissues develop. The compounds reach maximum levels approximately 40–50 days after fruit set [47, 48]. Upon maturation, capsaicinoids levels in most cultivars show a noticeable variable rate of decline associated with an increase in the activity of peroxidase enzymes in ripe fruit [49, 50]. Our results have also indicated that capsaicin levels in GM stage were higher than in RR stage, and vermicompost fertilization does not alter this behavior. This is apparently the first report in Chiltepin pepper of fertilization treatment affecting the metabolism of capsaicinoids responsible for pungency. We can deduce that, in RR stage, the peroxidases may be involved in the degradation of capsaicinoids.

3.4. Spectrophotometer Compared to HPLC. It is assumed that spectrophotometric estimation of the capsaicinoids is inaccurate because of interference of other compounds present in the extract. However, in a previous study, high levels of agreement were found between colorimetric and chromatographic techniques [21]. Although HPLC measurements are in general more precise and reproducible than spectrophotometric methods, these techniques can be an alternative to determine the total capsaicinoids, in spite of the fact that their efficacy is limited, perhaps partly because of carotenoids and chlorophylls. Thus, such testing requires standardized and reproducible techniques.

The agreement between quantification by HPLC-DAD and spectrophotometric method was evaluated by the graphical investigation of difference-plots [51] and by Deming

regression analysis [41]. Difference-plots are useful tools to display differences between methods, but they do not provide statistical tools for inference of the differences. The Deming regression analysis is suitable if measurement errors are proportional and the procedure accounts for errors in both methods [3]. In this case, samples were not analyzed in replicates and equal variance between the two methods was assumed. Medcalc was used for Deming regression analysis and difference-plots.

In difference-plot (Figure 3(a)) and Deming regressions (Figure 3(b)) for capsaicinoids in Chiltepin fruit extracts, the differences between the two methods (HPLC values subtracted from spectrophotometer values) were plotted against the mean concentrations shown by the two methods (Figure 3). The mean differences are shown (solid lines), as are the limits of agreement (broken lines), corresponding to mean ±2 SD. Concentrations quantified by HPLC-DAD and spectrophotometer are plotted against each other (dots) for total capsaicinoids (Figure 3(a)) and the Deming regression (capsaicinoids: $y = 17.59 + 0.716x$) is indicated (unbroken lines), as the identity lines (broken lines).

When comparing simple spectrophotometric and HPLC quantification methods, identical trends among varieties after the performance of means separation were revelated between total capsaicinoids concentrations (spectrophotometer) and total capsaicinoids concentration (HPLC) (Figure 4). A high linear relationship ($r = 0.913$; $P < 0.001$) was observed after correlation analyses. Differences between spectrometric measurements and HPLC analyses in total capsaicinoids content among different samples have shown slightly higher 2 to 26% difference ranging 2–24 mg/kg^{-1} dry wt (Table 1).

Total capsaicinoids quantified by HPLC correlated closely with spectrophotometer values. The correlation coefficient was 0.91 ($P < 0.001$) for capsaicinoids in Chiltepin pepper fruit, indicating a strong positive association between these
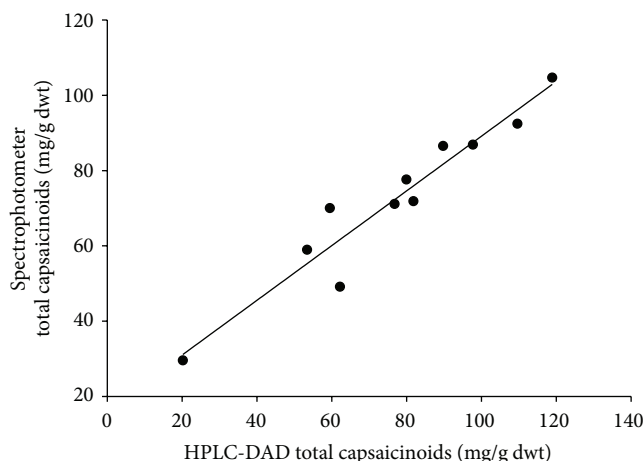
FIGURE 4: Correlation between cumulative capsaicinoids of Chiltepin acetonitrile extract separated by HPLC and total capsaicinoids by spectrophotometer ($r = 0.91$), $n = 11$.

values. The simple spectrophotometric procedure proved to be a valid, efficient, and cost-effective method for the quantification of total capsaicinoids in these conditions, which in fact adequately represent total capsaicinoids content in hot chili pepper extracts.

4. Conclusion

This study describes a spectrophotometric method for the precise determination of total capsaicinoids in pepper fruit samples and yields a close estimation, but it is not as accurate as HPLC-DAD. Further investigation is needed to understand the origin of these analytical errors. The method is an effective mean for quality control and is a viable alternative to the existing analytical methods for routine analyses, allowing a rapid and accessible quantitation of capsaicinoids in fruits samples without any time-consuming sample separation. In addition, our results indicate that, in contrast to other studies where capsaicin content was generally higher in ripe fruit than green fruit, Chiltepin red ripening fruits showed lower levels of capsaicin and dihydrocapsaicin than green mature fruits in all samples. Vermicompost treatment affected capsaicin and dihydrocapsaicin levels and favors the accumulation of capsaicin.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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