Article

Development and validation of an analytical method by HPLC-DAD for determination of caffeine in products based on guarana extracts (*Paullinia cupana*)

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Guarana (*Paullinia cupana*) is a native plant from the Amazon whose seeds contain a high concentration of caffeine. Aqueous extract of guarana is widely used in the world. In this study, the objective was to develop and validate a High-Performance Liquid Chromatography method for the determination of caffeine in extracts and commercial beverages based on guarana. A sensitive, simple, and viable high performance liquid chromatographic method without the need of an analyte extraction procedure was developed and validated according to Brazilian and international requirements. The method presented high performance, fulfilling Brazilian and international requirements, in addition to allowing product compliance tests. Results confirmed high selectivity and linearity (>0.999) between 5 to 135 ug/mL, with no significant matrix effect. Detection and quantification limits were 0.02 μg/mL and 2 μg/mL, respectively. Precision was less than 4 %, and accuracy varied from 99.9-120 %. Applicability of the method was demonstrated by conducting a limited evaluation in products containing caffeine. Commercial extracts showed quite different caffeine levels, while carbonated drinks follow Brazilian and American recommendations. Our results indicate that the developed method can be used to evaluate the quality of the guarana extract and of products containing caffeine.

Keywords: *Paullinia cupana*. Caffeine. Guarana extract. High performance liquid chromatography.

INTRODUCTION

Paullinia cupana Kunth belongs to the Sapindaceae family, which is popularly known as guarana. Commercial cultivation of guarana occurs mainly in Brazil, of which Empresa Brasileira de Pesquisa Agropecuária (Embrapa) Amazônia Ocidental maintains a breeding program. Out of the 3,280 tons of seeds produced annually, according to

the Instituto Brasileiro de Geografia e Estatística (IBGE), 70 % are directed to the soft drink industry in the form of extracts/syrups (IBGE, 2017). The other 30 % is sold by drugstores and health food stores, mainly in the form of roasted and powdered seeds (Marques *et al.*, 2019). Due to the high concentration of caffeine, guarana is widely used as raw material for the manufacture of juices, energy drinks, and carbonated drinks (Ângelo *et al.,* 2008).

Currently, about 120,000 tons of caffeine per year are consumed worldwide (Latosińska, Latosińska, 2017). Caffeine (1,3,7-trimethylxanthine) is a natural compound based on xanthine. It is classified as an alkaloid and has heterocyclic nitrogen (Fekry *et al.,* 2020). This substance is a natural stimulant found in teas, coffees, sodas, and chocolates. It is considered to be a stimulant, in part, because of its potential to cause positive behavioral effects

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at low doses (Turak, Güzel, Dinç, 2017). This compound has several physiological effects, including increased blood pressure, mood improvement, pain perception, and central nervous system stimulation (Fekry *et al.,* 2020; Arazi, Hoseinihaji, Eghbali, 2016).

Although caffeine is legally used in food, it can be harmful or even toxic after prolonged ingestion, being associated with irritability, insomnia, and tachycardia. In recent years, the addition of caffeine to improve product content has been reported, a practice known as sophistication (Do, Santi I, Reich, 2019). In many cases this addition can exceed the security concentration limits of this substance (Sereshti, Samadi, 2014). Thus, regulations limiting its use have been established in many countries, such as in the USA, where the Food and Drug Administration (FDA) limits the maximum permitted concentration of caffeine in these products to 200 mg/L (Fekry *et al.,* 2020). In this way, in order to verify if these products fulfil the regulatory requirements and also to maintain food high quality, several quantitative methods have been developed for determining caffeine.

Over the decades, the most popular method has been High Performance Liquid Chromatography with a Diode Array Detector (HPLC-DAD) which is used to identify and determine substances in natural products, because it enables reduced analysis time, lower consumable consumption, and lower cost per analysis than the methods that use gas chromatography and/or mass spectrometry (Bertolín, Joy, Blanco, 2019).

Some authors have proposed methods to analyze caffeine in beverages and medications (Sun *et al.,* 2019; Machado *et al.,* 2018; da Silva *et al.,* 2017). However, few methods have been developed and applied to guarana seed extract, which is the raw material for most of these products. In this context, the analysis of guarana seed extract caffeine-containing products becomes extremely important in order to ensure that the levels meet regulatory requirements and are safe for consumers (Sereshti, Samadi, 2014; Sousa *et al.,* 2011). Thus, it becomes necessary to build a simple, fast, accurate, and efficient analysis method in the shortest possible time. From this, the objective of this study was to develop and validate an analytical method by HPLC-

DAD for the quantitative determination of caffeine in commercial extracts of *P. cupana*. The method was validated according Brazilian, American, and European regulations (Brasil, 2011; Brasil, 2017; FDA, 2015; Kazusaki *et al.,* 2012; European Commission, 2017; Magnusson, Ornemark, 2014) and the Harmonized Tripartite Guideline (ICH, 2005).

The aim of this study was to develop a simple analytical method in order to identify and quantify the amount of caffeine in guarana extracts. The development of this method aims to contribute to the quality control of herbal medicines, beverages, and other products based on guarana extract or that complement the caffeine content with guarana extracts, since the validation of analytical methods applied to natural products is still the key point for obtaining standardized extracts.

MATERIAL AND METHODS

Reagents and standards

The solvents and reagents used in this study were acetonitrile, methanol, formic acid, and ultra-purified water (Milli-Q® IQ 7003/05/10/15 Water Purification Systems). All solvents and reagents employed had either pre-analysis purity or chromatographic purity (HPLC). All solvents and reagents used were purchased commercially from Tedia® and/or Sigma-Aldrich®. The 0.22-µm PTFE syringe filters were purchased commercially from Filtrilo®. The sterile syringes of 1, 5, and 10 mL were purchased commercially from the BD company.

Samples

Commercial samples of *P. cupana* extracts in liquid form were purchased from a medium-sized Brazilian industry (Midwest region of Minas Gerais, Brazil). Commercial products (cola, guarana, and energy drink) were purchased from small commercial establishments (Divinópolis, Minas Gerais, Brazil). Three samples were evaluated in triplicate. Caffeine standard was purchased commercially and certified by CSPC Innovation Pharmaceutical.

Preparation of samples

Samples of commercial extracts of *P. cupana* were diluted 1:100 or 1:1000 in methanol or acetonitrile. Soft drink and energy drink samples were degassed in an ultrasonic bath for 60 min and then diluted 1:10 or 1:100 in methanol. All samples were transferred to a vial using a 0.22-µm PTFE syringe filter before injection into the liquid chromatographic system.

Preparation of the standard solution

The stock solution was prepared with a mass of 50 mg caffeine in methanol at a concentration of 0.5 mg/mL. By gradual dilution of the stock solution, levels covering the linear range and estimated caffeine levels in the sample were performed.

Instrumentation and chromatographic conditions

High speed analysis by HPLC-DAD was performed using the Prominence HPLC chromatograph system (Shimadzu, Kyoto, Japan). The chromatograph system was comprised of a binary pump system (LC-20AD) coupled to a diode array detector (SPD-M20A), autosampler (SIL-20AHT), communicator (CBM-20A), and degasser, and was controlled by LabSolutions software version 1.25 (Shimadzu, Kyoto, Japan). The experimental work was performed in an air-conditioned room maintained at 14 ± 2 ºC.

Method development

Pre-filtered samples (5 µL) were injected into a Waters C_{18} column (5 µm, 150 × 4.6 mm). The mobile phase system consisted of acetonitrile: ultra-purified water (80:20 v:v), and was run in isocratic mode at a flow rate of 1.2 mL/min through the column. The run time was 10 min per injection and the elute was monitored at a wavelength of 280 nm (Brasil, 2013).

Optimized method

The chromatographic conditions adopted were as follows: Waters C_{18} column (5 µm, 150 × 4.6 mm) eluted with the mobile phase system in a gradient mode composed of ultra-purified water acidified with 0.1 % formic acid (A) and methanol (B). The gradient program was as follows: 0 min, 45 %B; 0.01-5 min, 45 to 90 %B; 5-6 min, 90 to 45 %B; 6-7 min, 45 %B. The analysis time was 7 min. The flow rate was 1.2 mL/min. The injection volume was 20 µL. An Ultraviolet/Diode Array Detector (UV/DAD) was used for identification and quantification.

Method Validation

The validation of the analytical method used to quantify caffeine in commercial extracts of *P. cupana* was performed according to the parameters recommended by the FDA (FDA, 2015; Kazusaki *et al.,* 2012), the SANTE/11813/2017 (European Commission, 2017), the Lab Guide to Method Validation by Eurachem (Magnusson, Ornemark, 2014), the guide of the Instituto Nacional de Metrologia, Normalização e Qualidade Industrial (Inmetro) – Guidelines on Validation of Analytical Methods (DOQ-CGCRE-008) (Brasil, 2011), the Resolução da Diretoria Colegiada (RDC) Nº 166 of 2017 of the Brazilian Health Regulatory Agency (ANVISA) (Brasil, 2017), and the Harmonised Tripartite Guideline (ICH, 2005).

The evaluated parameters were selectivity, linearity, matrix effect, precision, accuracy, and robustness.

System Suitability Specifications

The FDA suggests evaluation of chromatographic parameters to certify that the equipment used complies with analyte separation and analysis (FDA, 2015; Kazusaki *et al.,* 2012). The chromatographic parameters, such as retention factor (*k*), resolution (Rs), separation factor (α), and number of theoretical plates (N), were used to evaluate the chromatographic separation.

Selectivity

In this study, selectivity was demonstrated by analyzing the patterns of caffeine and tannins injected alone and together, as well as *P. cupana* extracts. In addition, LabSolutions software peak purity analysis was used to evaluate the chromatographic peak purity.

Linearity and matrix effect

The linearity test was performed by drawing the analytical curve by the standard addition method in solvent (methanol) in the range of 5 to 135 µg/mL. Linearity between peak area and concentration was determined using a calibration curve obtained with standard solution at 8 concentration levels (5, 10, 20, 40, 80, 100, 125, and 135 µg/mL) with three independent replicates of each level. The solutions were analyzed in random order within each day.

Regression parameters were estimated by the Ordinary Least Squares Regression (OLS) method. Outliers were diagnosed by the standard Jackknife residue test (Bollinger *et al.,* 1981), and applied successively until new extreme values were not detected or up to a maximum exclusion of 22 % in the original number of outcomes. The assumptions regarding the regression analysis were verified by the Ryan-Joiner test for residual normality (Ryan, Joiner, 1976), Durbin-Watson test for residue independence (Watson, Durbin, 1950), and the Brown-Forsythe test (or modified Levene) for residue homoscedasticity (Brown, Forsythe, 1974). F tests were adopted to verify the adequacy of the fit to the linear model by means of the regression significance, and the linearity deviation evaluated against the pure error (Draper, Smith, 1998). Linearity was also statistically evaluated by analysis of variance (ANOVA) to assess linearity deviation and regression significance.

In addition to visual analysis, the interference of the matrix on the method accuracy was evaluated by the F test (Snedecor, Cochran, 1991) to verify whether the inclinations and intersections would be compared by the t test with the combined variances or distinct variances (Armitage *et al.,* 2001). The matrix effect was evaluated by the tilt and parallelism t-test.

Sensitivity

The detection limit (LOD) and the theoretical quantitation limit (LOQ$_t$) were determined from the caffeine standard calibration curve. The LOD was obtained by multiplying the standard deviation of the replicates ($n = 10$) of the blank by the value of t (*student*) considering 95 % confidence. The LOD was calculated by summing the mean concentrations obtained by the white replicates and the t (*student*) value of the replicates, multiplied by the standard deviation (SD). The LOQ$_t$ was obtained by multiplying the SD of the blank (methanol) replicate concentrations ($n = 10$) by 10 (Brasil, 2017; Magnusson, Ornemark, 2014).

The LOQ$_t$ value found mathematically (0.10 µg/mL) was evaluated experimentally (LOQ), as well as a value below (0.05 µg/mL) and above (0.15 µg/mL). To find the experimental LOQ ($n = 7$), an evaluation of the concentrations below the first point of the best linear curve were performed until a Coefficient of Variation (CV) less than or equal to 5 % was obtained.

Accuracy and Precision

Precision was assessed by repeatability and intermediate precision (Brasil, 2017). For repeatability, the same analyst performed 21 determinations on the same day, which were comprised of three different concentrations: low (15 µg/mL), medium (60 µg/mL), and high (130 µg/mL) with 7 replicates at each level on three separate days. The mean, SD, and CV of the replicates were calculated at each level on separate and inter-days. It was considered acceptable when the CV was less than or equal to 5 %. Accuracy was assessed by the recovery test (Brasil, 2017). Recovery was considered acceptable if it was within the range of 80 to 120 % of the expected value.

Robustness

Robustness of a parameter was considered satisfactory if the CV was less than or equal to 5 % and the recovery was within the range of 80 to 120 % of the expected value. Additionally, the influence of the six analytical parameters on retention time (RT), retention

factor (k), resolution (Rs), and number of theoretical plates (N) was evaluated (Table SI).

Statistical analysis

Statistical tests used in the analytical validation as the linearity parameter were: OLS, Standardized Jackknife Residue Test – Outliers, Ryan Joiner Test, Durbin-Watson Test, Brown-Forsythe Test, and ANOVA. In addition, tests for the effects of the parameter matrix (OLS, F test, and t test for slope and parallelism, and interception testing) were performed in Excel 19 (Microsoft Office 2019, Microsoft, California, EUA, 2019) and Origin Lab 8.6 (Origin Lab Corporation, Massachusetts, USA).

RESULTS AND DISCUSSION

The use of chromatographic techniques for the separation and quantification of compounds of plant origin has become one of the most used techniques in laboratories and in research. The use of selective, sensitive, and validated analytical methods for the quantitative evaluation of commercial products is crucial for the quality control and adequate inspection of these products (Ribani *et al.,* 2004).

Initially, the method was based on the chromatographic conditions described in the rules suggested by the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA) (Brasil, 2013). The method uses acetonitrile, which is a high-cost solvent and presents a high risk of toxicity (Klein, Longhini, de Mello, 2012). In this sense, a new method was developed using methanol and water, a mobile phase that is more ecological, simple, easily found commercially, and has a lower cost of acquisition. In addition, this mobile phase showed good separation and resolution of chromatographic peak.

Some methods for determining caffeine depend on programming the mobile phases in isocratic mode, a chromatographic column greater than 20 cm, and a long analysis time (Machado *et al.,* 2018). The MAPA method was based on programming the mobile phases in isogradient mode (acetonitrile:water 20:80 v:v) in 10 min of analysis. The development and optimization of the new conditions allowed to reduce the analysis time to 7 min, to reduce the cost of consumables, and to allow a better separation of the components of the samples. It was possible to obtain the chromatographic peak corresponding to caffeine in approximately 3 min (Figure 1).
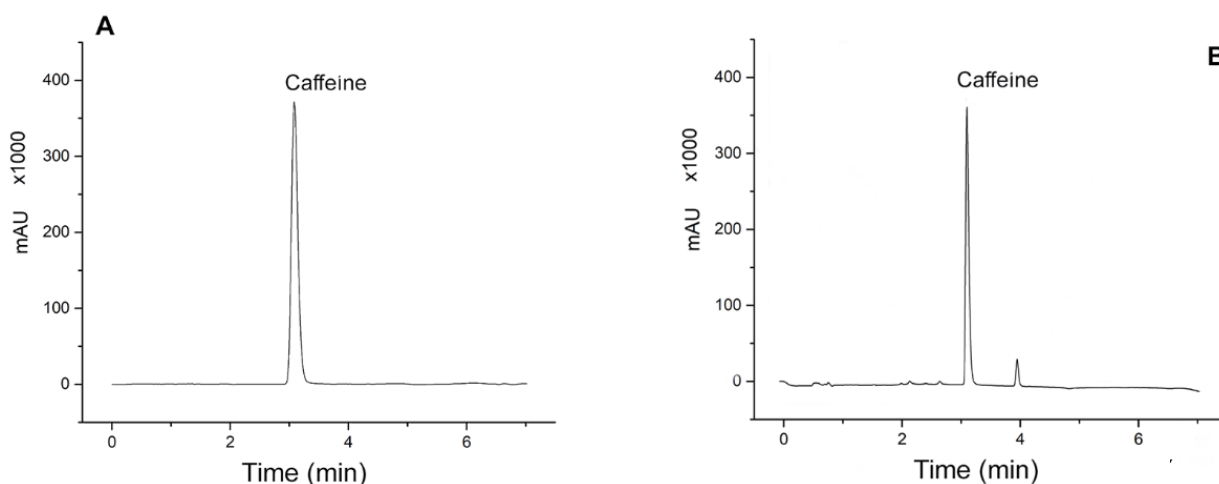
FIGURE 1 - Chromatographic profile of the caffeine in a standard sample (A) and in the commercial extract (B) at 270 nm.

The compounds were analyzed by scanning the UV spectrum in the 200-600 nm range. The scan determined that caffeine has a maximum absorbance close to 270 nm, and therefore was used in all subsequent analyses. Methylxanthines have their maximum and most selective absorbance at 280 nm. The 280 nm band is associated with electronic benzoyl transitions with a catechin ring, increasing the selectivity of the method due to the smaller number of organic compounds that absorb this wavelength (Yonekura, Tamura, 2019). Thus, current methods employ 280 nm. However, our results have shown that 270 nm has better precision and accuracy.

During validation, the FDA suggests evaluating the chromatographic parameters as a criterion to evaluate the efficiency of the equipment (FDA, 2015). The chromatographic parameters established to evaluate the equipment and chromatographic conditions are shown in Table I. The results found for caffeine were acceptable according to the regulation, with excellent resolution (Rs > 2), a satisfactory retention factor, and high number of theoretical plates (N > 2000) as suggested by the FDA (Magnusson, Ornemark, 2014). Selectivity is an important parameter that refers to the ability of the analytical method to differentiate the signal related to the analyte of interest from the signals generated by interferents present in the matrix. Selectivity was assessed by injecting standard caffeine solutions at known concentrations, as well as a mixture of a guarana extract sample to observe possible changes, interferences, and chromatographic separation behaviors. The UV spectra obtained in this study are similar to those found by Klein, Longhini and de Mello, (2012), and Bae *et al.* (2015).

TABLE I - Chromatographic parameters for caffeine

Chromatographic parameter	Caffeíne[1]
Retention factor (*k*)	1.46 ± 0.512
Resolution (Rs)	4.484 ± 0.432
Number of theoretical plates (N)	3162.8 ± 26.3
Retention Time (RT)	3.086 ± 0.014
UV (λ max) nm	203 and 272

[1]Values expressed as mean ± standard deviation (SD) (n = 7)

Linearity was initially assessed using R^2, but it must be proven by statistical tests (ANVISA, 2017). The initially drawn curves showed R^2 values suggestive of linearity ($R^2 > 0.99$), but they were not statistically linear. According to the Analytical Methods Committee, a regression coefficient value next to the unit is not necessarily the result of a linear relationship, and as a consequence statistical treatment for the lack of adjustment must be applied (Klein, Longhini, de Mello, 2012).

A test was prepared with 8 concentration levels (5, 10, 20, 40, 80, 100, 125, and 135 µg/mL) in triplicate for the elaboration of the linear range, but the curve obtained deviated from linearity. The linear curve established was 10 to 135 µg/mL (10, 20, 40, 80, 125, and 135 µg/mL). This study highlights the importance of treatment and statistical evaluation to confirm linearity, because if the treatment were not carried out, it would not result in true concentrations.

Five extreme values were detected both in the solvent curve and in the matrix curve by the standardized Jackknife test. However, only four values were removed due to the allowed elimination limit, which is equivalent to 22 % (Horwitz, 2003). The graphs of the regression residuals, with indications of the extreme values detected and removed, are shown in Figure 2.

The premise that the residues follow normal distribution (Figure 3), independence (Figure 4), and are homoscedastic (Figure 5) was confirmed for the solvent and matrix curve. After verifying the premise of OLS, the following regression equations were retrieved: Area = 52508 [Caffeine] – 112551 ($R^2 = 0.9986$) for the curve in solvent; and Area = 51475 [Caffeine] + 1.23×10^6 ($R^2 = 0.9975$). The data were evaluated, as well as adjusted to the linear model, as shown in Table II. The significance of the regression and non-significant linearity deviations indicated linearity in the range of 10 to 135 µg/mL. The graphs of the solvent and matrix curves after linearity assessments are shown in Figure 6.
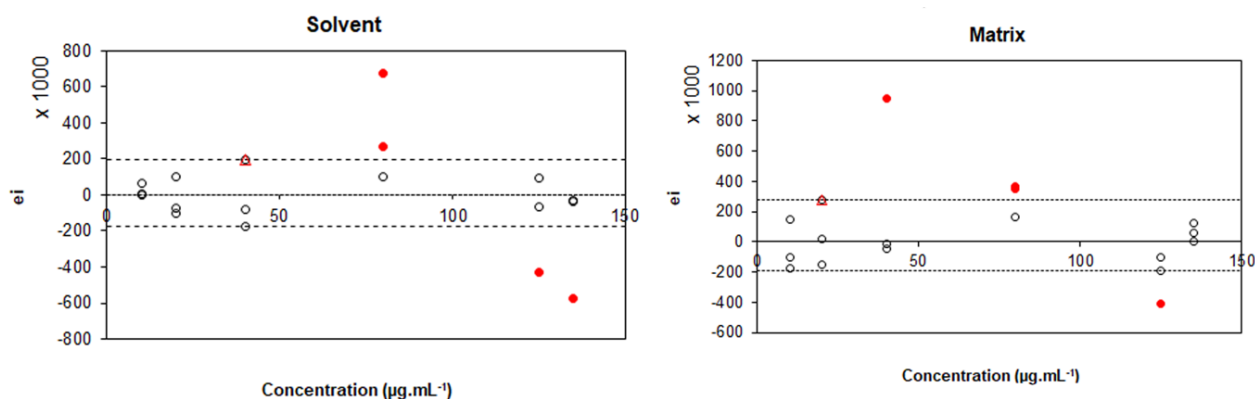

FIGURE 2 - Exploratory graphs of the residues from the regression of the caffeine curves (10 to 135 µg/mL) in solvent and matrix with indication of the respective extreme values diagnosed by the standardized Jackknife residue tests.

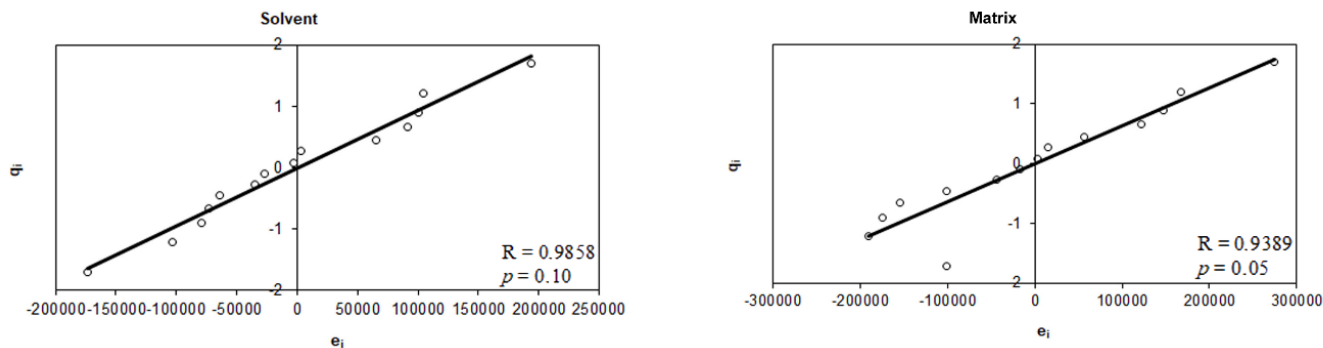• = extreme value eliminated; Δ = extreme value not eliminated.

FIGURE 3 - Plots of normal probability of caffeine curves (10 to 135 µg/mL) in solvents and matrices.

e_i = residual of the regression, q_i = expected normal value, R = Ryan-Joiner correlation coefficient, p = significance.
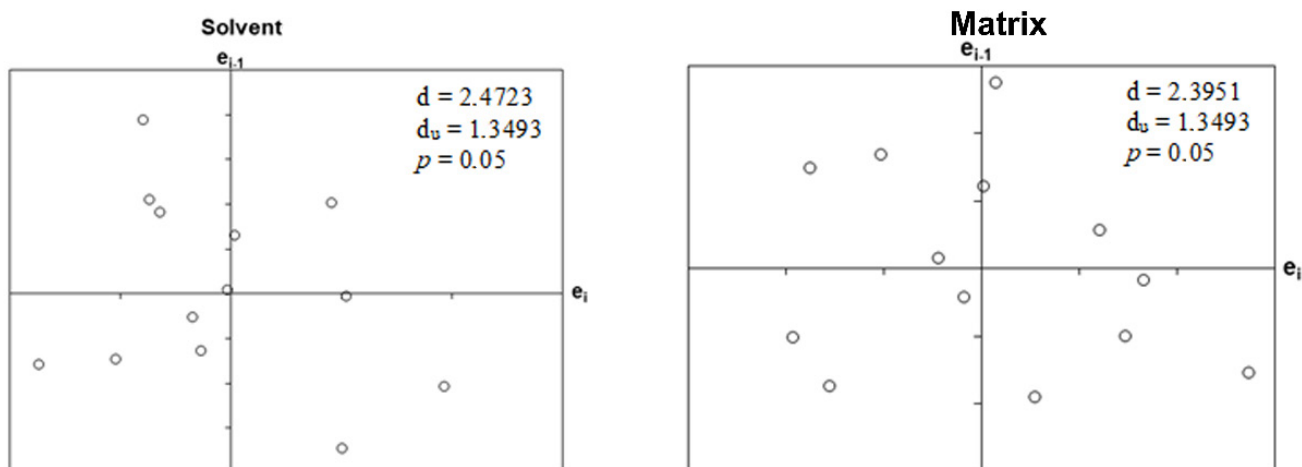
FIGURE 4 - Durbin-Watson graphs of the solvent and matrix curve.

e_i = residual of the regression, d = Durbin-Watson statistic, d_u = upper critical limit, p = significance.
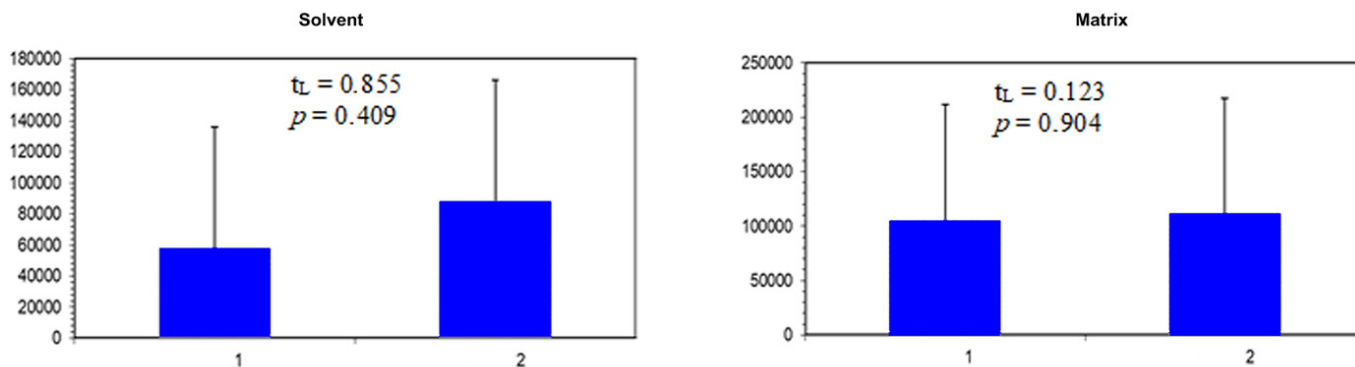

FIGURE 5 - Representation of the homogeneity of the variances of the caffeine curve (10 to 135 µg·mL^{-1}) in solvent and matrix by modified Levene test. t_L = Levene's t-statistic; p = significance; Group 1 = 80, 125, 135 µg/mL; Group 2 = 10, 20, 40 µg/mL.

TABLE II - Evaluation of the linearity for the caffeine curve (10 to 135 µg/mL) in solvent and matrix obtained in the matrix effects evaluation tests

Statistic	Solvent Curve	Matrix Curve
Number of observations		
N	14	14
Normality		
R	0.9858	0.9389
p	p = 0.10	p>0.05
$R_{critical}$	0.9481	0.9351
Homoscedasticity		
t_L	0.855	0.123
p	0.409	0.904

TABLE II - Evaluation of the linearity for the caffeine curve (10 to 135 µg/mL) in solvent and matrix obtained in the matrix effects evaluation tests

Statistic	Solvent Curve	Matrix Curve
Independence		
d	2.4723	2.3951
p	0.05	0.05
dU	1.3493	1.3493
Regression		
F	8.55×10^3	4.76×10^3
p	1.71×10^{-18}	5.69×10^{-17}
Fcritical	4.747	4.747
Deviation from linearity		
F	0.297	1.175
p	8.72×10^{-1}	3.91×10^{-1}
Fcritical	3.837	3.837

n = number of observations; R = Ryan-Joiner correlation coefficient; p = significance; tL = Levene's t statistic; d = Durbin-Watson statistic; d_U = Durbin-Watson upper limit; F = ratio between variances
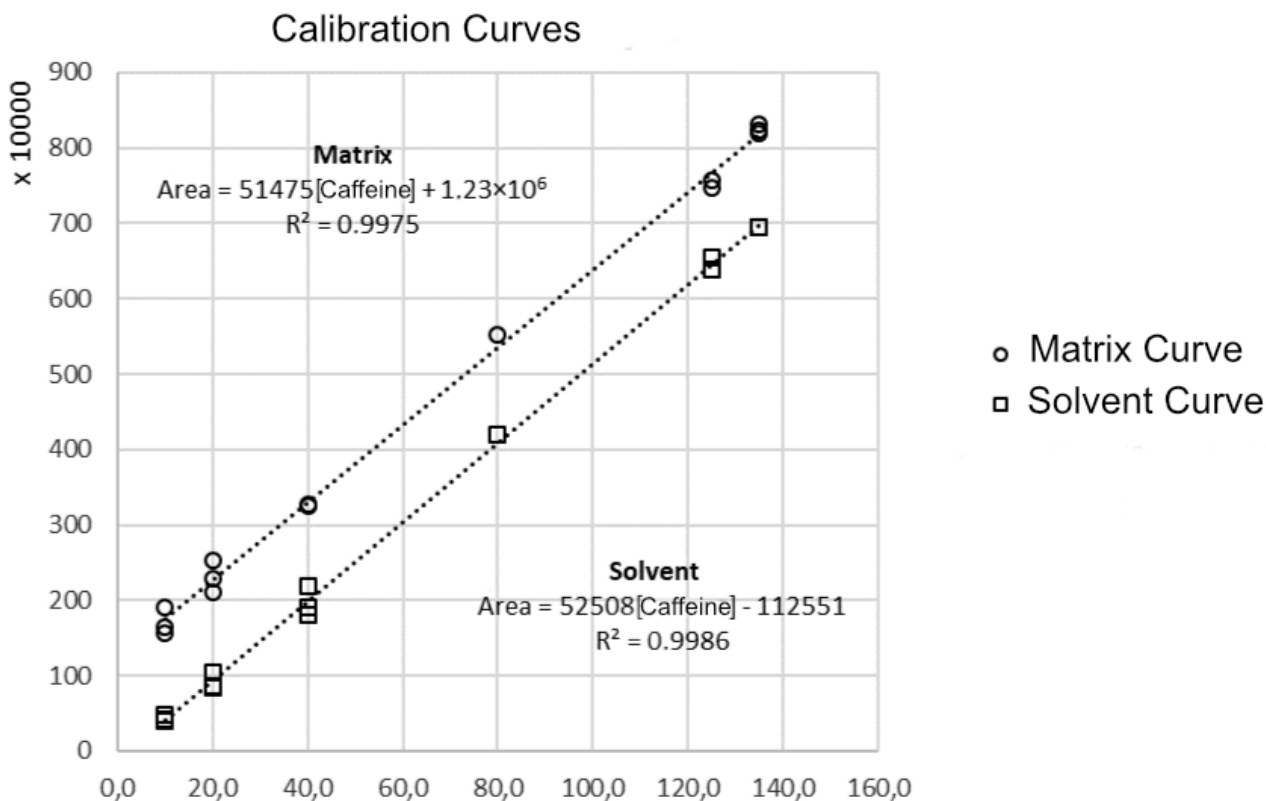

FIGURE 6 - Calibration curves of the analyte in solvent and matrix obtained for caffeine in assays evaluating matrix effects in the range of 10 to 135 µg/mL with the respective equations and determination coefficients.

The inclination of the straight lines with and without the matrix was evaluated by the t-test of inclination and parallelism. The variance test (F test) showed homoscedasticity (F = 1.98 < $F_{critical}$ = 2.68), so the combined variance test was conducted. Through these results, it can be seen that the lines have statistically equal slopes (t'_b = 1.08 < $t_{critical}$ = 2.06).

The LOD value obtained was 0.02 μg/mL and 0.10 μg/mL for the LOQ_t. The LOQ_t value was considered to be a theoretical value, as it was obtained from linear equations and could not correspond to the experimental reality. The experimental LOQ determined was 2 μg/mL, with a CV of 2.4 % and recovery of 119.8 %.

Limits close to or lower than those reached in this validation stage were reported by authors who adopted the signal/noise ratio or visual analysis as a criterion, without experimental determination. (Viana *et al.,*2018) obtained LOD (0.05 μg/mL) and LOQ_t (0.16 μg/mL), which are very close to those obtained in this study. The limits found by (Turak, Güzel, Dinç, 2017) were 1.44 μg/mL for LOD and 4.78 μg/mL for LOQ_t. Machado *et al.,* (2018) found LOD and LOQ_t values much higher than those found in this study, 2.63 μg/mL and 0.86 μg/mL, respectively. The method proposed by Yonekura, Tamura, (2019) for samples from permeability studies obtained values about 80 to 360 times lower than those found in the literature, with 0.043 nmol/mL for LOD and 0.144 nmol/mL for LOQ_t. The values found in our study are very low and indicate that even a very small amount of caffeine can be detected using the method developed, thus demonstrating a high sensitivity of the method.

The method of quantifying caffeine in guarana extracts proved to be precise and accurate in all chromatographic analyses. The results obtained demonstrated high precision and accuracy in the established range with CV less than 4 % and recovery within the expected range.

The developed and optimized method had a working range of 2 to 135 μg/mL. This range covers the Brazilian recommendations of MAPA for guarana seed or equivalent in a standardized extract with at least 1.2 % caffeine (Armenta, Garrigues, De La Guardia, 2005)

and is beyond the maximum limit (0.02 %) allowed by the FDA for carbonated drinks (Kole, Barnhill, 2013).

The scope of the method developed in our study is high, mainly due to the dilution factors used, which allows the samples to be kept within the linear range of the analytical method. The robustness of a method is the ability to remain unaffected by small changes in the chromatographic parameters. An assessment of robustness must be considered during the development phase of a method, and the method must show reliability of the analysis in relation to variations in chromatographic parameters (Huber, 1998).

Our method has no sample processing step, so there was no need to test variations resulting from this step. The pH of the mobile phase was not considered, since small pH variations were allowed due to the preparation of the acidified water solution with 0.1 % formic acid. The column temperature was also not evaluated, as it is related to room temperature. In addition to these parameters, different batches or manufacturers of solvents were not evaluated, due to the use of different batches and manufacturers of solvents throughout the experimental procedure. When there was a decrease in the flow of the mobile phase, the recovery obtained was greater than the acceptable range, thus, this parameter was not considered to be robust. In general, the method proved to be robust for the quantification of caffeine under the conditions evaluated.

The room temperature stability test of the analytical solutions demonstrated that the stock solution starts the crystallization process after 25 hours from preparation. However, there were no signs of crystallization or pattern precipitation in the aliquot vials, even after this period. But to ensure data reliability, all solutions containing a caffeine standard were prepared on the day of the analysis.

The method developed was applied to perform the determination of caffeine in several types of commercialized guarana extracts, and additionally in different soft drinks and energy drinks in order to evaluate the applicability of the method as well as the quality of these products. Preparation of 1.2 % caffeine solutions in acetonitrile and dilution in methanol (1:100 v: v) was also carried out. The results obtained are described in Table III.

TABLE III - Caffeine content in the evaluated samples

Food Commodity	Caffeine[1] (mg/100 mL)
E_1	970.00 ± 20.00
E_2	1180.00 ± 30.00
E_3	1830.00 ± 30.00
E_4	1730.00 ± 30.00
E_5	933.20 ± 35.02
E_6	1940.00 ± 70.00
E_7	1830.00 ± 10.00
Caffeine Standard (1.2%)	1297.54 ± 26.90
Energy drink	28.40 ± 3.56
Coke drink	9.69 ± 0.11
Guarana drink 1	1.12 ± 0.24
Guarana drink 2	6.56 ± 0.02

E_1; E_2; E_3; E_4; E_5; E_6; E_7 = Sample of commercial extracts

[1]Caffeíne content expressed as mean ± standard deviation

The results of caffeine present in cola soft drinks agree with the limits established by regulatory agencies (Brasil, 2022). In Brazil, ANVISA (2022) established a maximum of 35mg/100 mL of caffeine in energy drinks. The energy drink evaluated still has problems, because it has a value lower than that described on the label (73.85 mg/250 mL *vs* 83.2 mg/250 mL). This had already been discovered Armenta, Garrigues, De La Guardia, (2005) and by Nedeljković *et al.,* (2017). The soft drinks showed values very similar to those found by Nour, Trandafir and Ionica (2010). The method proved to be applicable for analysis in carbonated and energy drinks containing caffeine, presenting results similar to those described in the literature.

Paullinia cupana seed extract is highly valued, mainly due to its stimulating properties promoted by the high caffeine content, which can reach 6 % (Schimpl *et al.,* 2013). The difference in caffeine concentration in guarana (*P. cupana*) seeds may be due to the geography of plant cultivation. (da Silva *et al.,* 2017) showed significant differences between components of samples produced in the states of Bahia and Amazonas in Brazil. Another factor that can alter the caffeine content is the processing of the seeds, which can increase it up to three times, depending on the extraction treatment applied (Schimpl *et al.,* 2013). The differences found between the caffeine content in the commercialized guarana extracts and the minimum content required by the legislation show that the method developed has the potential to contribute to quality control in the food and pharmaceutical industries.

CONCLUSION

The HPLC-DAD method was developed to quantify caffeine in commercially available guarana (*P. cupana*) extracts and in commercial drinks. The method has been validated in accordance with international and Brazilian regulations. The developed method proved to be simple, sensitive, precise, linear, reproducible, specific, and with robust stability. It only requires a 7-min run, so it offers the high throughput needed to process a large number of samples. It is economical because it consumes less solvent due to the short analysis time. These results indicate that this method is suitable for the determination of caffeine in different matrices.

The developed method shows a linear range from 10 to 135 µg/mL, precision less than 4 %, and accuracy in the range of 99.9 to 120 %. LOD was 0.02 µg/mL, LOQ_t was 0.10 µg/mL, and the experimental LOQ was 2.00 µg/mL. With a sample injection volume of 20 µL, the method consumes little solvent. The best wavelength was at 270 nm, which showed better sensitivity and specificity for caffeine. Another advantage presented by this method is the simple sample preparation, reducing the cost and time required for analysis.

Our results indicate that the developed method can be used sensitively and accurately to quantify caffeine in guarana extracts and drinks. As far as we know, this is the first validated method that is simultaneously applicable to guarana extract and drinks containing caffeine.

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TABLE SI - Parameters evaluated on the robustness of the method

Parameters		
Composition of the mobile phases	40 to 95% Methanol	50 to 85%Methanol
Wavelength	260 nm, 280 nm and 285 nm	
Flow rate	1 mL.min⁻¹	1.3 mL.min⁻¹
Total time	5 minutes	9 minutes
Column	Kinetex C18 (100 × 2.1 mm 5µm, Phenomenex)*	

* The flow and time of analysis were changed proportionally: 100×2.1 mm 5 µm: Flow of 0.5 mL.min⁻¹and 4.5 minutes of running.

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