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Studies on the Phytochemical Variability of Xanthine Alkaloids from the Leaves of *Ilex guayusa* Loes. in the Amazon Region of Ecuador

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Abstract

Ilex guayusa Loes., known as guayusa, is a plant that has been used for generations by the indigenous peoples of the Ecuadorian Amazon for its medicinal properties, notably its stimulating effects. This bioactivity is primarily due to the presence of xanthine alkaloids such as caffeine, theobromine, and theophylline. In this study, the concentration of each alkaloid was determined in thirty populations throughout the Amazon region, with data ranging from 2065.0 mg/100 g to 364.8 mg/100 g for caffeine; 225.8 mg/100 g and 74.6 mg/100 g for theobromine; and 4.9 mg/100 g and 1.5 mg/100 g for theophylline. A statistical analysis was conducted on twenty-four guayusa populations by integrating alkaloid concentrations with altitude, climatic, and soil variables. Caffeine showed high variability among populations and was only weakly associated with elevation, whereas theobromine and theophylline were determined primarily by climatic and soil factors. These results indicate that methylxanthine accumulation in *Ilex guayusa* is driven by the combined influence of climatic and edaphic conditions rather than by a single environmental factor.

Keywords: *Ilex guayusa*; caffeine; theobromine; environmental variability

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1. Introduction

The Amazon is home to the greatest plant biodiversity on the planet, accounting for 30% of all species [1–3]. In Ecuador, the Amazon region covers approximately half of the country's total territory [4,5], and its natural boundary is the eastern foothills of the Andes mountain range, resulting in the presence of various types of ecosystems and climatic zones within the region, including: lowland evergreen forests, lowland evergreen forests flooded by whitewater, lowland evergreen forests flooded by blackwater, lowland floodplain palm forests, lowland lacustrine grasslands, piedmont evergreen forests, low montane evergreen forests, and low montane moist shrublands [6].

Some medicinal species, such as guayusa (*Ilex guayusa* Loes.), grow in more than one of the ecosystems mentioned above; therefore, a study of the variability of secondary metabolites based on location is warranted. Guayusa is a plant that has been cultivated for

hundreds of years and is frequently used by the indigenous and mestizo cultures inhabiting the Ecuadorian Amazon rainforest [7,8].

Guayusa contains several notable secondary metabolites, including alkaloids, phenolic compounds, and terpenes [7], the most important of which, both in terms of concentration and bioactivity, are xanthine-type alkaloids such as caffeine, theobromine, and theophylline; caffeine can be found in high concentrations ranging from 1% to 3% [9], depending on the plant and the type of extraction method used. Caffeine is widely consumed worldwide for its stimulating properties, which can be both physical and mental [10–16], making it one of the most widely consumed alkaloids [17,18]. Theobromine is an alkaloid found in cacao (*Theobroma cacao*); like caffeine, it is a stimulant with effects on the cardiovascular system and overall well-being [19–22]. Theophylline is present in smaller amounts, with concentrations below 0.1%; its best-known property is acting as a bronchodilator [23,24].

The chemical composition varies and depends, among other factors, on altitude, ambient temperature, humidity, light intensity, soil type, and precipitation patterns [25–27]. Because guayusa grows throughout the Amazon region of Ecuador, it is important to analyze the concentration of each alkaloid, considering all provinces and various localities where the plant is cultivated and used for food and pharmaceutical purposes.

To supplement the above, we should mention that guayusa is considered one of the plant-based bioresources with the greatest commercial and economic potential within Ecuador's biodiversity, generating approximately 12 million dollars in revenue [7], which makes it very important to identify the areas with the highest production of methylxanthines.

In this study, 30 guayusa samples were analyzed, collected from communities in Ecuador's six Amazonian provinces; national parks and territories inhabited by uncontacted indigenous peoples were not included.

2. Materials and Methods

2.1. Reagents and Standards

Caffeine, Sigma-Aldrich St. Louis MO. USA, code C0750; Theobromine, Sigma-Aldrich, code T4500; Theophylline, Phyto Lab Reno NV. USA, code 82668; Ammonium hydroxide solution reagent, Sigma-Aldrich, code 221228; Dichloromethane anhydrous, Sigma-Aldrich, code 270997; Tetrahydrofuran 99.8%, Sigma-Aldrich, code 34865; Acetonitrile 99.8%, Sigma-Aldrich, code 34851.

2.2. Plant Material

Plant material was collected at 30 sites across the six provinces of Ecuador's Amazon region; no samples were collected in Yasuní National Park due to difficult access and the presence of indigenous peoples living in voluntary isolation within the park [28,29]. All of the locations are inhabited, either by indigenous peoples or by mestizo settlers. The plants collected were cultivated in small family farms. Details and locations of the collection sites are shown in Table 1 and Figure 1.

Table 1. Information on the origin of the Guayusa samples.

Sample Number	Place	Location	Elevation (m.a.s.l.)	Province
1	Jondachi	0°44′00.8″ S 77°47′12.1″ W	1153	Napo
2	Wawa Sumaco	0°43′28.1″ S 77°34′32.0″ W	1131	Napo
3	Atenas	0°00′49.1″ S 77°25′58.1″ W	965	Sucumbios

Table 1. *Cont.*

Sample Number	Place	Location	Elevation (m.a.s.l.)	Province
4	Lumbaqui	0°03′19.0″ N 77°19′54.2″ W	502	Sucumbios
5	Lago Agrio	0°06′03.7″ N 76°53′10.7″ W	300	Sucumbios
6	Tena	0°59′42.1″ S 77°48′21.9″ W	545	Napo
7	Joya de los Sachas	0°18′52.7″ S 76°52′15.9″ W	283	Orellana
8	El Coca	0°25′47.6″ S 76°59′26.8″ W	271	Orellana
9	Estrella Yacu	0°29′18.5″ S 77°06′27.4″ W	304	Orellana
10	Capihuara	0°32′30.0″ S 77°13′04.1″ W	327	Orellana
11	Loreto	0°41′32.7″ S 77°18′48.9″ W	404	Orellana
12	Comuna 24 de Mayo	0°44′46.9″ S 77°28′53.6″ W	632	Orellana
13	Cotapino	0°44′24.9″ S 77°31′15.8″ W	1033	Orellana
14	Archidona	0°54′25.6″ S 77°48′14.8″ W	593	Napo
15	Cotundo	0°49′51.8″ S 77°46′16.0″ W	857	Napo
16	San Pedro de Cofanes	0°09′44.6″ S 76°51′00.1″ W	289	Sucumbios
17	Pacayacu	0°02′29.7″ S 76°35′27.4″ W	261	Sucumbios
18	Shell, Comunidad Shacha Runa	1°29′25.6″ S 78°03′42.4″ W	1048	Pastaza
19	Arajuno, Centro de Salud	1°14′31.4″ S 77°41′46.0″ W	627	Pastaza
20	Arajuno, San Jose de Curaray, Comunidad Bataborro	1°27′45.6″ S 76°39′40.7″ W	240	Pastaza
21	Teniente Hugo Ortiz, Comunidad Buayacun	1°22′11.5″ S 77°52′50.6″ W	957	Pastaza
22	Palora, Finca Miguel Angel	1°43′26.6″ S 77°58′01.0″ W	907	Morona Santiago
23	Macas, Sevilla Don Bosco	2°18′40.9″ S 78°06′13.4″ W	975	Morona Santiago
24	Taisha	2°23′16.0″ S 77°29′56.9″ W	446	Morona Santiago
25	Sucua	2°27′18.5″ S 78°09′54.7″ W	830	Morona Santiago
26	Yantzaza, Roldan Guaillas CS	3°49′06.5″ S 78°45′13.1″ W	802	Zamora Chinchipe
27	Nangaritza, Zurmi PS	4°06′07.6″ S 78°39′33.0″ W	871	Zamora Chinchipe
28	Zumba HB	4°51′47.1″ S 79°08′07.7″ W	1226	Zamora Chinchipe
29	Palora	1°42′34.8″ S 77°59′37.4″ W	889	Morona Santiago
30	Cuchaentza	2°07′21.5″ S 77°51′51.8″ W	1011	Morona Santiago

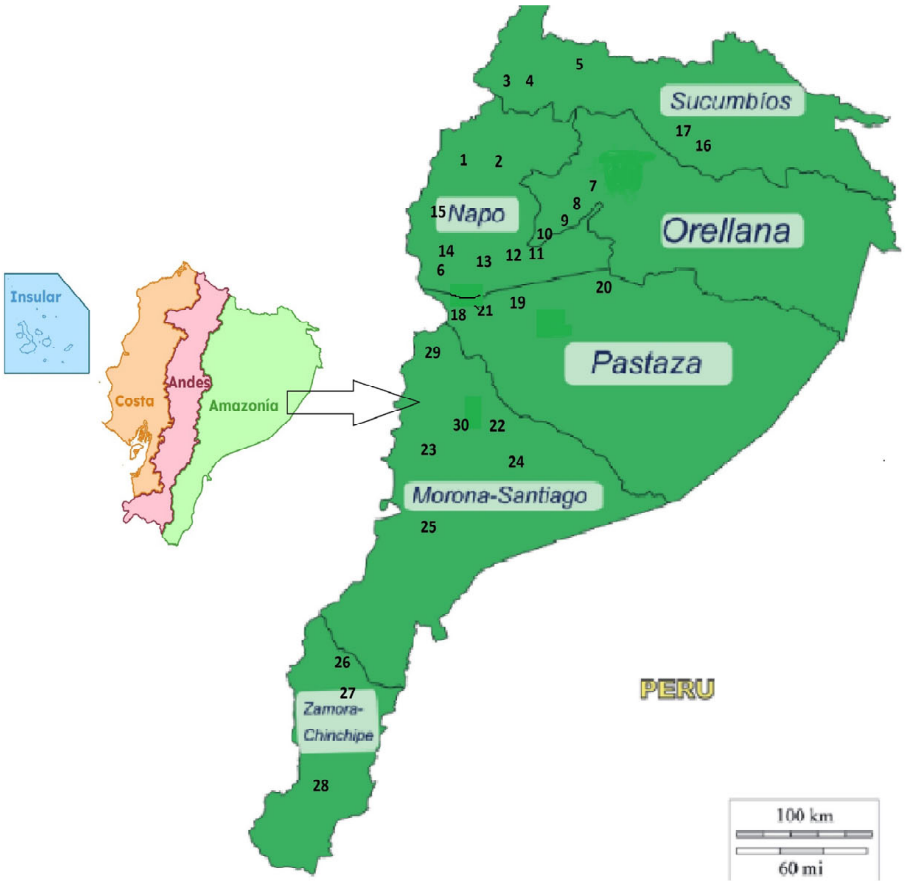

Figure 1. Location of guayusa samples in the Ecuadorian Amazon.

The collected specimens were identified at the herbarium of the Salesian Polytechnic University by botanist Marco Cerna.

2.3. Processing of Guayusa Simples

The guayusa leaves were carefully selected, discarding those that were damaged or stained. The plant material was washed and disinfected with a 1% sodium hypochlorite solution; the samples were then dried in a Binder KBF 240 oven at 60 °C for 48 h; finally, the plant material larger than 5 mm was ground in a mill. The material was stored at 4 °C in amber-colored containers.

2.4. Extraction of Alkaloids

Five grams of dried, crushed plant material were weighed on a Mettler Toledo ME204T/00 balance. The material was placed in an Erlenmeyer flask containing 50 mL of distilled water and shaken for 24 h in a Thermo Scientific 4310 orbital shaker. It was then filtered and made up to a volume of 50 mL with distilled water. Two milliliters of the aqueous extract were taken, 5 mL of 10% ammonium hydroxide were added, and the mixture was placed in a separatory funnel; finally, the xanthine alkaloids were extracted with 3 mL of dichloromethane on three occasions. The organic phase was transferred to a container and finally made up to 10 mL for injection into the HPLC, according to the methodology proposed by Radice & Vidari 2007 [30].

2.5. HPLC Analysis

The chromatographic conditions were adapted from Srdjenovic et al. (2008) [31] and modified for the present study. Analyses were performed using a Waters HPLC system equipped with a 1525 binary pump, a 2998 diode array detector, and Empower 3 software. Detection was carried out at 275 nm using a Hypersil GOLD LC-18 column (150 mm × 4.6 mm). Mobile phase A consisted of 1% tetrahydrofuran (THF), while mobile phase B was acetonitrile, using an A:B ratio of 90:10. The injection volume was 20 µL, the flow rate was 1 mL/min, and the total analysis time was 10 min. Calibration curves for caffeine, theobromine, and theophylline were experimentally obtained in the present study using six concentration levels (5, 10, 25, 50, 100, and 250 mg/L), with three replicates at each concentration level. The experimentally evaluated calibration range was 5–250 mg/L for all three analytes.

The limits of detection (LOD) and quantification (LOQ) were estimated from the calibration data obtained in the present study according to LOD = 3.3 S_a/m and LOQ = 10 S_a/m, where S_a is the standard deviation of the intercept of the respective calibration curve and m is its slope. The calculated LOD values were 0.28, 1.19, and 0.12 mg/L for caffeine, theobromine, and theophylline, respectively, whereas the corresponding LOQ values were 0.83, 3.61, and 0.35 mg/L. These calculated LOD and LOQ values should be distinguished from the experimentally evaluated calibration range (5–250 mg/L). Recovery was not experimentally determined in the present study. Therefore, LOD, LOQ, recovery, linear range, and correlation coefficient values previously reported by Srdjenovic et al. (2008) [31] are not used as validation parameters for the present method, since they were obtained under the chromatographic conditions of the original method rather than under the modified conditions applied in this study.

2.6. Data Filtering and Sample Size

Initially, 30 *Ilex guayusa* sampling sites were considered. Before conducting the statistical analyses, integrity and consistency criteria were applied to the environmental and analytical data. Sites lacking edaphic information in SoilGrids or presenting outlier values in methylxanthine concentrations were excluded. Outliers were identified using a statistical criterion based on a Z-score threshold of $|Z| > 2$, complemented by a Student's *t*-test to assess significant differences from the variable mean ($p < 0.05$). The application of these criteria allowed only sites with complete edaphic information and without outlier concentration values to be retained, thereby minimizing potential distortions in model estimates. As a result of this filtering process, six sites—Jondachi, Tena, El Coca, Capihuara, Sucua, and Zumba HB—were excluded, yielding a final dataset of 24 sites for subsequent analyses.

2.7. Study Species and Sampling

This study was conducted on natural populations of *Ilex guayusa* Loes. distributed throughout Ecuador. Twenty-four sampling sites were selected to capture environmental variability across the species' range. At each site, geographic coordinates (UTM) and elevation were recorded. Leaf samples were collected and subsequently analyzed to quantify three methylxanthines: caffeine, theobromine, and theophylline.

2.8. Environmental Variables

2.8.1. Bioclimatic Data

Bioclimatic variables (BIO1–BIO19) were obtained from the WorldClim database [32], which provides high-resolution spatial information. These variables represent temperature and precipitation patterns relevant to plant ecological responses.

2.8.2. Soil Data

Soil properties were obtained from SoilGrids and included sand and silt content, bulk density (bdod), soil pH (phh2o), cation exchange capacity (cec), soil organic carbon (soc), and nitrogen content [33].

2.9. Statistics

All analyses were performed in the R statistical environment (version 4.2.1). The relationship between methylxanthine composition and environmental variables was evaluated using redundancy analysis (RDA), implemented with the vegan package. Separate analyses were performed for bioclimatic and edaphic variables. Variables were selected using a forward-selection procedure with a significance threshold of $p < 0.05$. Model significance was assessed using permutation tests with 999 permutations.

The spatial structure of methylxanthine concentrations was evaluated using Moran's I, implemented with the spdep package. For this purpose, a spatial weight matrix based on the four nearest neighbors ($k = 4$) was constructed and row-standardized. Statistical significance was assessed using permutation tests.

The relationship between elevation and methylxanthine concentrations was evaluated using linear and quadratic models. Model fitting and statistical tests were performed using base R functions. Model performance was compared using the adjusted coefficient of determination (adjusted R^2) and analysis of variance (ANOVA).

Environmental variables were analyzed using their original values. Additionally, principal component analysis (PCA) was explored as a dimensionality-reduction strategy. However, models based on the first two principal components (PC1 and PC2) produced results consistent with those obtained using the original variables. Because individual predictors offer greater ecological interpretability, subsequent analyses were performed using the original variables.

3. Results

3.1. Determination of Caffeine, Theobromine, and Theophylline

Caffeine, theobromine, and theophylline were detected in all 30 Guayusa samples. Caffeine is the most abundant alkaloid, with the highest concentration of 2065.0 mg/100 g found in the community of Taisha and the lowest of 364.8 mg/100 g in San José de Arajuno. The second most important alkaloid is theobromine, with the highest values reported in the community of Cotundo at 225.8 mg/100 g and the lowest in Jondachi at 74.6 mg/100 g. The concentration of theophylline is quite low compared to the two previous alkaloids, with just 4.9 mg/100 g in Cotundo and 1.5 mg/100 g in Jondachi. Figure 2 shows the chromatograms of the standards for each alkaloid and the chromatogram of one of the samples analyzed. Table 2 shows all the concentration values obtained for caffeine, theobromine, and theophylline for thirty guayusa samples analyzed.

3.2. Analysis of Environmental, Soil Composition, and Geographic Factors Affecting the Presence of Xanthic Alkaloids

3.2.1. Effect of Altitude on Alkaloid Concentrations in *Ilex guayusa*

The quadratic models revealed that altitude has a selective and compound-dependent effect on alkaloid variability in Ecuadorian populations of *Ilex guayusa*. Of the three methylxanthines analyzed, only caffeine showed a significant response to the altitudinal gradient.

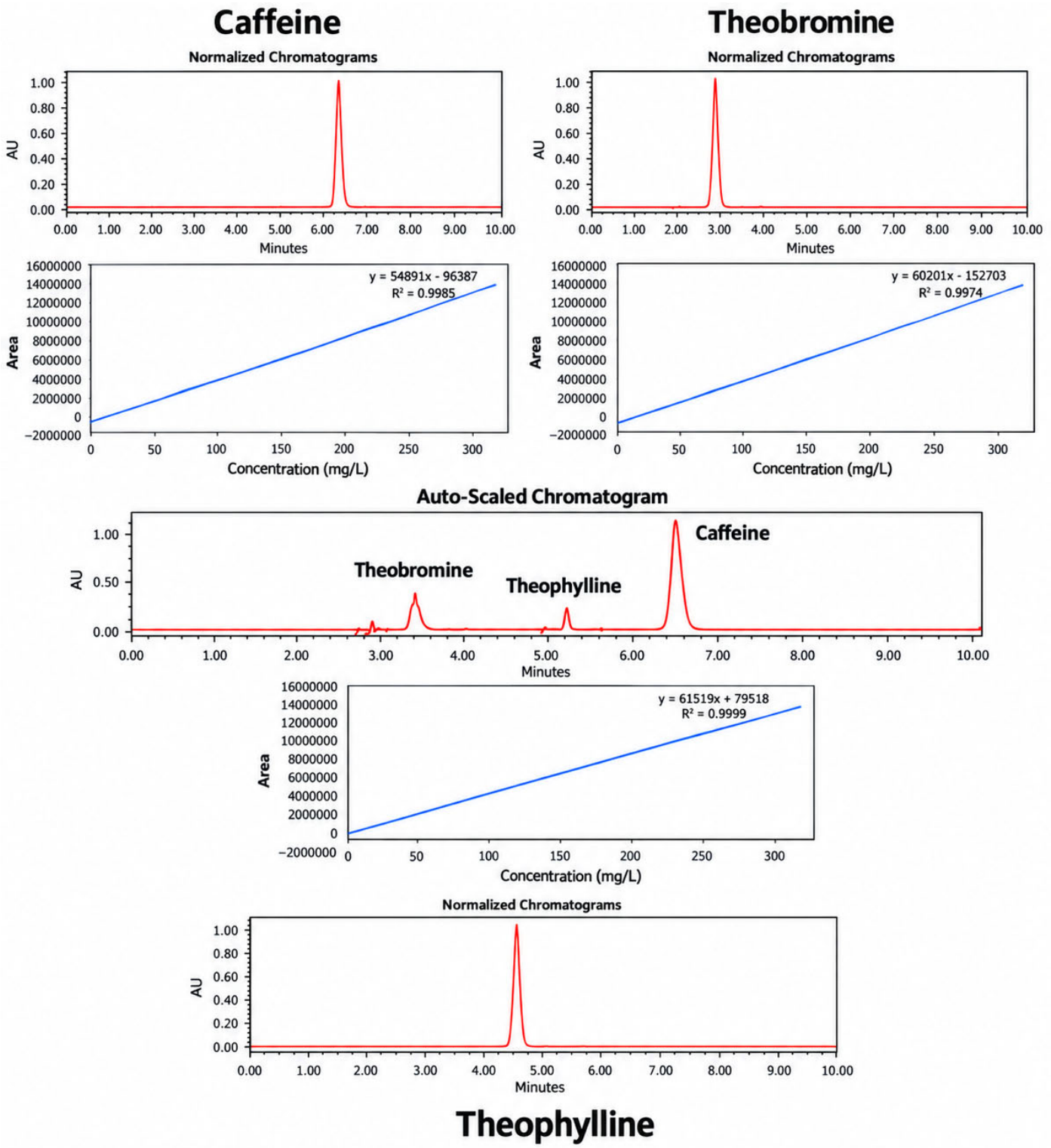

Figure 2. Comparative chromatographic separation of the alkaloids caffeine, theobromine, theophylline, and a guayusa sample. Calibration curve data: caffeine R^2 = 0.999, Y = 54,556X – 86,926; theobromine R^2 = 0.999, Y = 60,170X + 120,103; theophylline R^2 = 0.999, Y = 61,605X + 64,852.

Table 2. Concentrations of caffeine, theobromine, and theophylline in guayusa leaves from 30 locations in the Amazon region of Ecuador. Mean SD (n = 3), $p < 0.05$.

Sample Number	Place	mg Caffeine/kg	mg Theobromine/kg	mg Theophylline/kg
1	Jondachi	6310 ± 40	750 ± 30	15 ± 1
2	Wawa Sumaco	5760 ± 50	2160 ± 20	45 ± 2
3	Atenas	5310 ± 280	1890 ± 110	34 ± 5
4	Lumbaqui	14,000 ± 470	2240 ± 80	49 ± 4
5	Lago Agrio	6150 ± 160	2120 ± 60	43 ± 2
6	Tena	6210 ± 160	2120 ± 60	46 ± 3
7	Joya de los Sachas	7320 ± 420	2080 ± 140	41 ± 5
8	El Coca	6900 ± 210	2100 ± 70	43 ± 3
9	Estrella Yacu	15,950 ± 600	1990 ± 80	40 ± 3
10	Capihuara	5240 ± 300	1940 ± 120	40 ± 5
11	Loreto	6080 ± 200	2110 ± 70	46 ± 4
12	Comuna 24 de Mayo	13,210 ± 330	2090 ± 50	46 ± 3
13	Cotapino	6760 ± 340	2030 ± 110	41 ± 5
14	Archidona	9720 ± 820	2120 ± 200	47 ± 9
15	Cotundo	6720 ± 180	2260 ± 60	49 ± 3
16	San Pedro de Cofanes	8310 ± 390	2070 ± 100	44 ± 5
17	Pacayacu	7244 ± 155	1713 ± 85	26 ± 2
18	Shell, Comunidad Shacha Runa	11,560 ± 570	1840 ± 90	31 ± 5
19	Arajuno, Centro de Salud	11,310 ± 870	1730 ± 140	28 ± 4
20	Arajuno, San Jose de Curaray, Comunidad Bataborro	3650 ± 310	1840 ± 160	31 ± 7
21	Teniente Hugo Ortiz, Comunidad Buayacun	10,570 ± 560	1750 ± 100	28 ± 4
22	Palora, Finca Miguel Angel	7810 ± 220	1550 ± 50	19 ± 3
23	Macas, Sevilla Don Bosco	4890 ± 140	1710 ± 50	26 ± 3
24	Taisha	20,660 ± 800	1640 ± 70	24 ± 3
25	Sucua	11,880 ± 140	1670 ± 20	25 ± 1
26	Yantzaza, Roldan Guaillas CS	12,850 ± 310	1870 ± 50	33 ± 2
27	Nangaritza, Zurmi PS	15,860 ± 920	1870 ± 100	27 ± 5
28	Zumba HB	7720 ± 260	1690 ± 60	24 ± 3
29	Palora	8240 ± 380	1680 ± 80	23 ± 4
30	Cuchaentza	5840 ± 110	1690 ± 40	24 ± 1

Caffeine showed a significant unimodal relationship with altitude (adjusted $R^2 = 0.188$, $p = 0.043$), with both the linear and quadratic terms being significant ($p < 0.05$). The negative quadratic coefficient indicates a maximum concentration at around 629 m above sea level, followed by a decrease at higher elevations.

In contrast, neither theobromine nor theophylline showed significant relationships with altitude (adjusted $R^2 \leq 0$; $p \geq 0.43$), and the inclusion of quadratic terms did not improve the fit of the models. Figure 3 illustrates the relationship between altitude and the concentration of each of the methylxanthines. These results indicate that altitude explains only a modest fraction of the variability in caffeine and has no detectable effects on the other methylxanthines.

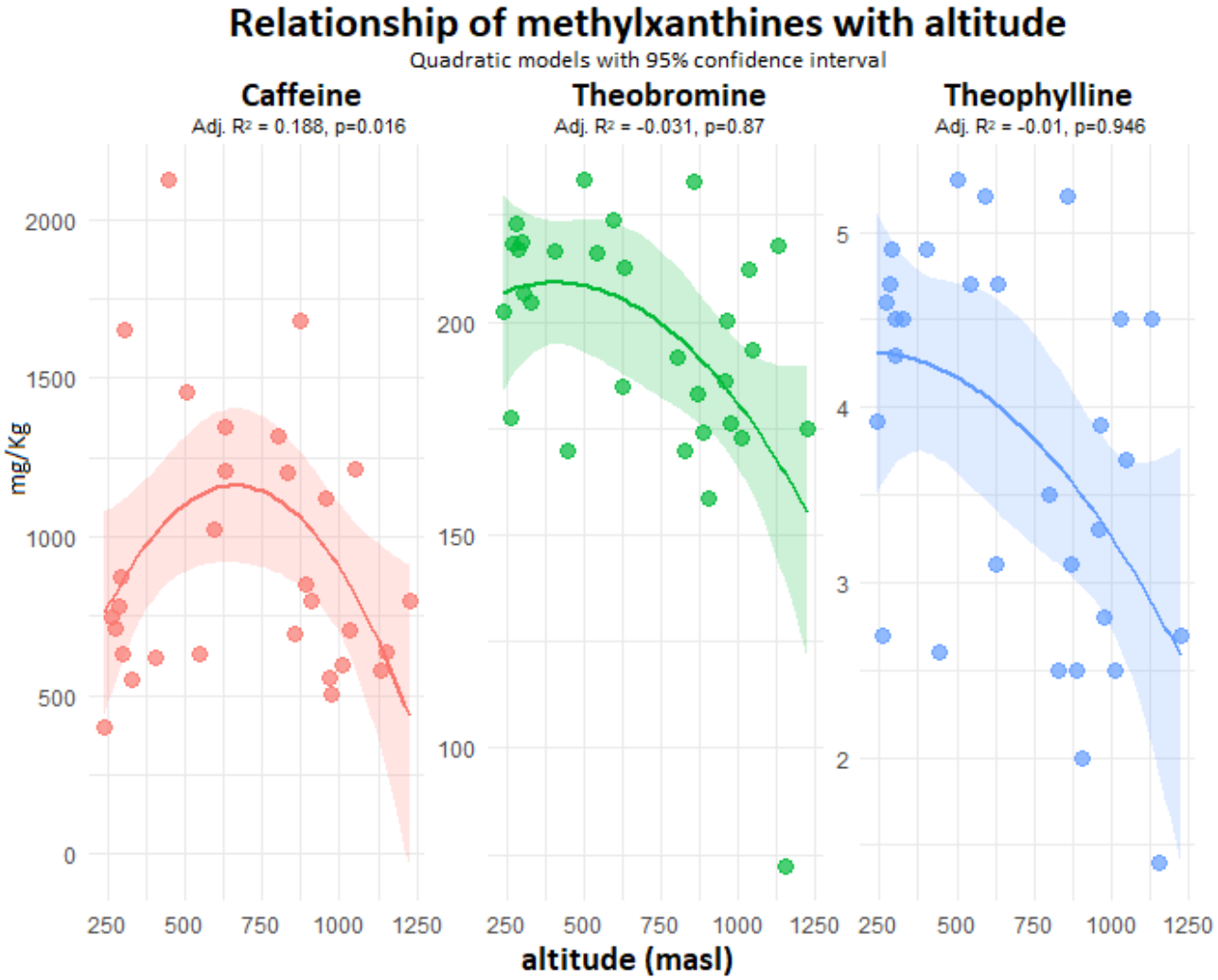

Figure 3. Relationship between methylxanthine concentration and altitude.

3.2.2. Effects of Bioclimatic Variables

Redundancy analysis (RDA) identified isothermality (BIO3) and precipitation seasonality (BIO15) as the only significant bioclimatic predictors ($p = 0.001$), explaining 40% of the total variance (adjusted $R^2 = 0.356$). Both variables showed strong positive associations with theobromine and theophylline:

- Theobromine: r = 0.638 (BIO15), r = 0.683 (BIO3);
- Theophylline: r = 0.686 (BIO15), r = 0.707 (BIO3).

In contrast, caffeine showed weak and nonsignificant correlations with these variables ($|r| < 0.20$).

The high loadings of BIO15 and BIO3 on the first axis of the RDA indicate that climatic variability is a major factor in the composition of methylxanthines, particularly for theobromine and theophylline. This climate-driven variability is evident in Figure 4.

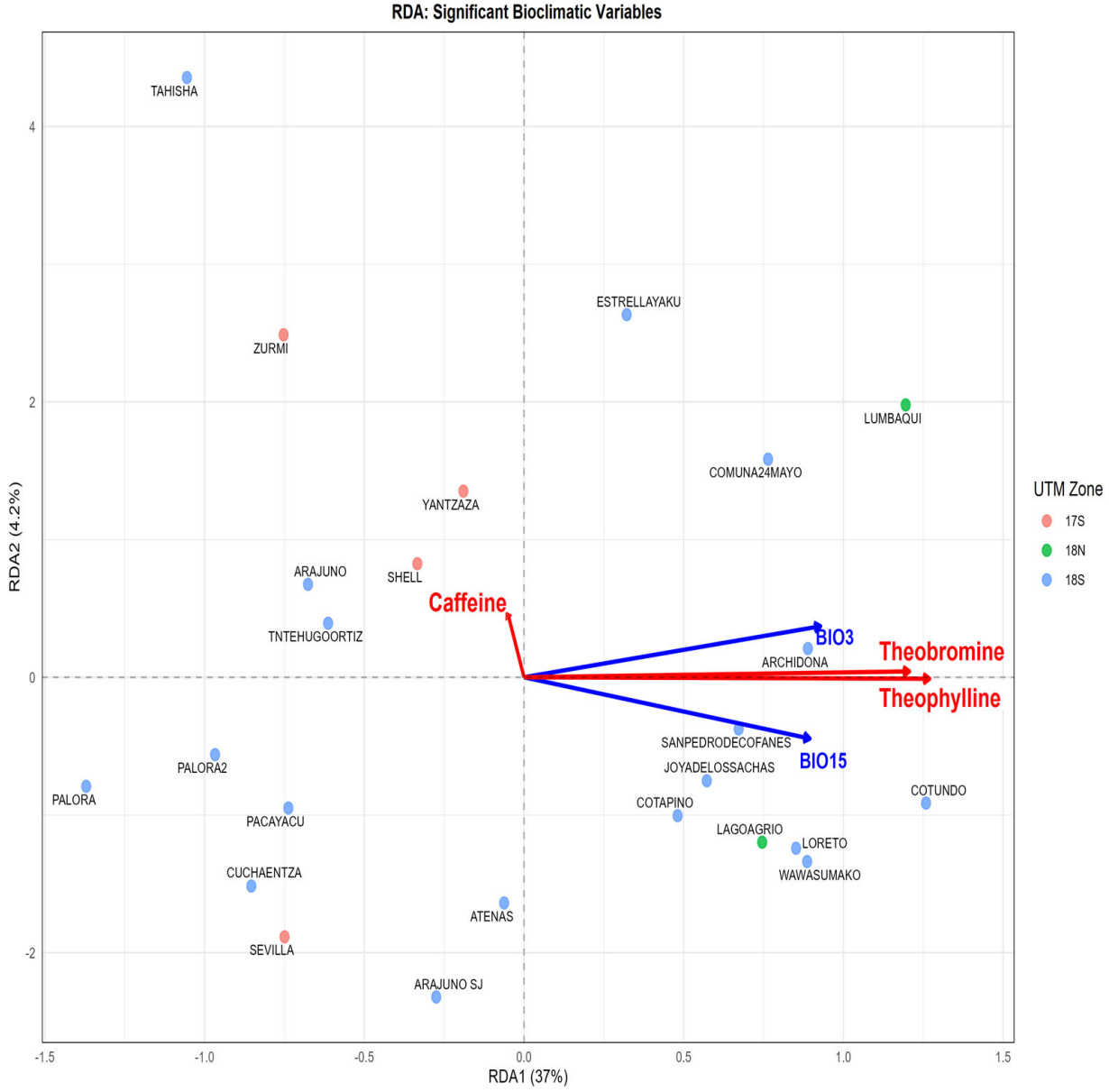

Figure 4. RDA of Bioclimatic Variables and Alkaloid Profiles across UTM Zones.

3.2.3. Effects of Edaphic Variables

The edaphic RDA identified bulk density (bdod) and soil organic carbon (soc) as significant predictors ($p = 0.003$), explaining 30% of the total variance (adjusted $R^2 = 0.266$).

Both variables showed consistent negative associations with theobromine and theophylline: Theobromine: $r = -0.505$ (bdod), $r = -0.465$ (soc); Theophylline: $r = -0.504$ (bdod), $r = -0.471$ (soc).

Caffeine showed weak correlations with soil variables ($|r| \leq 0.26$), indicating a limited response to soil conditions.

The similar contributions of bdod and soc suggest that soil structure and organic matter act together to modulate alkaloid expression. The identified effects of soil variability are shown in Figure 5.

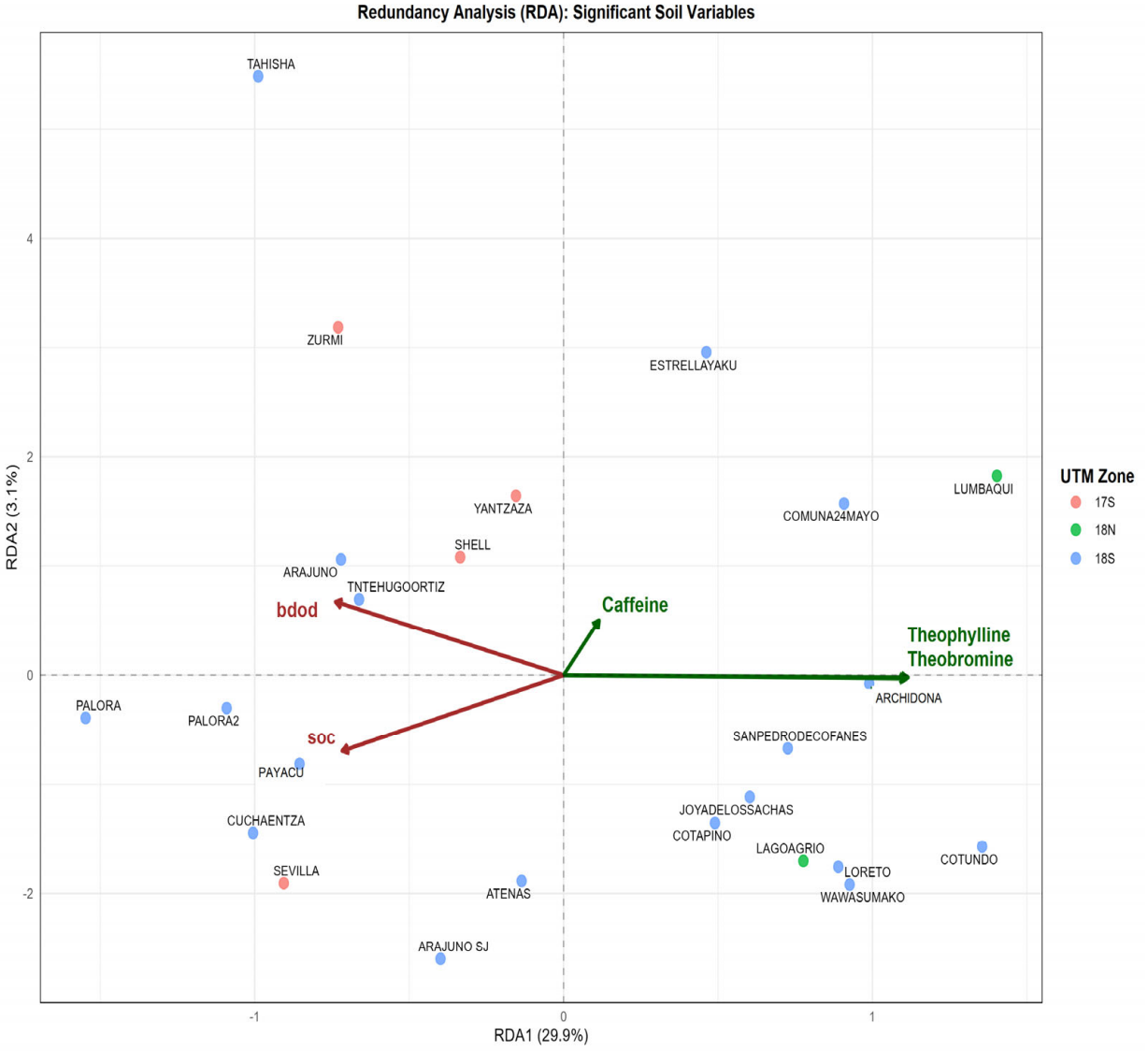

Figure 5. Redundancy analysis (RDA) biplot of significant soil variables and methylxanthine concentrations (caffeine, theophylline, theobromine) across sampling sites.

3.2.4. Integrated Environmental Effects

Bioclimatic and edaphic variables together explained 62.2% of the total variance, with 35.6% attributed to climate and 26.6% to soil.

These results indicate the following:

- Climatic factors are the primary determinants of variability in theobromine and theophylline;
- Soil properties act as secondary modulators;
- Caffeine shows a weak association with both environmental components.

The results are shown graphically in Figure 6.

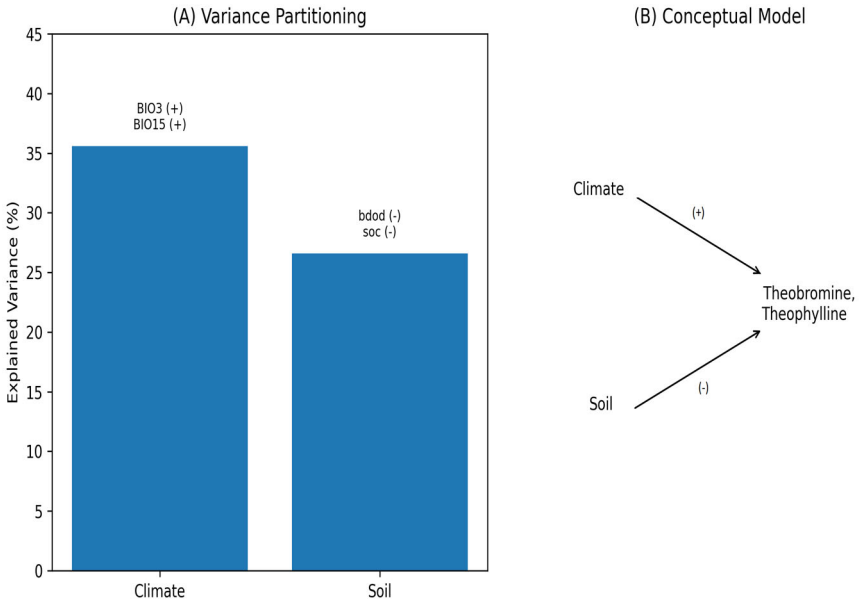

Figure 6. Variance partitioning and conceptual model of climate and soil effects on methylxanthine concentration.

3.2.5. Spatial Structure of Alkaloid Variability

Spatial autocorrelation analysis revealed a strong and significant spatial structure (Moran's I = 0.719, $p < 0.001$), indicating that alkaloid concentrations in *Ilex guayusa* are highly structured across the Ecuadorian landscape, as shown in Figure 7.

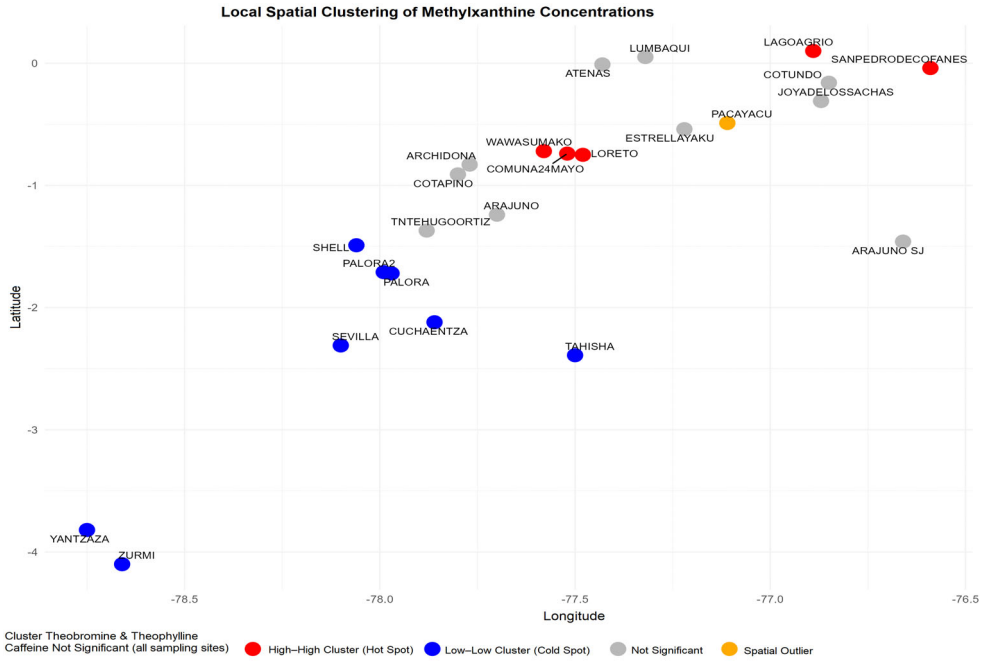

Figure 7. Local spatial clustering of Methylxanthine concentrations.

This spatial pattern is consistent with the distribution of climatic and edaphic variables, suggesting that environmental heterogeneity underlies the observed spatial clustering, particularly in the case of theobromine and theophylline.

4. Discussion

Some findings from other studies on caffeine show maximum concentrations of around 3% [34,35]; in this study the highest value found was 2065.5 mg/100 g, approximately 2.07%. This difference stems from the extraction methodology, since other studies have used more efficient methods such as decoction or ultrasound-assisted extraction; despite this, the caffeine ranges are consistent with those reported in most previous studies [8,34–36]. Previous studies have reported theobromine levels ranging from 0.008% to 0.12% [8,36], which are lower than those reported in this study, where the percentage range is 0.08% to 0.23%. The reported concentration of theophylline is very low, which is consistent with the findings of this study, where the values range from 0.0015% to 0.0049%, while the literature indicates concentrations ranging from 0.002% to 0.005% [8]. The studies are consistent with previous research conducted on various guayusa samples.

4.1. Compound-Specific Environmental Regulations

The results indicate that the accumulation of methylxanthines in *Ilex guayusa* is not governed by a single common environmental gradient, but rather by compound-specific controls. Among the three alkaloids analyzed, caffeine showed a weak association with altitude, while theobromine and theophylline showed stronger relationships with climatic and edaphic variables. This pattern supports a model of differentiated metabolic regulation, in which different branches of the methylxanthine biosynthetic pathway respond independently to environmental stimuli.

This interpretation is consistent with the general evidence that plant secondary metabolism is highly sensitive to abiotic stress signals such as temperature, radiation, and water availability, which selectively regulate different classes of metabolites based on their ecological function [37]. In systems such as tea, it has been shown that environmental variation modifies secondary metabolite profiles in a compound-specific manner, depending on climatic and edaphic conditions [38]. In *I. guayusa*, recent studies have shown that light exposure, soil conditions, and leaf age collectively influence methylxanthine content [39].

4.2. Altitude as an Indirect Environmental Factor Affecting Caffeine Content

Although elevation significantly explained the variation in caffeine concentration, its explanatory power was limited (<20%), indicating that elevation acts primarily as a composite environmental indicator rather than as a direct causal factor. In tropical systems, altitudinal gradients generate simultaneous changes in temperature, solar radiation, and moisture availability, creating a set of environmental conditions that influence plant physiology and the synthesis of secondary metabolites [40]. Altitudinal gradients affect the production of secondary metabolites in plants by modifying both biotic and abiotic conditions [41].

This indirect role of altitude has been extensively documented. In tea, environmental factors associated with elevation influence secondary metabolites through complex interactions rather than through direct altitudinal effects [38]. Similarly, in coffee, biochemical composition is determined by climatic factors linked to altitude rather than by elevation alone [42,43]. The unimodal response observed for caffeine suggests that intermediate environmental conditions may optimize its biosynthesis.

4.3. Climate Control of Theobromine and Theophylline Accumulation

In contrast to caffeine, the absence of altitude-related effects for theobromine and theophylline suggests that other environmental variables better explain their variation. This is consistent with studies showing that environmental gradients can shape phenotypic

variation [44]. Furthermore, genetic evidence in *I. guayusa* indicates that variables such as isothermality and thermal seasonality (BIO3 and BIO4) are key determinants of population structure [45], which reinforces the interpretation of altitude as an indirect gradient. This suggests that climatic gradients simultaneously influence metabolic expression and genetic differentiation.

The strong influence of isothermality (BIO3) and precipitation seasonality (BIO15) highlights the central role of climate variability in the composition of methylxanthines. Both variables were positively associated with theobromine and theophylline, suggesting that these compounds accumulate preferentially in environments with relative thermal stability and marked seasonal rainfall patterns. In contrast, caffeine was not significantly associated with any of the bioclimatic factors, suggesting distinct environmental regulation within the same group of alkaloids.

This pattern is consistent with evidence showing that climate is a key determinant of secondary chemistry in plants. Geographic, climatic, and edaphic factors have been linked to variation in metabolite profiles across various species [46]. Similarly, metabolomic studies of species in the genus *Ilex* have revealed chemodiversity associated with environmental conditions [9], while in *Theobroma cacao*, regional climatic variability has been shown to significantly affect metabolomic composition [47].

4.4. The Role of Soil in Metabolic Modulation

Soil variables, particularly bulk density and soil organic carbon, were significant predictors of alkaloid variability. Their negative association with theobromine and theophylline suggests that less is invested in these compounds under more favorable soil conditions.

This interpretation is consistent with ecological theories of resource allocation, which propose that plants balance their investment between growth and defense based on resource availability [48,49]. Under favorable conditions, plants tend to prioritize growth over the production of secondary metabolites.

However, the effect of soil depends on the environmental context. As has been described in *Camellia sinensis* L. crops, soil nutrients and management practices influence chemical quality, but their effects depend on interactions with other environmental factors [50]. In *I. guayusa*, it has been shown that soil properties interact with light and plant age to determine methylxanthine content [39], suggesting that soil acts as a modulator within a multifactorial system. Similarly, in *Ilex paraguariensis*, methylxanthine concentrations vary significantly depending on the developmental stage of the leaf, supporting the existence of differential regulation [51] that may be associated with soil nutritional characteristics.

4.5. Spatial Structure and Environmental Heterogeneity

The strong spatial autocorrelation observed indicates that chemically similar populations tend to cluster geographically. The geographic structuring of metabolic profiles has been extensively documented, showing that geographic location acts as an integrator of multiple environmental gradients [52]. In *I. guayusa*, the spatial distribution of climatic and edaphic variables likely explains the observed clustering.

Genetic evidence supports this interpretation. The presence of genetic clusters and the role of the environment in their formation indicate that spatial patterns reflect environment-induced genetic differentiation [45]. This behavior has also been reported in other plant species, for which climatic heterogeneity proved to be a determining factor in patterns of genetic differentiation and in the spatial distribution of populations [53].

4.6. Integration of Genetic and Environmental Variability

The proportion of unexplained variance is consistent with what is expected in ecological studies. Genotype × environment interactions are fundamental to phenotypic variation, especially in secondary metabolites [54].

In *I. guayusa*, moderate genetic diversity, high clonal diversity, and the existence of distinct genetic clusters have been reported [45]. This suggests that genetic structure contributes to the observed chemical variability.

Therefore, variation in methylxanthines can be interpreted as the result of the interaction between environmental gradients and underlying genetic diversity [55].

5. Conclusions

Overall, this study demonstrates that alkaloid variability in Ecuadorian populations of *Ilex guayusa* is primarily determined by climatic and edaphic factors, while altitude plays a secondary role specific to certain compounds.

The observed spatial pattern reflects environmental gradients and genetic differentiation. From an applied perspective, these results suggest that management practices such as planting within an optimal altitude range and soil selection—along with other factors described, such as shade control, site selection, and harvest timing—can influence phytochemical composition.

In another *Ilex* species, yerba mate (*I. paraguariensis*), it has been demonstrated that environmental conditions and cultivation systems affect chemical properties [55,56]. Similar results have been reported for *I. guayusa* [39], and its ethnobotanical significance underscores the importance of optimizing its production [57].

In conclusion, the integration of climatic, edaphic, spatial, and genetic factors is essential for understanding and optimizing secondary metabolism in *Ilex guayusa*, which will benefit the plant's emerging production and industrialization efforts aimed at improving the economic well-being of Amazonian communities and promoting the development of supplements and energy drinks.

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