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FREEZE-DRIED EXTRACT OF *ILEX GUAYUSA*: A POTENTIAL INGREDIENT FOR ENERGY DRINK

EXTRACTO LIOFILIZADO DE *ILEX GUAYUSA*: POTENCIAL INGREDIENTE PARA BEBIDAS ENERGIZANTES

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Abstract

The objective of this study is to produce and analyze a freeze-dried extract of *Ilex guayusa*, with a high caffeine concentration potentially used as ingredient in energy drinks. The caffeine extraction from dried leaves was done by ultrasounds, using water as solvent. The parameters considered were time, citric acid concentration, ultrasonic amplitude, solid/liquid ratio. Significant factors were optimized using a Box-Behnken response surface design. Physicochemical parameters and heavy metals were performed according to (Servicio Ecuatoriano de Normalización (INEN), 2013). The shelf life was determined by accelerated temperature tests. The results showed that time, citric acid concentration and ultrasonic amplitude had significant effects on caffeine extraction, while the solid-liquid ratio was not significant. The optimum conditions for caffeine extraction were a time of 26.94 minutes, a citric acid concentration of 2.72 %, an ultrasonic amplitude of 68.29 % and caffeine concentration of 3.36 %. The physicochemical parameters of the freeze-dried product were: moisture of 1.2±0.1 %, total ash of 11.87±0.12 %, pH of 5.1±0.2, hot solubility of 17±1 s and cold solubility of 1.8±0.2 minutes. The levels of heavy metals found were zinc (19.16±0.04 mg/kg), copper (12.35±0.23 mg/kg) and tin (8.64±0.17 mg/kg). The shelf life of the freeze-dried extract at 20 °C was 3.5 months. This paper provides valuable information for developing a freeze-dried product rich in caffeine.

Keywords: Citric acid, caffeine, aqueous extract, ultrasound extraction, optimization.

Resumen

El objetivo de este estudio es producir y analizar un extracto liofilizado de *Ilex guayusa*, con elevada concentración de cafeína y con potencial de uso como ingrediente de bebidas energizantes. La extracción de cafeína desde hojas secas se realizó mediante ultrasonido en medio acuoso. Los parámetros considerados fueron: tiempo, concentración de ácido cítrico, amplitud ultrasónica, relación sólido-líquido. Los factores significativos se optimizaron mediante un diseño de superficie de respuesta Box-Behnken. Se evaluaron los parámetros fisicoquímicos y los metales pesados según la norma (Servicio Ecuatoriano de Normalización (INEN), 2013). La vida útil se determinó mediante pruebas aceleradas de temperatura. Los resultados mostraron que el tiempo, la concentración de ácido cítrico y la amplitud ultrasónica tuvieron efectos significativos sobre la extracción de cafeína, mientras que la relación sólido-líquido no fue significativa. Las condiciones óptimas para la extracción de cafeína fueron en un tiempo de 26.94 min, una concentración de ácido cítrico del 2.72 %, una amplitud ultrasónica de 68.29 % y una concentración de cafeína de 3.36 %. Los parámetros fisicoquímicos del liofilizado fueron: humedad (1.2±0.1 %), cenizas totales (11.87±0.12 %), pH (5.1±0.2), solubilidad en caliente (17±1 s) y solubilidad en frío (1.8±0.2 minutos). Los niveles de metales pesados fueron: zinc (19.16±0.04 mg/kg), cobre (12.35±0.23 mg/kg) y estaño (8.64±0.17 mg/kg). La vida útil del producto liofilizado a 20 °C fue de 3.5 meses. El presente estudio proporciona información valiosa para el desarrollo de un producto liofilizado rico en cafeína.

Palabras clave: Ácido cítrico, cafeína, extracto acuoso, extracción con ultrasonido, optimización.

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1 Introduction

Ilex guayusa Loes. is a plant with great cultural and medicinal importance in the Amazonian regions of Ecuador, Peru, Colombia, and Bolivia, where it grows at altitudes ranging from 200 to 2000 meters above sea level. This species, which can reach heights of up to 30 meters, has been used for centuries by Indigenous peoples due to its various medicinal properties, including antipyretic, diuretic, and analgesic uses, as well as applications in the treatment of arthritis and the improvement of fertility (Meneses et al., 2024). However, the most popular use of *I. guayusa* is its stimulant content, mainly derived from its high concentration of caffeine, which exceeds 3 % in the leaves (Negrin et al., 2019).

The growing global demand for natural products with functional properties has driven interest in *I. guayusa*, whose leaves not only contain caffeine but also a variety of bioactive compounds, such as antioxidants, anti-inflammatory, and antibacterial compounds (Cakmak et al., 2023).
Recent research has identified the presence of theobromine, theophylline, and chlorogenic acids, which provide additional health benefits beyond the stimulant action of caffeine (Chianese et al., 2019; Cadena-Carrera et al., 2019).

Among the phenolic compounds detected in *I. guayusa* leaf extracts, 14 compounds have been identified, including chlorogenic acid derivatives and flavonoids such as quercetin, which reinforces its potential as a functional ingredient in the formulation of new products (García-Ruiz et al., 2017).

Caffeine extraction has traditionally been carried out through several methods, including extraction with organic solvents, maceration extraction, and Soxhlet extraction. Although these methods are effective, many have significant disadvantages, such as the use of toxic solvents, long processing times, and high temperatures that can degrade bioactive compounds (Ivanović et al., 2020; Lezoul et al., 2020). In recent years, considerable emphasis has been placed on developing more efficient and environmentally friendly extraction techniques. Ultrasound-assisted extraction (UAE) has gained popularity due to its ability to improve extraction efficiency through acoustic cavitation, a process that generates micro-bubbles in the liquid medium, facilitating the rupture of cell walls and the release of bioactive compounds such as caffeine (Kumar et al., 2020).

UAE offers several advantages over conventional techniques, including shorter extraction times, lower temperatures, and the possibility of using safer solvents such as water, making it a sustainable option for the food and pharmaceutical industries (Carreira-Casais et al., 2021). In addition, the combination of citric acid as an acidifying agent further enhances the extraction of alkaloids, since these compounds in acidic media release more easily (Yang et al., 2024). This makes UAE an ideal technique to maximize the extraction of caffeine from *I. guayusa* leaves.

Likewise, the growing demand for natural and functional products has highlighted the importance of developing healthy alternatives to commercial energy drinks, which often contain synthetic additives. A lyophilized product based on *I. guayusa* would not only take advantage of the bioactive properties of the plant but would also respond to current market demands for natural energy products that offer additional health benefits, such as antioxidants and anti-inflammatory compounds. *I. guayusa*, due to its caffeine content and other beneficial polyphenolic compounds, represents a potential option for creating an innovative product that can be reconstituted as an energy drink. The objective of the present study is to formulate and evaluate the shelf life of a lyophilized *I. guayusa* intended for the preparation of an energy drink.

2 Materials and Methods

2.1 Raw material and sample preparation

I. guayusa leaves were purchased dried at "Los plátanos" market in the city of Puyo, Ecuador, located at coordinates 1.4837° S and 78.0026° W (Bromatology Laboratory of Universidad Estatal Amazónica, km 2 ½ vía Tena, canton and province of Pastaza). The leaves were ground in a mill (KitchenAid brand, model BCG111OB) and sieved until a particle diameter smaller than 0.5 mm was obtained. Citric acid (purity >99 %) of food grade, used to favor caffeine extraction, was purchased from the chemical store "QUIMICOSAS" located in the city of Quito, Ecuador.

2.2 Ultrasound-assisted extraction

A solid–liquid extraction was carried out using a thermostatic ultrasonic bath (Wisd.23 brand, model WUC-DO6H). The extractions were performed according to the conditions established for each experiment. Samples of pulverized *I. guayusa* were weighed in 200 mL flasks and 100 mL of a citric acid solution (m/v) dissolved in water were added. The flasks were placed in the equipment previously calibrated at a constant temperature of 50 °C. The extracts obtained were filtered using Whatman No. 4 paper. The determination of caffeine content was performed immediately.

2.3 Spectrophotometric determination of caffeine

Caffeine determination was performed by spectrometry, according to the methodology employed by Luna-Fox et al. (2024). 1 mL of NaOH (0.1 M) was added to 10 mL of guayusa aqueous extract to increase the pH of the solution. Caffeine was extracted in two fractions of 15 mL of chloroform each. The obtained extracts were combined and then evaporated in a water bath. The resulting caffeine was dissolved in 50 mL of hot water (at a temperature between 60 and 90 °C) and allowed to cool. Subsequently, the solution was transferred to a 100 mL flask, adjusting the final volume with distilled water. 5 mL of this solution were placed in a 25 mL volumetric flask and 1 mL of HCl (0.01 M) was added; it was then completed with distilled water. Finally, the absorbance of the samples was measured at 275 nm in a UV–vis spectrophotometer (Perkin Elmer brand, model Lambda 20) and the concentration was determined using the calibration curve obtained from nine concentrations (1, 2, 3, 5, 10, 12, 16, 20 and 25 mg/mL; $R^2 = 0.9991$), according to equation (1). The results were expressed as a percentage on a dry matter basis.

$$A = 0.006C + 0.0011 \tag{1}$$

Where C: caffeine concentration in the sample (mg/mL) and A: absorbance of the sample.

2.4 Experimental design

The experiment was carried out in two stages. In the initial phase, a two-level factorial design with four factors (Table 1) was used, comprising 36 experiments: 16 trials with two replicates each and four repetitions at the central point to evaluate the curvature of the model. The influence of extraction time, citric acid concentration, ultrasonic amplitude, and the solid/liquid ratio on the caffeine content in *I. guayusa* leaves was analyzed. To identify significant and non-significant variables, a standardized effects diagram was employed, while the selection of relevant variables was consolidated through the Pareto diagram and the Bonferroni limit (Anderson and Whitcomb, 2016).

In the second phase, the significant variables were analyzed to determine the optimal levels of the studied variables to optimize caffeine extraction. For this purpose, a Box–Behnken design (Response Surface Methodology – RSM) was used, implemented through the software Design Expert version 13.0.5.0 (trial version, Stat-Ease Inc., Minneapolis, MN, USA). The experimental results were fitted using the following second-order polynomial model:

$$y = \beta_0 + \sum_{i=1}^{n} \beta_{ii} x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j \tag{2}$$

Where y indicates the estimated response, while β_0, β_{ii} and β_{ij} represent the regression coefficients associated with the mean, linear, and quadratic terms, respectively. These were determined from the experimental results using the least squares method. The independent variables x_i and x_j were coded within an interval from -1 to 1.

Table 1. Levels of the studied variables.

Study variable		Levels		
	Code	Low -1	Central 0	High 1
Time (min)	A	15	22.5	30
Citric acid (%)	B	0	1.5	3
Ultrasonic width (%)	C	30	50	70
Solid-liquid relationship (m/v)	D	5	10	15

2.5 Model validation

To corroborate the model, the values of the adjusted R^2 and predicted R^2 coefficients were examined. The validity of each experimental series, along with that of the model, was verified through an analysis of variance (ANOVA) (Dinardo et al., 2019). The optimization of caffeine extraction from dried *I. guayusa* leaves was performed considering extraction time, citric acid concentration, and ultrasonic amplitude as independent factors. The ideal extraction conditions were established using the predictive equation of the response surface methodology.

2.6 Preparation of the lyophilized product

The lyophilized product was obtained from the aqueous extract produced under the optimal extraction conditions, which was processed in a lyophilizer (SCANVAC brand, model COOLSAFE). The operating conditions were condenser temperature of 90 °C, pressure of 0.105 Torr, and a processing time of 7 days.

2.7 Determination of physicochemical parameters and heavy metals in the lyophilized extract

Moisture, pH, solubility in hot and cold water, total ash content, and heavy metals (lead, cadmium, zinc, copper, arsenic, and tin) were analyzed according to the procedures established by the Ecuadorian technical standard (NTE) INEN 1122:2013 corresponding to freeze-dried coffee. The analyses were carried out using an atomic absorption spectrophotometer (Perkin Elmer, Model A Analyst 800, dimensions 1524.00 × 1016.00 × 812.80 mm, automatic sampler AS800, weight 136.08 kg). The results of this study were compared with the permissible limit proposed by NTE INEN 1122:2013 for soluble coffee, since it is the product that most closely resembles the one obtained in this study. Furthermore, there is currently no INEN standard for *I. guayusa*. Table 2 shows the standard used for each analysis.

2.8 Determination of shelf life

The shelf life of the lyophilized extract was determined through accelerated temperature tests considering moisture as the measurement parameter. For this purpose, 1 g of lyophilized extract was weighed in triplicate in glass containers with screw caps and placed in an oven (MEMMERT brand, model SFE700) at 30, 40, and 50 °C. The moisture content was evaluated at 0, 7, 14, 21, and 28 days. The data were fitted to the differential equation (3) proposed by Herrera et al. (2022).

$$\frac{dH}{dt} = kH^n \tag{3}$$

Where H is the moisture content of the freeze-dried extract, k represents the reaction kinetic constant, t indicates time, and n indicates the reaction order.

The experimental results in this study followed a first-order reaction. Therefore, by integrating equation (3) from a moisture content of H_0 to H, and from an initial time of 0 to t, equation (4) was obtained, which allowed the shelf life of the freeze-dried extract to be determined.

$$\ln(H) = \ln(H_0) + kt \tag{4}$$

The reaction kinetic constant (k) was determined using the linearized Arrhenius equation (5) by plotting $\ln(k)$ against the inverse of the temperature $(1/T)$.

$$\ln(k) = \ln(k_0) - \frac{E_a}{RT} \tag{5}$$

Where k_0 is the pre-exponential factor, E_a represents the activation energy (kJ/mol), R is the universal gas constant (8.314 kJ/molK) and T is the absolute temperature (K). The shelf life was estimated at a temperature of 20 °C. The data were processed using Origin Pro 2022 software (OriginLab, Northampton, MA, USA).

Table 2. Physicochemical and heavy metal analyses according to NTE INEN 1122:2013

Analysis	Test method
H (%)	NTE INEN-ISO 3726
CT (%)	NTE INEN 1117
pH	NTE INEN 389
SC (s)	Cup test
SF (min)	Cup test
Pb (mg/kg)	Atomic absorption spectrophotometry
Cd (mg/kg)	
Zn (mg/kg)	
Cu (mg/kg)	
As (mg/kg)	
Sn (mg/kg)	

H: moisture, CT: total ash, SC: solubility in hot water, SF: solubility in cold water, Pb: lead, Cd: cadmium, Zn: zinc, Cu: copper, As: arsenic, Sn: tin.

3 Results and Discussion

3.1 Effect of the study factors on caffeine extraction

The estimation of standardized effects, both positive and negative, in the UAE of caffeine is shown in Figure 1A, where the significant variables correspond to the highest values of the standardized effects (≥ 0.5) according to what was mentioned by Held et al. (2023). On the other hand, the non-significant effects (< 0.5) followed a normal distribution with a mean of zero and constant variation. This indicates that when plotting the effects on a normal probability plot, the non-significant effects are located on a straight line, while the active effects deviate from this line of normality.

The Pareto diagram, together with the Bonferroni limit, reinforced the selection of significant factors. As indicated in Figure 1B, extraction time (A), citric acid concentration (B), and ultrasonic amplitude (C) were located above the Bonferroni limit, indicating that they were significant variables, whereas the solid–liquid ratio was located below this limit, showing that it did not have statistical influence. Likewise, according to the results shown in Table 3, the significant variables ($p < 0.05$) and non-significant variables ($p > 0.05$) are confirmed. The interactions between the studied variables (A, B, C, and D) were also not significant. The factorial model presented a good fit, supported by a coefficient of determination (R^2) equal to 0.9934. In addition, the difference between the adjusted R^2 (0.9881) and the predicted R^2 (0.9757) was less than 0.2, which is considered adequate according to Anderson and Whitcomb (2016) and Arteaga-Crespo et al. (2019).

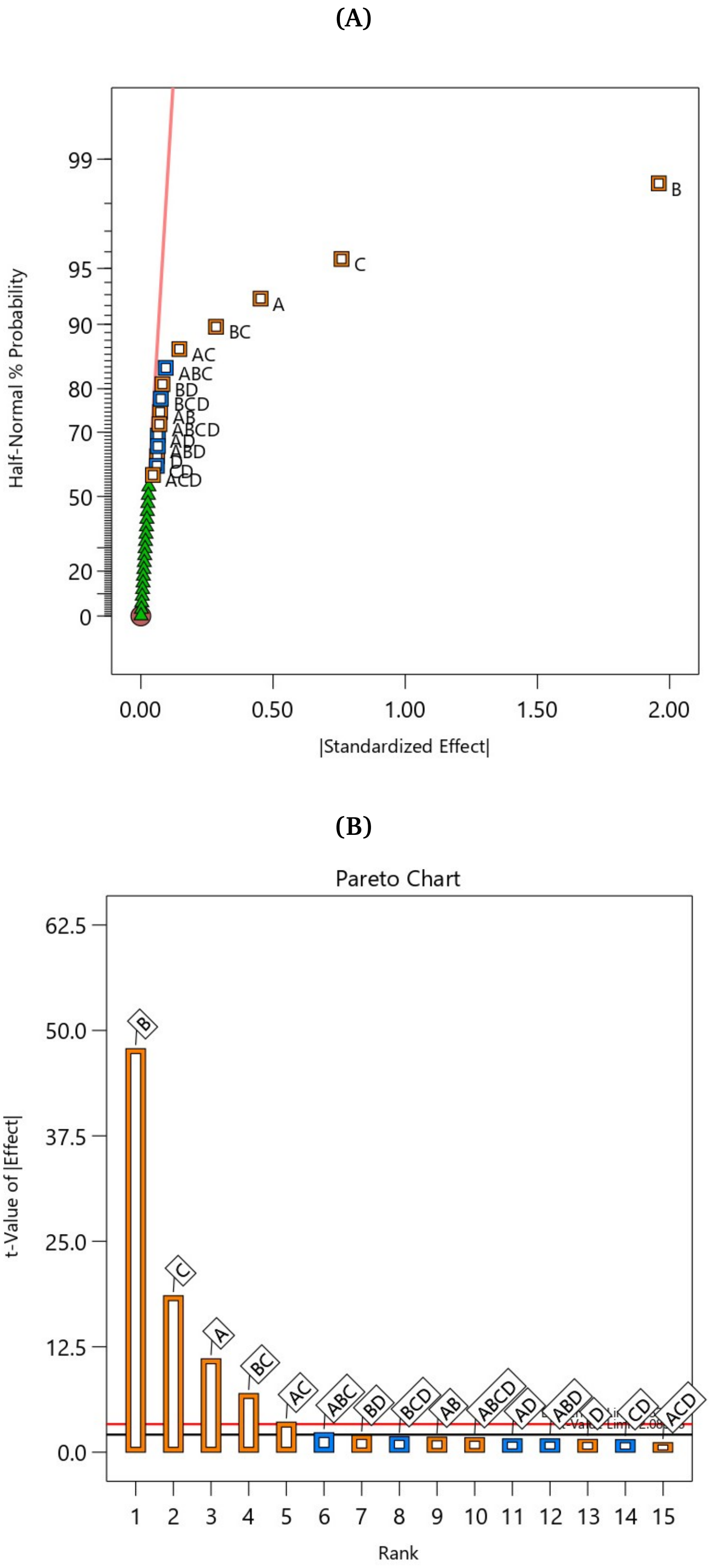

Figure 1. Positive (orange) and negative (blue) effects on caffeine extraction. Half-normal distribution plot (A), Pareto diagram (B).

Previous studies have highlighted the positive influence of extraction time and ultrasonic amplitude on caffeine extraction due to their effect on cell rupture and mass transfer. Hassan and Yaqoobi (2023) observed that a longer extraction time and higher ultrasonic amplitude significantly improved the yield in the UAE of caffeine from coffee beans, since these factors facilitate the release of this compound by increasing acoustic cavitation. Similarly, Serna-Jiménez et al. (2023) reported a proportional relationship between extraction time and caffeine recovery in coffee pulp, due to a longer contact between the solvent and the plant material, a factor that optimizes the solubilization of this alkaloid.

The significant effect of citric acid on caffeine extraction can be explained by its ability to acidify the medium, optimizing the solubility and release of caffeine. In acidic media, the interaction of the solvent with the plant material is strengthened, facilitating extraction. Yang et al. (2024) supported this observation by demonstrating that a eutectic solvent composed of polypropylene glycol, polyethylene glycol, and citric acid showed remarkable efficiency in extracting caffeine from tea residues through microwave irradiation, because the acidic environment favors its solubility and extraction.

The solid/liquid ratio factor did not show a statistical influence on caffeine extraction; this can be explained because when this factor increases, the amount of solvent used was not able to adequately cover all the plant material, limiting mass transfer and reducing the efficiency of the process. This suggests that, beyond a certain limit, the available solvent becomes insufficient to effectively extract caffeine. Similar results were reported by Abreu-Naranjo et al. (2018), who found that the solid/liquid ratio also had no significant effect on the extraction of bioactive compounds from the bark of *Maytenus macrocarpa*, which reinforces the idea that this factor may lose relevance when the proportion of solvent and plant material is not optimized.

Table 3. ANOVA for the effect of the study factors on caffeine extraction.

Caffeine	Sum of squares	df	Mean square	F value	P value	
Model	38.33	15	2.56	19.07	< 0.0001	Significant
A–Time	1.20	1	1.20	8.92	0.0076	
B–Citric acid	30.56	1	30.56	228.07	< 0.0001	
C–Amplitude	3.74	1	3.74	27.91	< 0.0001	
D–S/L ratio	0.0004	1	0.0004	0.0028	0.9582	
AB	0.0025	1	0.0025	0.0189	0.8921	
AC	0.3026	1	0.3026	2.26	0.1493	
AD	0.0007	1	0.0007	0.0052	0.9435	
BC	0.3970	1	0.3970	2.96	0.1014	
BD	0.0055	1	0.0055	0.0412	0.8413	
CD	0.0003	1	0.0003	0.0019	0.9656	
ABC	0.0118	1	0.0118	0.0881	0.7699	
ABD	0.0007	1	0.0007	0.0052	0.9435	
ACD	0.0006	1	0.0006	0.0047	0.9459	
BCD	0.0033	1	0.0033	0.0244	0.8776	
ABCD	0.0019	1	0.0019	0.0141	0.9068	
Residual	2.55	19	0.1340			
Lack of fit	0.0042	1	0.0022	1.45	0.895	Not significant
Pure error	0.0170	18	0.0009			

3.2 Optimization of the extraction process

An optimization design with 17 experiments was used to determine the optimal levels of extraction time, citric acid concentration, and ultrasonic amplitude, which were the significant variables. The objective of the optimization of the extraction process was to determine the best combination of these three variables to maximize caffeine extraction. Four mathematical models (Table 4) generated by the Design-Expert software were analyzed, where the quadratic model presented the best results, with an R^2 value equal to 0.9895. This value shows that 98.95 % of the total variation in caffeine extraction was determined by the studied variables.

Table 4. Mathematical models generated by the Design-Expert software

Model	p-value	Lack of fit	Adjusted R^2	Predicted R^2	
Linear	0.0014	0.0033	0.6153	0.4103	
2FI	0.5796	0.0023	0.5855	-0.0627	
Quadratic	$<$ **0.0001**	**0.3733**	**0.9761**	**0.9072**	**Suggested**
Cubic	0.3733	0.0087	0.9793	-0.1258	

Table 5. Caffeine concentration obtained experimentally

Caffeine (%)	Time (min)	Citric acid (%)	Amplitude (%)
3.24	22.50	3.00	70
3.08	30.00	1.50	70
2.68	15.00	1.50	70
2.64	30.00	3.00	50
2.61	22.50	1.50	50
2.50	22.50	1.50	50
2.50	22.50	1.50	50
2.45	22.50	1.50	50
2.36	22.50	1.50	50
2.22	15.00	3.00	50
2.15	30.00	1.50	30
1.98	15.00	1.50	30
1.88	22.50	3.00	30
1.60	22.50	0.00	70
1.37	22.50	0.00	30
1.22	30.00	0.00	50
0.98	15.00	0.00	50

The quadratic model in terms of the study variables was the following:

$$\textbf{Caffeine} = 0.66 + 0.10A + 0.62B - 0.03C + 0.40 \times 10^{-2}AB + 3.83 \times 10^{-4}AC + 9.42 \times 10^{-3}BC - 2.39 \times 10^{-3}A^2 - 2.60B^2 + 3.08 \times 10^{-4}C^2 \quad (6)$$

Where A, B, and C represent extraction time, citric acid concentration, and ultrasonic amplitude, respectively. The experimental results of caffeine content are shown in Table 5, and those estimated by the quadratic model were compared in Figure 2. The distribution of points validated the model's ability to cover the entire range of experiments analyzed. This pattern confirms the suitability of the model to cover the entire data interval, suggesting that it can be successfully applied.

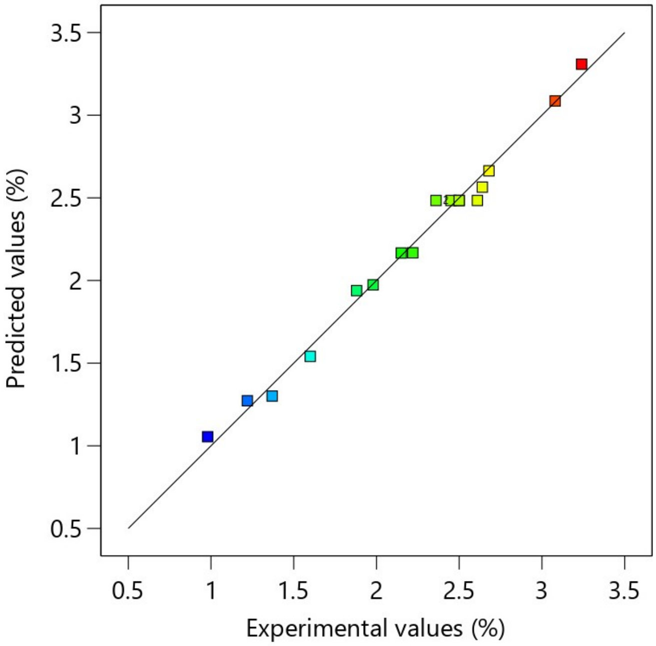

Figure 2. Experimental vs predicted caffeine results.

The ideal extraction conditions, which resulted in the maximum caffeine content (3.36 %), were the following: extraction time of 26.94 minutes, citric acid at 2.72 %, and ultrasonic amplitude of 68.29 %. Under these experimental conditions, the obtained caffeine content was 3.22, which coincided with that predicted by the quadratic model.

The statistical significance of the regression equation corresponding to the quadratic model of the response surface methodology was evaluated by the F test and ANOVA, whose results are presented in Table 6.

The result of Fisher's F test was 75.53, with a p-value lower than 0.0001, which demonstrates that the model is highly significant. In addition, the coefficient of determination (R^2) was 0.9895, reflecting a high agreement between the experimental values and those predicted by the model. The adjusted R^2 reached a value of 0.9761, indicating that the quadratic model was able to predict 97.61 % of the variation in caffeine extraction. On the other hand, the lack of fit was not statistically significant, with an F value of 1.37 and a p-value of 0.3733. These results indicate that the lack of fit did not have a significant impact on the results obtained. The graphical representations of the quadratic equation generated by the Design-Expert software are shown through a 3D response surface (Figure 3A) and 2D contour plots (Figure 3B). The optimal levels of the investigated variables were determined from the analysis of these graphs.

Table 6. ANOVA for the quadratic model of the RSM.

Caffeine	Sum of squares	df	Mean square	F value	P value	
Model	6.30	9	0.7001	73.53	< 0.0001	Significant
A-Time	0.1891	1	0.1891	19.86	0.0029	
B-Citric acid	2.89	1	2.89	303.76	< 0.0001	
C-Amplitude	1.30	1	1.30	136.13	< 0.0001	
AB	0.0081	1	0.0081	0.8508	0.3870	
AC	0.0132	1	0.0132	1.39	0.2771	
BC	0.3192	1	0.3192	33.53	0.0007	
A^2	0.0762	1	0.0762	8.00	0.0255	
B^2	1.44	1	1.44	151.09	< 0.0001	
C^2	0.0637	1	0.0637	6.69	0.0361	
Residual	0.0666	7	0.0095			
Lack of fit	0.0337	3	0.0112	1.37	0.3733	Not significant
Pure error	0.0329	4	0.0082			

$R^2 = 0.9895$; Adjusted $R^2 = 0.9761$; Predicted $R^2 = 0.9071$

The caffeine content present in dried *I. guayusa* leaves has been the subject of several studies, with results showing variability in the concentration of this compound. In a recent study by Luna-Fox et al. (2024), caffeine levels ranging between 0.24 % and 1.52 % were reported in aqueous extracts obtained by the ultrasound-assisted extraction technique. However, Cadena-Carrera et al. (2022) found a higher yield ($2.27 \pm 0.05\,\%$) using ethanol as a solvent and the supercritical fluid extraction technique. This difference in the obtained values can be due to several factors, such as the type of extraction technique, the solvent used, the particle size of the plant material, and environmental conditions. Previous research has indicated that variables such as climatic conditions and soil characteristics, as observed in the studies of Repajić et al. (2021) and Mazumder et al. (2020), also play a crucial role in the concentration of bioactive compounds, which demonstrates the complexity of the factors that affect the extraction of these compounds.

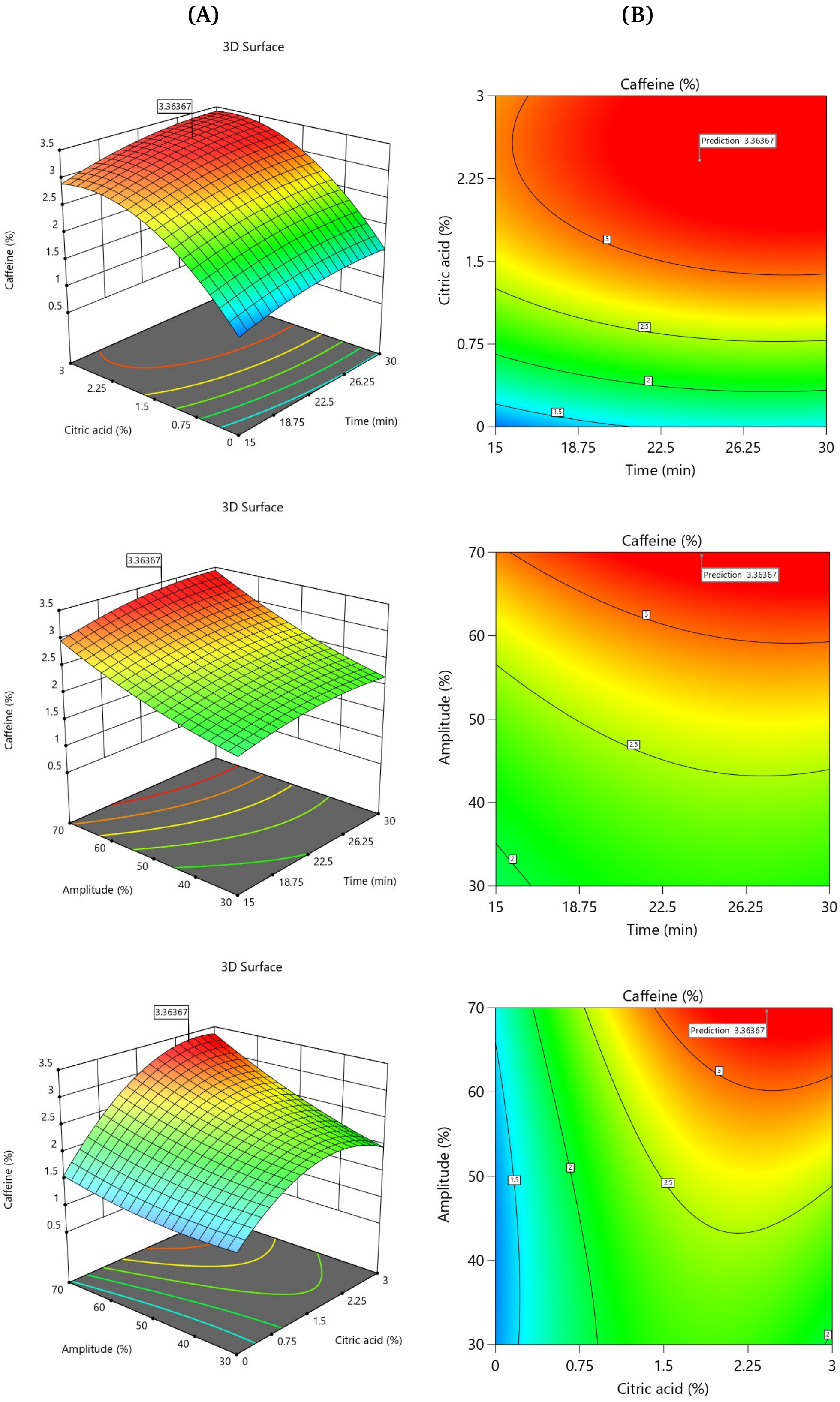

Figure 3. Optimal conditions for caffeine extraction. Response surface plots (A), contour plots (B).

3.3 Physicochemical parameters of the lyophilized extract

The lyophilized product was prepared from the aqueous extract obtained under the optimal extraction conditions defined by the quadratic model of the RSM. The lyophilized extract was processed to ensure that it complied with the specifications established in the NTE INEN 1122:2013 standard for freeze-dried coffee, which regulates key parameters such as pH, moisture, total ash content, and solubility in hot and cold water.

In this study, the results obtained for each of these parameters were adjusted to the specifications of the NTE INEN 1122:2013 standard (Table 7). The pH of the lyophilized extract was 5.1 ± 0.2, which is within the permitted range. Moisture was $1.2 \pm 0.1\,\%$, well below the maximum limit of 3.5 %, indicating a good lyophilization process that ensures low water activity and greater product stability. The total ash content was $11.87 \pm 0.12\,\%$, reflecting proper extract quality without excessive presence of mineral impurities. Regarding solubility, the lyophilized extract showed a good dissolution capacity, with a solubility time in hot water of 17 ± 1.0 s and in cold water of 1.8 ± 0.2 min, values that are below the limits established by the standard. These results reflect the high quality of the lyophilized extract obtained under the optimal extraction conditions. This regulatory compliance is an important indicator of the suitability of the extraction and lyophilization process and suggests that the produced lyophilized extract has characteristics that make it suitable for commercialization and consumption, ensuring quality and safety standards for the final consumer.

Table 7. Acceptable physicochemical parameters according to NTE INEN 1122:2013.

Analysis	Lyophilized extract of *I. guayusa*	Freeze-dried coffee	
		Minimum	**Maximum**
H (%)	1.2±0.1	-	3.5
CT (%)	11.87±0.12	-	14
pH	5.1±0.2	4.7	5.5
SC (s)	17±1	-	30
SF (min)	1.8±0.2	-	3

H: moisture, CT: total ash, SC: solubility in hot water, SF: solubility in cold water.

3.4 Heavy metal content in the lyophilized extract

The NTE INEN 1122:2013 standard establishes specific limits for the presence of heavy metals in freeze-dried coffee to guarantee the safety and quality of the product. These values were used as a reference to evaluate the heavy metal content in the lyophilized extract of *I. guayusa* in this study.

The results obtained showed that the heavy metal content in the lyophilized extract strictly complied with the limits established by the standard (Table 8). In particular, the levels of zinc, copper, and tin were 19.16 ± 0.04 mg/kg, 12.35 ± 0.23 mg/kg, and 8.64 ± 0.17 mg/kg, respectively, all below the maximum permitted limit. In addition, the presence of lead, cadmium, and arsenic was not detected, which highlights the quality and safety of the product.

Table 8. Maximum acceptable values of heavy metals according to NTE INEN 1122:2013

Metal analyzed	Lyophilized extract of *I. guayusa* (mg/kg)	Freeze-dried coffee (mg/kg)
Pb	ND	1
Cd	ND	0.1
Zn	19.16±0.04	50
Cu	12.35±0.23	20
As	ND	0.5
Sn	8.64±0.17	20

Pb: lead, Cd: cadmium, Zn: zinc, Cu: copper, As: arsenic, Sn: tin, ND: not detected.

Different studies have indicated that the presence of these metals may have toxic and cumulative effects in the human body. Lead, for example, is a neurotoxin that can affect the central nervous system, causing cognitive and behavioral disorders, especially in children (Sobin et al., 2023). Cadmium accumulates mainly in the kidneys and can cause renal damage, in addition to being associated with a higher risk of cancer in the lungs and other organs (Howard et al., 2023). Regarding arsenic, prolonged exposure may cause problems in the skin and respiratory system and increase the risk of developing cancer (Chappells and Dummer, 2024). On the other hand, Yan et al. (2023) have indicated that zinc, although essential in small amounts for health, at elevated levels may interfere with the absorption of other minerals and cause adverse effects such as nausea, vomiting, and liver dysfunction. Copper, in turn, is vital for biological functions, but at high concentrations it may damage the liver and nervous system (Min et al., 2024). Tin, although less studied, at high concentrations may cause gastrointestinal discomfort and affect kidney function (Liu et al., 2022). In this study, the absence of lead, cadmium, and arsenic, and the low concentration of zinc, copper, and tin in the lyophilized extract of *I. guayusa* highlight the safety and quality of the product, minimizing any risk to consumer health.

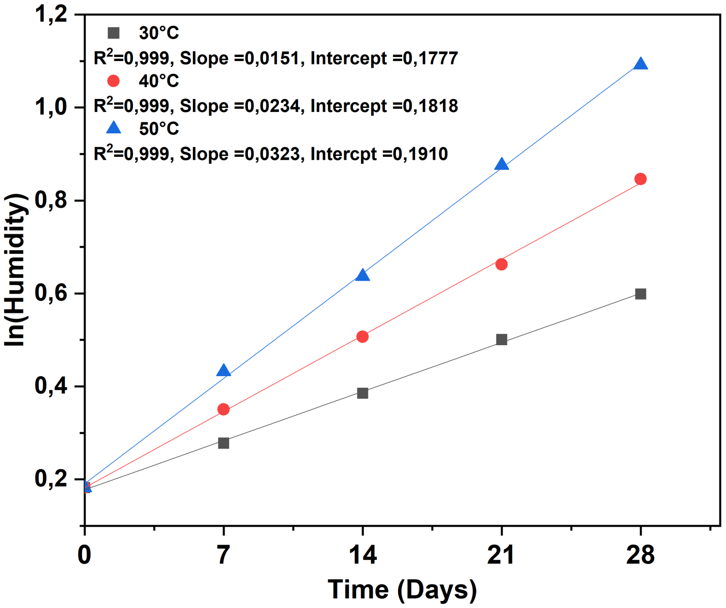

Figure 4. Relationship between ln (moisture) as a function of storage time (days) of the lyophilized extract of *I. guayusa* a 30, 40 y 50 °C.

3.5 Determination of shelf life

The relationship between the moisture content of the lyophilized extract and time was calculated by plotting $\ln(\text{moisture})$ as a function of time, according to Equation 4 (Figure 4). The results showed behavior consistent with a first-order reaction, which was confirmed by a coefficient of determination (R^2) of 0.999 for the curves obtained at 30, 40, and 50 °C (Table 9). The kinetic reaction constant (k) was represented by the slope of the line in each case.

Table 9. k values for storage temperatures.

Temperature (°C)	R^2	k	Intercept
30	0.999	0.0151	0.1777
40	0.999	0.0234	0.1818
50	0.999	0.0323	0.1910

K: kinetic reaction constant

Temperature had a strong influence on the kinetic reaction constant, reflected in a proportional increase in its value as temperature increased. This behavior can be explained by the increase in the kinetic energy of the reacting molecules, which favors a greater number of effective collisions, overcoming the energy barrier of the chemical process (Karal et al., 2024).

This trend is consistent with the Arrhenius equation, which states that the reaction constant depends exponentially on temperature. Similar results were reported by Isuiza et al. (2018), who documented a proportional increase in the reaction constant as temperature increased in similar kinetic processes. These authors concluded that this phenomenon was attributable to the decrease in activation energy and the enhancement of the molecular dynamics of the system.

The linearized Arrhenius equation was used to calculate the activation energy (E_a) and the kinetic reaction constant at a temperature of 20 °C to estimate the shelf life of the lyophilized extract. In Figure 5, the relationship between $\ln(k)$ and the inverse of absolute temperature is presented, where the data showed a good fit, with an R^2 of 0.995.

The shelf life at 20°C was calculated using Equation 4, taking moisture as the critical parameter according to the NTE INEN 1122:2013 standard for freeze-dried coffee, which establishes a maximum moisture limit of 3.5 %. The estimated shelf life is detailed in Table 10.

Table 10. Activation energy, kinetic reaction constant at 20 °C, and shelf life of the lyophilized extract.

Ea (kJ/mol)	k_0	k_{20}	H_0 (%)	$H_{\text{máx}}$ (%)	$t_{20\,^\circ\text{C}}$ (days)	$t_{20\,^\circ\text{C}}$ (months)	First-order equation used
30.097	3 361.02	0.0101	1.2	3.5	106	3.5	$\ln(H) = \ln(H_0) + kt$

E_a: activation energy, k_0: pre-exponential factor, $k_{20\,^\circ\text{C}}$: kinetic reaction constant at 20 °C, H_0: initial moisture of the lyophilized extract, $H_{\text{máx}}$: maximum acceptable moisture, $t_{20\,^\circ\text{C}}$: shelf life of the lyophilized extract.

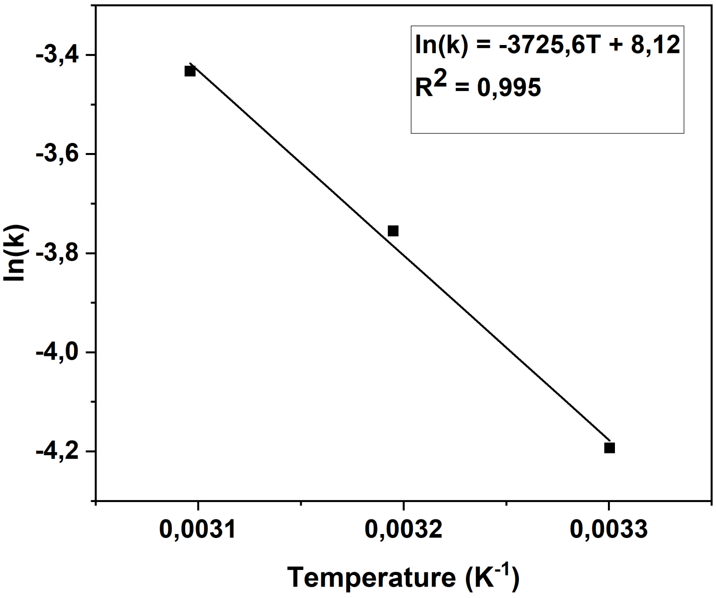

Figure 5. Relationship between the kinetic reaction constant (k) and the inverse of absolute temperature.

Currently, no previous studies have been identified that determine the activation energy and shelf life of a lyophilized product derived from aqueous extracts of dried *I. guayusa* leaves. However, studies conducted on other lyophilized products provide a reference framework to compare the obtained results. Isuiza et al. (2018) and López-Quiroga et al. (2019) reported activation energy values ranging between 18.3 and 31.35 kJ/mol, a range within which the activation energy determined for the *I. guayusa* lyophilized product in this study falls. This result indicates an energetic behavior comparable with other lyophilized products of plant origin.

Regarding shelf life, the 106 days estimated for the lyophilized extract of *I. guayusa* are similar to those reported by González-Pérez et al. (2024) for a product made from apple (105 days). This finding positions the lyophilized extract of *I. guayusa* as a competitive product in terms of storage stability. Setiawan et al. (2022) determined a shorter shelf life (59 days) for a coconut-based lyophilized beverage, which highlights the variability in preservation times among lyophilized products and reveals the influence of factors such as chemical composition, processing methods, and packaging materials.

The results of this research provide a starting point for future studies aimed at optimizing the stability of *I. guayusa* lyophilized products and extending their storage time. In addition, this study contributes to filling a gap in the literature by establishing key parameters for the development of innovative products based on *I. guayusa*.

4 Conclusions

Citric acid concentration was the factor that most influenced caffeine extraction from dried *I. guayusa* leaves, followed by ultrasonic amplitude and extraction time. The solid–liquid ratio did not show a significant effect.

A lyophilized product was prepared from the aqueous extract of *I. guayusa* obtained under the optimal extraction conditions. The final product complied with the physicochemical parameters and heavy metal limits established in the NTE INEN 1122:2013 standard.

The results of this research provide valuable information for the development of a lyophilized product rich in natural caffeine, positioning it as a potential alternative to products that use synthetic caffeine. It is necessary to study the long-term stability of the product under different storage conditions to assess its commercial viability.

Declaration on the use of Artificial Intelligence

The authors DECLARE that, during the preparation of the article entitled "Freeze-dried extract of *Ilex guayusa*: a potential ingredient for energy drink", no generative artificial intelligence tools or automated assistance systems were used for the writing, analysis, data interpretation, content generation, translation, or editing of the manuscript.

The authors assume full responsibility for the content, originality, integrity, and final version of the article.

Author Contributions

L.S.: Conceptualization, investigation, writing – original draft, writing – review and editing. **S.L.F.:** Formal analysis, writing – original draft. **M.R.:** Methodology, project administration, writing – original draft, writing – review and editing.

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