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ORIGINAL ARTICLE

Identification and Quantification Bioactive Compounds on Liquid Extract of *Capsicum chinense* Jacq. Biquinho variety: Standardization and Quality Control is Frontier to Acceptance of Herbal Products on Market

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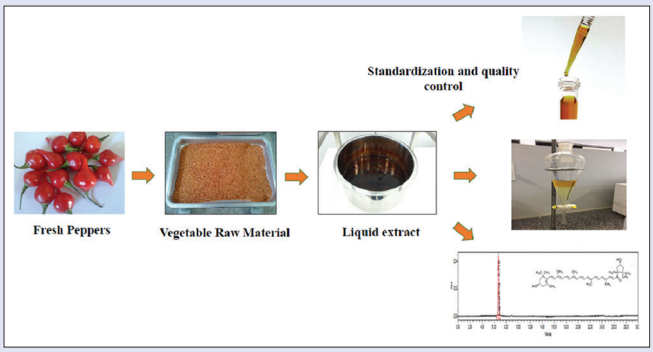
ABSTRACT

Introduction: The peppers of the *Capsicum* genus (*Solanaceae*) are originating in the Americas and have aroused great interest from cosmetics and food industries because these species are rich in antioxidant compounds. **Objectives:** The aim of the present study was to develop and characterize the liquid extract of *Capsicum chinense* Jacq. Biquinho variety standardized in capsanthin. **Materials and Methods:** The liquid extract of the pepper was obtained by percolation and characterized about the solids content, pH, density and viscosity. Subsequently, the total carotenoids were determined by spectrophotometry in the pepper liquid extract, and an analytical method by high performance liquid chromatography was validated to quantify the capsanthin in the same sample. **Results:** The extract showed the following physicochemical properties: 19.16% ±1.03% (w/w) to solids content, a pH of 3.7 ± 0.06, density of 1.040 ± 0.0014 g/mL, and a viscosity of 3.22 mPas. The concentration of total carotenoids was 84.403 mg expressed as β-carotene/100 g of the extract. **Conclusion:** The analytical method developed was considered simple, fast, selective, linear (in the range of 0.48 to 7.2 µg/mL for the liquid extract), precise, exact, and robust, and the found content of capsanthin was 1.13%. The analytical method used for the quantification of capsanthin was validated, proving the simplicity and speed of analysis of the evaluated sample.

Key words: Antioxidants, capsanthin, carotenoids, high-performance liquid chromatography, pepper, *Solanaceae*

SUMMARY

- The fruits of *Capsicum chinense* Jacq. Biquinho variety in the last stage of maturity reveal a large percentage of the carotenoid called capsanthin. Standardization and quality control tests have shown that the liquid extract of this pepper, standardized in capsanthin by high-performance liquid chromatography, may be an alternative as a natural antioxidant for cosmetics and food industries with potential applicability in various types of formulations.



Abbreviations used: HPLC: High-performance liquid chromatography; PDA: Photodiode array; k: Capacity factor; RS: Peak resolution; TF: Tailing factor; N: Theoretical plates; LD: Limit of detection; LQ: Limit of quantification; RSD: Relative standard deviation.

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INTRODUCTION

Peppers are widely used in gastronomy and modern medicine, and basically, there are two widely known genera, *Piper* and *Capsicum*.[1] The genus *Capsicum*, which originates from Central and South America, belongs to the *Solanaceae* family and includes 20 species, represented by peppers of important economic value. These species are known to be good sources of several nutrients, such as Vitamin C, phenolics, flavonoids, and carotenoids.[2,3] The *Capsicum chinense* Jacq. is popularly known as "Biquinho pepper," and this cultivar has gained much popularity in the last years for being tasty, aromatic, and without the characteristic pungency of other peppers.[4] The fruits of *C. chinense* Jacq. Biquinho variety in the last stage of maturation reveal a large percentage of the carotenoid called capsanthin[5] [Figure 1], which has been widely used in culinary to conserve and enhance the taste of food

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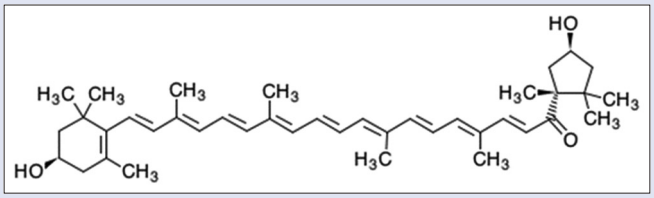

Figure 1: Chemical structure of capsanthin

and as dye in the cosmetic industry. The capsanthin is easily metabolized by the body being absorbed by the intestinal mucosa and presents greater antioxidant activity in relation to the other xanthophylls of the carotenoid group.[6]

The challenges to acceptance of herbal products are the lack of standardization of parameters and quality, such as color, odor, and concentration of secondary metabolites. The herbal extracts, especially of peppers, can be incorporated in various cosmetic and food preparations;[7] consequently, standardization of these extracts is necessary to guarantee the quality and reproducibility, as well as to maintain the stability of the effects and provide user safety.[8] Among the chromatographic techniques, the high-performance liquid chromatography (HPLC) coupled to photodiode array (PDA) has been shown to be a suitable method for the analysis of capsanthin, mainly due to the high selectivity, sensitivity, specificity, and accuracy.[9] Considering the great potential of the carotenoids from *Capsicum* peppers, the aim of this study was to develop and validate a rapid and simple HPLC-PDA method for capsanthin quantification on liquid extract of the *C. chinense* Jacq. Biquinho variety.

MATERIALS AND METHODS

Chemical and reagents

Capsanthin standard (95%) was purchased from Sigma-Aldrich® (Sigma-Aldrich Brazil Ltd., São Paulo, Brazil). All other chemicals used were analytical grade or HPLC grade reagents.

Plant material

The fruits of *C. chinense* Jacq., Biquinho variety, were purchased from the company Rei da Pimenta located in Goiânia Goiás State, Brazil, in July 2016. A voucher specimen was deposited at the Herbarium of Federal University of Goiás, under code number 65904. The fruits were cleaned with distilled water, dried at 45°C for 10 days, and ground a knife mill (Tecnal Ltda., Brazil). The powdered material was stored at room temperature and protected from moisture and heat. The technique of granulometric evaluation by sieving was applied posteriorly, where the granulometric distribution of the obtained powder allowed classifying it as coarse powder.[10]

Liquid extract obtainment

The liquid extract of *C. chinense* Jacq. Biquinho variety was obtained according to the methodology proposed by Santamaría *et al.* and Moresco.[11,12] The preparation of the extract was started with the dynamic maceration technique, at room temperature for 24 h, using 4 kg of vegetable raw material and 25 L of 96% (v/v) ethanol as extractive solvent. Subsequently, the macerated mixture was subjected to the percolation process until exhaustion. Finally, the percolated extract was concentrated by forced evaporation of the solvent using a mechanical evaporator at room temperature.

Pharmacognostic characterization

The physicochemical characterization of the liquid extract was carried out by determinations of the pH, relative density, viscosity, and total solid content, in accordance with the methodologies recommended in National Health Surveillance Agency.[10] All analyses were performed in triplicate.

Total carotenoid determination

Total carotenoid content was measured according to the methodology described by Rodriguez-Amaya.[13] The carotenoid concentration was expressed in mg of β-carotene/100 g of the extract of *C. chinense* Jacq. Biquinho variety at 450 nm, using a UV-Vis spectrophotometer Varian Cary 50. All analyses were performed in triplicate.

High-performance liquid chromatography-photodiode array for capsanthin quantification

HPLC analysis was performed on a Waters® model e2695 (Milford, Massachusetts, USA) equipped with a 2998 matrix photodiode detector (PDA) and Empower® 2.0 software (Waters Corporation, Milford, Connecticut, USA). A column C_{18} (250 mm × 4.6 mm, 5 μm) was used, and the mobile phase was composed of Acetone HPLC grade (A) and ultrapure water (B), using the following gradient elution: linear gradient of 75% A and 25% B in 5 min, linear gradient of 95% A and 5% B in 10 min, linear gradient of 95% A and 5% B in 17 min, convex gradient of 100% A and 0% B in 22 min, and finally linear gradient of 75% A and 25% B in 27 min, as described by Míngguez-Mosquera and Hornero-Méndez,[14] with slight modifications. The flow rate was 1.5 mL/min, the injection volume was 20 μL, the running time was 30 min, and the detection wavelength was 450 nm.

Preparation of standard solution for calibration curve construction

To the construction of the calibration curve, a standard solution of capsanthin was prepared, in triplicate, by dissolving 1 mg of reference standard in mobile phase to obtain a standard solution of 20 μg/mL. The solution was sonicated for 30 min.

Preparation of the sample for quantification of capsanthin

Liquid extract of *C. chinense* Jacq. Biquinho variety (1.35g) was transferred to 5 mL volumetric flasks, solubilized in acetone, and submitted to sonication for 30 min. Aliquots of this solution were diluted in acetone to obtain concentrations between 26.93 and 404 μg/mL. Subsequently, the solutions were analyzed by HPLC.

Evaluation of the method for quantification of capsanthin

The system suitability parameters were evaluated to ensure that the chromatographic system used was able to generate results of acceptable precision and accuracy. These parameters were capacity factor (k), peak resolution (RS), tailing factor (TF), and theoretical plates (N), which were analyzed according to specifications established by Food and Drug Administration (FDA).[15]

Validation of high-performance liquid chromatography-photodiode array method for capsanthin quantification

The validation of the analytical method for the quantification of capsanthin in the liquid extract of the pepper *C. chinense* Jacq. Biquinho variety was carried out according to the criteria proposed by Brazilian Health Surveillance Agency (ANVISA).[16] The following parameters

were determined: selectivity, linearity, limit of detection (LD) and quantification (LQ), precision, accuracy, and robustness.

Selectivity

The selectivity of the method was determined by comparison between the chromatograms and ultraviolet (UV) absorption spectra (range from 300 to 500 nm) of the peaks obtained for both the pepper extract and the capsanthin standard (0.007 mg/mL).

Linearity

An analytical curve derived from the capsanthin standard diluted in methanol using seven different concentrations (0.48 to 7.2 µg/mL) obtained by HPLC was prepared in triplicate. The results were submitted to linear least squares regression analysis to obtain correlation coefficient (r) and standard curve equation.

Limit of detection and limit of quantification

Both LD and LQ for capsanthin were assessed by the residual standard deviation of the linear regression of the three analytical curves. Thus, LD and LQ were performed by means of Eqs. (1) and (2), where σ is the standard deviation of the intercept.

$$LD = \frac{3.3.\sigma}{IC} \quad (1)$$

$$LQ = 10.\frac{\sigma}{IC} \quad (2)$$

Precision

The precision of the analytical method was evaluated in terms of repeatability and intermediate precision, from the analysis of six determinations of the same theoretical concentration (270 mg/mL), performed on different days, with different analysts, but with the same equipment. The results were expressed by the percentage coefficient of variation obtained.

Repeatability

The evaluation of the repeatability was carried out from the elaboration of six solutions containing 270 mg/mL of the extract diluted in acetone submitted to an ultrasonic bath for 30 min. The samples were prepared in triplicate, filtered through a 0.45 µm membrane and injected into the chromatograph.

Intermediate precision

The intermediate precision was performed by different analysts on different days, with sample preparation and analyses performed according to the methodology described for the evaluation of repeatability.

Accuracy

The accuracy was evaluated in terms of recovery using the standard addition method and three concentration levels: low 135 mg/mL, medium 270 mg/mL, and high 404 mg/mL. The result obtained for the recovery was expressed as the relationship between the obtained concentration (Co) and theoretical concentration (Ct), expressed as percentage. The Eq. (3) expresses the relationship between these terms.

$$\text{Accuracy}(\%) = \left(\frac{Co}{Ct}\right) \times 100 \quad (3)$$

Robustness

The robustness of the method was evaluated by the modification of column temperature (28°C and 30°C) and mobile phase flow rate (1.4 mL/min and 1.6 mL/min).

Statistical analysis

All experimental data were submitted to an analysis of variance, with $P < 0.05$ considered statistically significant. The results were processed using software RStudio (R-Tools Technology, Richmond Hill, Ontario, Canada) version 3.4.0 (2017-04-21) and Microsoft Excel (2016).

RESULTS AND DISCUSSION

Pharmacognostic characterization

The granulometric evaluation of the powder material was classified as coarse powder, with a moisture content of 4.62%, showing if suitable for the extraction process, having the characteristics to avoid the formation of preferential channels or packaging of the sample. During the percolation process, because vegetable raw materials with very fine particles are not considered to be the most appropriate for the extraction process, due to the formation of preferential channels, it is difficult to diffuse the solvent in the plant material.[10,17,18] Studying the influence of powder granulometry on the oil extraction of *Raphanus sativus* L. using supercritical CO_2 concluded that a smaller diameter of the particle caused solid matrix compaction, making percolation of the supercritical fluid difficult, reducing the possibility of a satisfactory extraction yield.

The results obtained for the characterization of the liquid extract of the pepper *C. chinense* Jacq. Biquinho variety are shown in Table 1. The evaluation of the physicochemical properties of the extract is necessary because these data can provide useful information about the standardization of this material, ensuring the quality as well as its safety conditions.[8]

Total carotenoid determination

In the present study, the total carotenoid content obtained was 84.40 mg/100 g, expressed as β-carotene/100 g of extract. Parameters such as fruit age as well as drying temperature should be considered for the increasing of the carotenoid extraction. The degradation of these metabolites occurs above 40 °C; therefore, the exposure of the raw material to high temperatures could promote the exponential degradation of the carotenoids.[19,20] According to Sun *et al.*,[21] the carotenoid concentration can be affected by environmental conditions (such as high temperature, pH of the medium, and luminosity) and the maturation stage of the fruit as well as the signaling and modulation properties performed by these compounds during the development of the plant, directly influencing its concentration.[22]

In a study carried out with fruits of the *Capsicum* genus, Moresco[12] identified, by chromatographic separation, five types of carotenoids and verified that peppers of *C. chinense* species presented a higher content of these metabolites, obtaining a concentration of 10.68 mg/100 g of ethanolic extract.

Table 1: Results for the physicochemical characterization of the liquid extract of *Capsicum chinense* Jacq. Biquinho variety

Test description	Results
pH (hydrogenation potential)	3.7±0.06
Total solids content (%)	19.16±1.03
Relative density (g/mL)	1.04±0.0014
Viscosity (mPas)	3.22

In a study performed by Menichini *et al.*,[23] with fruits of *C. chinense* Jacq cv. variety Habanero, the samples analyzed presented a longer stage of maturation and the carotenoid concentration obtained was 62.7 mg/100 g in the ethanolic extract.[24] Reported that during the immature stage of the *C. annuum* fruits, the predominant carotenoids are lutein and beta-carotene, but in mature fruits, the concentration of these carotenoids decrease and the main carotenoid identified is capsanthin.

In general, the result determined in this article suggests that the liquid extract, obtained from *C. chinense* Jacq. variety Biquinho, presents good levels of total carotenoids, making this complex matrix an excellent source of carotenoids.

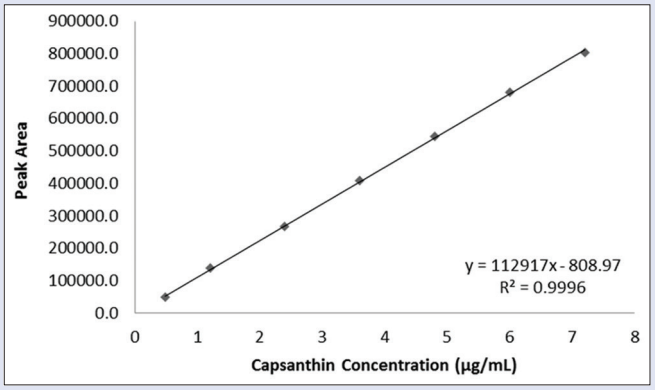

Figure 2: Calibration curve of the capsanthin standard

Calibration curve construction

The calibration curve constructed from the standard capsanthin solution demonstrated that peak area values are directly proportional to the analyte concentration in the sample. The obtained results were considered satisfactory, because the minimum acceptable criterion of the correlation coefficient (r) of the calibration curve is 0.99,[16] and an average value of 0.9996 was obtained in the experiment [Figure 2].

Evaluation of the method for capsanthin quantification

The system suitability parameters were in accordance with the parameters established by the Food and Drug Administration[15] and are available in Table 2. The method developed for the analysis of capsanthin by HPLC showed an ability to generate acceptable quality data when performing the tests of precision and accuracy.

The quantification of capsanthin in the liquid extract of *C. chinense* Jacq. Biquinho variety was performed based on the integrated areas of the six sample peaks and its identification was performed by comparison with the retention time [Figure 3] and UV spectra [Figure 4] in relation to capsanthin. The capsanthin content obtained in the liquid extract was 1.126% in relation to the total extract mass. The peak of capsanthin was identified by its absorption spectrum in the visible region with maximum λ at 301.2 and 475.6 nm [Figure 4] and by the retention time (6,71 min) compared to the values obtained for the capsanthin standard [Figure 3].

Using the analytical methodology for the analysis of capsanthin by HPLC in the liquid extract of Biquinho pepper proposed in this study, it was observed that the mobile phase used provided adequate resolution and symmetrical peaks, better separation of the analytes, and less interference.

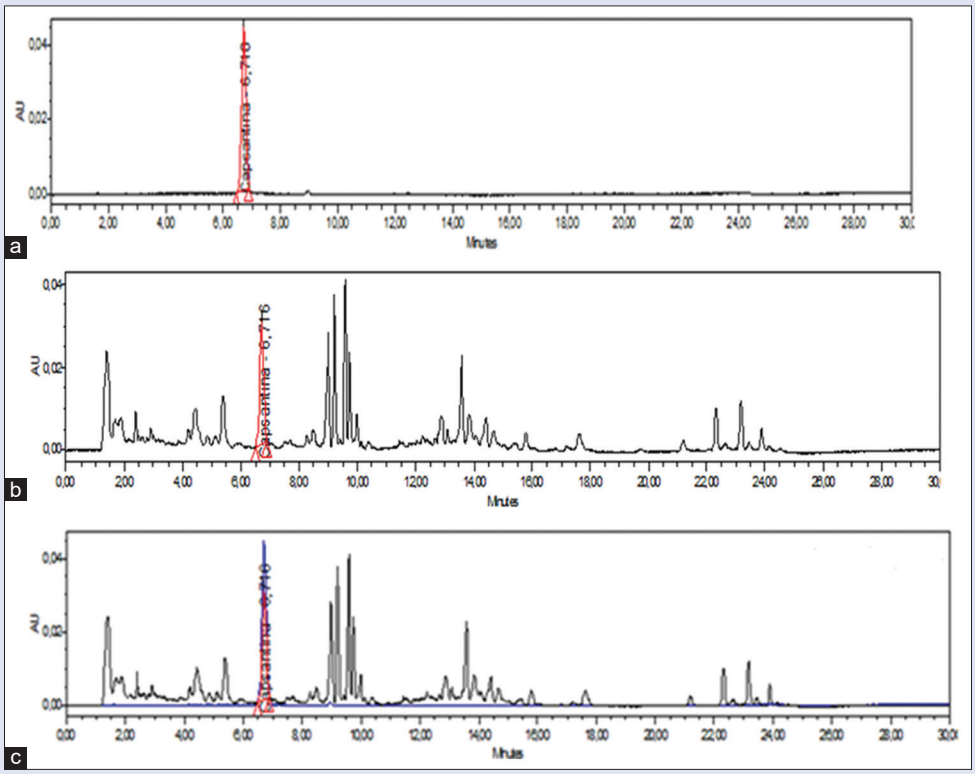

Figure 3: High-performance liquid chromatography-photodiode array chromatograms of (a) capsanthin standard; (b) liquid extract of *Capsicum chinense* Jacq. Biquinho variety; and (c) OVERLAP of a and b. Chromatographic conditions: Promosil C_{18} column, 250 mm × 4.6 mm, 5 µm, 29°C/Acetone: H_2O, gradient/1.5 mL/min

This behavior suggests that the carotenoid pigments present in the liquid extract of the pepper *C. chinense* Jacq., Biquinho variety, can be separated with gradient elution techniques in the reverse phase separation mode using a binary mixture of acetone and water as the mobile phase.

Validation of the analytical methodology

One of the limitations in analyzing herbal products is the chemical complexity, which increases the difficulty of quality control of these products.[25] Thus, the quality, efficacy, and safety of Biquinho pepper products may be assured using a validated method for capsanthin quantification as the chemical marker in the raw material and in intermediate products, such the liquid extract.

The selectivity was proven for the method proposed in this study, because the analytical response observed was due exclusively to capsanthin and could be observed by comparing the sample and standard scanning chromatogram at the retention time corresponding to the capsanthin peak in 6.71 min for standard and 6.72 min for the sample without the presence of interfering substances [Figure 3]. Furthermore, well-resolved peaks reveal the specificity of this method.

The linearity test was performed through the elaboration of analytical curve for the capsanthin standard in the concentration range of 0.48–7.20 µg/mL. The linear equation obtained was $y = 112917x - 808.97$ ($n = 3$) and the resulting correlation coefficient 0.9996 allows estimating the quality of the curve obtained, since a correlation coefficient greater than 0.999 is considered as evidence of an ideal fit of the data for the regression line [Figure 2].[16] From the parameters of the calibration curve, the quantification limit (LQ), equivalent to the lowest amount of capsanthin determined with acceptable precision and accuracy, corresponded to 1.46 µg/mL, and LD equivalent to the lowest level to be detected was 0.48 µg/mL. The results obtained showed good sensitivity of the proposed method.

The precision was evaluated by the proximity of the results obtained in the analyses of six determinations to 100% of the test concentration. The assay performed for repeatability analysis on day 1 showed a relative standard deviation (RSD) of 3.52%, the analysis of day 2 showed a RSD value of 2.25%, and for intermediate precision, the RSD was 3.01%. In all tests performed, it was observed that the method presented precision values consistent with the limits established by the National Health Surveillance Agency (Brazil),[26] because even with the chemical complexity of the liquid extract of *C. chinense* Jacq. the results of relative standard deviation should not exceed 15%. According to Betz *et al*.,[27] the precision test is essential for the development of methodologies for products obtained from natural sources.

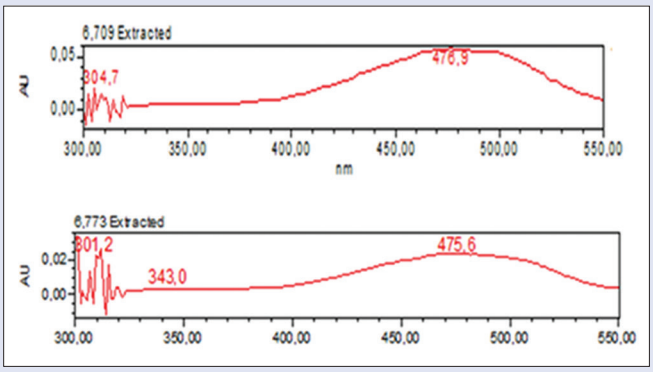

Figure 4: Absorption spectra in the UV-visible region of capsanthin in the standard and liquid extract of *Capsicum chinense* Jacq. pepper Biquinho variety

The accuracy of the method evaluated the recovery percentage obtained in each established concentration level for capsanthin, and three determinations were performed for each concentration: low (135 mg/mL), mean (270 mg/mL), and high (404 mg/mL). The mean recovery was 102.91% (RSD = 2.30) [Table 3]. According to Ribanni *et al*.,[28] acceptable recovery values depend on the analytical complexity and the sample and admit values ranging from 50% to 120% for precision with a standard deviation of ±15%.

The robustness showed that the small and deliberate modifications in the chromatographic conditions of the original method did not result in problems in the quantification of capsanthin, presenting a satisfactory robustness to the proposed method according to Table 4. It is said that a method is robust when it is unaffected by small and deliberate modifications in its parameters, on average a variation between ±2.0% and ± 5.0% in relation to the parameters initially adopted.[28]

The method developed in the current study proved to be selective, linear, precise, accurate, and robust, which, although being a gradient elution, can be considered simple and less expensive due to the use of a smaller amount of reagents and a short elution time. The method was also considered suitable to be used in the standardization and quality control of liquid extracts obtained from the peppers of the Biquinho variety, because once validated it guarantees the achievement of more reliable and consistent analytical results.

Due to the increasing demand for the use of natural raw materials in the manufacturing of processed products, the liquid extract of the pepper *C. chinense* Jacq. Biquinho variety, standardized in capsanthin, may be an alternative as assets antioxidant, anti-aging, and preservative, as capsanthin has greater antioxidant activity than other xanthophylls

Table 2: System suitability data of the high-performance liquid chromatography analytical method for quantification of capsanthin in the liquid extract of *Capsicum chinense* Jacq. Biquinho variety

Parameters	Results	Literature recommendations
Capacity factor (k)	7.05	>2
Resolution (Rs.)	12.90	≥2
TF	1.26	≤2
Theoretical plates (n)	11.500	>2000

TF: Tailing factor

Table 3: Results of the accuracy assay for the standard of capsanthin recovery test in liquid extract of *Capsicum chinense* Jacq. Biquinho variety

Standard concentrations added (mg/mL)	Recovery (%)	Average (%)±RSD (%)
135	105.72	102.91±2.30
270	99.66	
404	103.22	

RSD: Relative standard deviation

Table 4: Results obtained in the robustness test for the method of quantification of the capsanthin in the liquid extract of the pepper *Capsicum chinense* Jacq. Biquinho variety

Modified parameters	Average peak area (µv×S)	Average retention time (min)	SD	CV (%)
Original method	353343.667	6.46	0.03	0.44
Temperature 30°C	372065	6.13	0.16	2.58
Temperature 28°C	372241.667	6.23	0.03	1.83
Flow rate 1.6 mL/min	348642.667	5.92	0.19	3.11
Flow rate 1.4 mL/min	397440	6.66	0.26	4.08

SD: Standard deviation; CV: Coefficient of variation

due to their molecular structure, particularly the keto groups.[29] In general, capsanthin plays an important role for human health because of its antioxidant effect,[30] being a great ally in the prevention of certain types of cancer,[31] cardiovascular diseases,[32] eye disorders,[33] and degeneration and premature aging of the skin.[34]

CONCLUSION

The results showed that the liquid extract of *C. chinense* Jacq. Biquinho variety standardized in capsanthin is an alternative as a natural antioxidant active for cosmetic and food industries and can be used in diverse formulations. The method for capsanthin quantification was selective, sensitive, precise, accurate, robust, and linear over the concentration ranging between 0.48 and 7.2 µg/mL. These results reveal that the quantification method of capsanthin can be successfully applied on the routine quality control of *C. chinense* Jacq. Biquinho variety products.

This study was the first to report the validation of a method for the quantification of capsanthin in the liquid extract obtained from the Biquinho pepper, adding value to this raw material of vegetable origin, because it underwent a process of control of quality and standardization.

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Conflicts of interest

There are no conflicts of interest.

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