Physicochemical Characterization and Valorization of Processing Residues from Amazonian Guayusa (*Ilex guayusa* Loes.) within a Circular Economy Framework: A Case Study of Kallari Cooperative, Ecuador

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Article

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Abstract

Ilex guayusa is a culturally significant Amazonian plant cultivated in traditional chakra agroforestry systems. Processing generates substantial residues with potential valorization opportunities. This study quantified and characterized guayusa processing residues from Kallari cooperative (Napo, Ecuador) to identify circular economy applications. Residues were separated by particle size (425 μm, 300 μm, <300 μm) and analyzed for 20 replicates measuring density, pH, conductivity, moisture, ash, organic matter, fiber, protein, fat, total phenols, tannins, caffeine, and thermal properties. Granulometric analysis revealed 67.50 ± 0.94% coarse fraction (>425 μm), 11.94 ± 0.49% intermediate (300–425 μm), and 20.56 ± 0.91% fine (<300 μm), indicating 32.5% potential rejection. Fine residues showed mean density 0.27 g/cm³, pH 5.85, moisture 6.22%, high organic matter (92.37%), protein (1.61%), fat (6.78%), total phenols (15.70 units), tannins (9.41 units), and caffeine (1.89–2.09%). No significant differences were found between coarse and fine fractions for 12 parameters ($p > 0.05$). Guayusa residues retain 55–70% of caffeine and substantial phenolic compounds compared to whole leaves, demonstrating potential for food ingredient applications, functional product development, nutraceutical extracts, and bioenergy generation within circular bioeconomy models.

Keywords: ilex guayusa; processing residues; circular economy; bioactive compounds; caffeine; polyphenols; amazonian agroforestry; valorization; indigenous cooperatives

1. Introduction

Wayusa (*Ilex guayusa* Loes.), more commonly known as guayusa in Spanish, is an evergreen tree native to the western Amazon basin traditionally cultivated by Kichwa communities [1-3], with is natural distribution extending across Ecuador, southern Colombia, and northern Peru with its cultivation now predominantly concentrated in the Ecuadorian Amazon provinces of Napo, Pastaza, Sucumbíos, Morona Santiago, and Zamora Chinchipe [4-6], where it has been traditionally managed and consumed for centuries by Indigenous communities, particularly the Kichwa and Shuar peoples [3].

For centuries, *guayusa* has held profound cultural and spiritual significance among Amazonian indigenous communities, particularly the amazonian Kichwa people, who traditionally consume an infusion beverage of its leaves during early morning rituals known as *Waysa Upina* [7-9].

These ceremonial gatherings serve as vital spaces for intergenerational knowledge transmission, dream interpretation, and community organization, reinforcing the plant's role as a sacred element in indigenous cosmology [10, 11]. The cultivation and management of guayusa are intrinsically linked to the *chakra* agroforestry system, a traditional polyculture practice employed by Kichwa

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communities that integrates diverse plant species to promote agricultural biodiversity, ecological sustainability, and food sovereignty [8, 12, 13]. This socioecological system is primarily managed by women, locally referred as *mama chakra*, who assume a role in seed selection, plant propagation, intercropping design, and the intergenerational transmission of agroecological knowledge. within these agroforestry landscapes, that also function as buffer zones and natural corridors, reducing pressure on natural reserves and providing valuable resources for both local indigenous communities and wildlife [14], in this context guayusa functions not only as a culturally significant crop but also as an integral component of indigenous land management strategies that have shaped Amazonian ecosystems for generations [15, 16].

Guayusa leaves accumulates high concentrations of bioactive compounds, with caffeine as the dominant alkaloid typically 95 –136 mg g^{-1} in leaf extracts [17] exceeding levels found in yerba mate (Ilex paraguariensis) and approaching those of coffee [16, 18]. This makes guayusa both a potent natural stimulant and a subject of toxicological interest given caffeine's marked physiological effects [19, 20]. Beyond caffeine, the phytochemical profile includes theobromine, chlorogenic acid derivatives (predominantly 5-caffeoylquinic acid), dicaffeoylquinic acids, and a spectrum of flavonoids, tannins, terpenes, carotenes, and coumarins [8, 16]. García-Ruiz, Baenas [21] demonstrated that mono- and dicaffeoylquinic acid isomers account for a substantial fraction of total phenolics and directly correlate with peroxyl radical scavenging capacity in both biological membranes and food matrices. Caffeic acid serves as the principal cinnamate precursor for these chlorogenic derivatives, which collectively confer antioxidant activity superior to that of green tea [18]. Interestingly, pH modulation enhances this antioxidant efficiency [16], a property relevant to food processing and formulation design. Collectively, these bioactive constituents underpin guayusa's documented pharmacological activities: CNS stimulation, antioxidant protection, anti-aging effects, glycemic regulation, antimicrobial action, and cosmetic applications. The convergence of high caffeine content with a robust polyphenolic arsenal positions guayusa as a multipurpose botanical for functional foods, nutraceuticals, and pharmaceutical development.

Despite these advances, the agroindustrial consolidation of guayusa presents significant sustainability-related challenges. One major limitation lies in the difficulty of defining, measuring, and standardizing sustainability indicators, which are essential for conducting comprehensive situational assessments and evaluating the long-term viability of production systems. Moreover, industrial processing activities associated with food manufacturing may generate environmental externalities, including resource depletion, emissions, and contamination risks [22] .

A critical concern within food processing systems is the generation of substantial volumes of organic waste that are frequently underutilized or inadequately managed. Paradoxically, these by-products often retain valuable bioactive and functional compounds. Food by-products are increasingly conceptualized as sources of functional ingredients that can be recovered from processing residues and incorporated into new value-added products. In plant-based processing systems, such by-products represent a low-cost and sustainable alternative for the extraction of functional compounds with commercial potential [23].

In this context, the circular economy paradigm offers a conceptual and operational framework to transform agroindustrial residues from waste liabilities into value-generating inputs through cascading utilization strategies that maximize resource efficiency, minimize environmental impacts, and create closed-loop production systems [24, 25]. In the Amazonian bioeconomy context, residue valorization assumes particular significance as it aligns with principles of sustainable biodiversity utilization, indigenous economic empowerment, and climate change mitigation through reduced waste generation [26, 27]. Successful valorization models for botanically and chemically similar agroindustrial residues provide instructive precedents: coffee processing by-products (husks, pulp, silverskin) have been developed into functional food ingredients, antioxidant extracts, and biofuel substrates [28], tea processing waste has been valorized for polyphenol extraction and animal feed supplementation; and cocoa pod shells have been transformed into dietary fiber sources, natural colorants, and bioactive compound concentrates [29]. These case studies demonstrate that processing

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residues often retain significant portions of the bioactive compounds present in primary products, rendering them suitable for diverse applications in food, pharmaceutical, cosmetic, and energy sectors.

Notwithstanding growing scientific interest in guayusa leaf composition and bioactivity [3, 20, 30], the physicochemical characteristics, nutritional constituents, and valorization potential of processing by-products have yet to be thoroughly investigated in published literature. This knowledge gap constitutes a critical barrier to developing evidence-based circular economy strategies for guayusa producing cooperatives and limits opportunities to capture economic value from currently discarded biomass fractions. Characterization of residue properties is essential to identify retained bioactive compounds, assess quality parameters relevant to potential applications, and match residue characteristics to specific end-uses. Such information is particularly relevant for gastronomic innovation and functional food development, where bioactive-rich plant residues may serve as novel ingredients providing nutritional enhancement, natural flavoring, antioxidant fortification, or functional properties in food matrices [31].

Therefore, This study aimed to: (1) quantify residue generation by particle size fractions in the guayusa processing chain at Kallari cooperative; (2) comprehensively characterize the physicochemical properties, nutritional composition, and bioactive compound content of these residues; and (3) evaluate their potential for valorization within circular economy applications, particularly for gastronomic innovation and functional food development.

2. Materials and Methods

2.1. Study Site

This study was conducted in Kallari cooperative (Asociación Agroartesanal de Producción de Bienes Agrícolas y Pecuarios de Napo), located in Tena Canton, Napo Province, within the Ecuadorian Amazon region (0°59′S, 77°49′W; approximately 500 m above sea level). The region is characterized by a humid tropical climate with annual temperatures ranging from 23.5 to 24.6 °C. Precipitation is high throughout the year. The peak period occurs between Marh and May (414.3–451.7 mm), whereas relatively lower precipitation is recorded in August and September (264.5–298.4 mm) [32]. Kallari is a grassroots Indigenous cooperative comprising 333 smallholder producers dedicated to the cultivation of cacao, vanilla, and guayusa within traditional chakra agroforestry systems. These integrative administered production ecosystems span an estimated area of 7,791.17 hectares, with 673.68 hectares having certification as a climate-resilient agricultural land.

The cooperative local facilities in located in Tena for post-harvest handling, including drying, milling, and granulometric classification of guayusa leaves destined mainly for international export markets. However, the industrial processing chain—encompassing harvesting, washing, dehydration, milling, and particle-size classification—inevitably generates substantial quantities of plant residues that do not meet commercial export specifications. These by-products are typically rejected due to excessive particle size (>300 μm), heterogeneous granulometry, oxidative discoloration, stem contamination, or elevated moisture content. Preliminary internal assessments indicate that approximately 25–35% of the processed biomass is discarded during quality control procedures, representing not only economic losses for producer organizations but also an emerging environmental management challenge in remote Amazonian production contexts. At present, these residues are commonly disposed of through low-value practices such as composting or open burning, thereby limiting opportunities for the recovery and valorization of bioactive compounds retained within these plant materials.

2.2. Sample Preparation

Processed guayusa leaf samples were collected from Kallari's cooperative processing facility during December 2024. After industrial drying and milling operations residues were separated using

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a three-level sieve system with mesh sizes of 425 µm, 300 µm, and <300 µm, generating coarse, intermediate, and fine fractions respectively.

Fresh guayusa leaves (harvested from mature plants in member chakras) had undergone standardized post-harvest processing consisting of: (1) manual selection to remove damaged leaves and foreign material; (2) washing with potable water; (3) forced-air drying at 45–50°C for 18–24 hours until moisture content reached approximately 6–8%; and (4) mechanical milling using a hammer mill with initial coarse grinding followed by classification.

2.3. Quantification of Residue Generation by Granulometry

To quantify the proportional generation of each residue fraction in the processing chain, nine replicate sieving analyses were performed. For each replicate, precisely 160.0 g of dried, milled guayusa leaves (representing the output of industrial processing) were placed in the uppermost sieve (425 µm aperture) of a three-tiered sieve stack. The stack was subjected to mechanical agitation for 10 minutes at standardized intensity to ensure complete particle separation. Following sieving, the mass retained on each screen was determined gravimetrically using a calibrated analytical balance (±0.01 g): (1) Coarse residue (R_1): Material retained on 425 µm screen; (2) Intermediate residue (R_2): Material passing through 425 µm but retained on 300 µm screen; (3) Fine residue (R_3): Material passing through 300 µm screen.

The percentage of each fraction was calculated as:

$$\%\ Fraction = \frac{\text{Mass of fraction}}{\text{Total initial mass}}\ x\ 100$$

2.4. Physicochemical Characterization

Physicochemical analysis was performed on an intermediate-fine residue (<300 µm) and coarse residue (>425 µm) fractions to facilitate comparative analysis. A total of twenty (20) independent replicates of each fraction were analyzed. All analyses were performed in the Science Laboratory at Escuela Superior Politecnica de Chimborazo with established protocols.

2.4.1. Density Determination

The apparent bulk density of the solid residue fractions was assessed utilizing the graduated cylinder methodology. Dried samples, weighing between (15–17 g) were measured using an analytical balance (Camry EK3252, max 5 kg, ±0.01 g) to obtain mass (m), subsequently transferred into a 100 mL graduated cylinder (Glassco, ±1.0 mL, calibrated at 20°C) without compaction. The volume occupied by the sample (V) was recorded from cylinder's graduations. The apparent bulk density (ϱ) was calculated using the formula:

$$p = \frac{m}{V}$$

where density is expressed in g/cm³ (equivalent to g/mL). All measurements were conducted at standard laboratory ambient temperature (20 ± 2°C).

2.4.2. Electrical Conductivity Determination

Electrical conductivity was measured using a calibrated conductivity meter (Thermo Orion, AUTO-READ function) with automatic temperature compensation. The instrument was calibrated using standard conductivity solutions (84 µS/cm and 1,413 µS/cm at 25°C) before each measurement session.

Aqueous extracts were prepared following the same protocol described for pH determination (3.0 g sample + 45 mL distilled water, shaking 30 min, centrifugation 5 min at 3,000 rpm). The conductivity probe was immersed in the clarified supernatant, and readings were recorded after stabilization. Results are expressed in microsiemens per centimeter (µS/cm) at the measurement

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temperature (20 ± 2°C). The electrode was rinsed thoroughly with distilled water between measurements.

2.4.3. pH Determination

pH measurements were performed using a calibrated digital pH meter (Thermo Orion, AUTO-READ function with timed stabilization intervals). The instrument was calibrated daily using standard buffer solutions at pH 4.00, 7.00, and 10.00 (NIST-traceable) before each measurement session.

For solid residue samples, aqueous extracts were prepared by mixing 3.0 g of homogenized sample with 45 mL of distilled water (1:15 w/v ratio) in 50 mL centrifuge tubes. Samples were homogenized using an orbital shaker (ISOCIDE) at 200 rpm for 30 minutes at room temperature (20 ± 2°C), then centrifuged (ORTO Alresa centrifuge) at 3,000 rpm for 5 minutes. The supernatant (approximately 35 mL) was decanted into clean beakers, and the pH electrode was immersed directly in the supernatant. pH readings were recorded after signal stabilization (typically 30–60 seconds). The electrode was rinsed with distilled water and blotted dry between measurements.

2.4.4. Moisture Content and Dry Matter Determination

Moisture content of residue samples was determined by thermogravimetric analysis using a halogen moisture analyzer (RADWAG Balance and Scales, precision ±0.001%). Approximately 3.0 g of homogenized sample were placed on the aluminum pan after instrument taring. The analysis was performed under controlled heating conditions (105°C) with automatic termination when mass loss stabilized (change <0.1 mg over 90 seconds), indicating complete moisture evaporation. Moisture content was recorded directly as percentage mass loss. Dry matter content was calculated by difference:

$$\text{Dry matter (\%)} = 100 - \text{Moisture (\%)}$$

2.4.5. Ash Content Determination

Ash content (total inorganic residue) was determined by dry ashing following AOAC Official Method 923.03 with minor modifications. Empty porcelain crucibles were pre-dried in a forced-air oven (ESCO Isotherm, max 300°C) at 100°C for 1 hour, cooled in a desiccator containing anhydrous silica gel, and weighed to obtain tare mass (W_1).

Approximately 3.0–5.0 g of homogenized sample was accurately weighed into pre-tared crucibles (W_2 = mass of crucible + sample). Crucibles were initially heated gently on a hot plate under a fume hood (Fume Hood FH1000(X), 110 V ±10%) to volatilize organic compounds and prevent flaming, continuing until smoke emission ceased (typically 30–45 minutes). Subsequently, crucibles were transferred to a muffle furnace (AIKEN ROAD, max 1200°C) and subjected to incineration at 550 ± 25°C for 3 hours until a white or light gray ash residue was obtained, indicating complete combustion of organic matter. After ashing, crucibles were cooled to approximately 250°C in the furnace, then transferred to a desiccator and cooled to room temperature before final weighing (W_3 = mass of crucible + ash).

Ash content was calculated as:

$$Ash(\%) = \frac{(W^3 - W^1)}{(W_2 - W_1)]} \text{ x } 100$$

2.4.6. Organic Matter Determination

Organic matter content was calculated by difference, assuming complete recovery of inorganic material as ash:

$$\text{Organic matter (\%)} = 100 - \text{Ash (\%)}$$

2.4.7. Crude Fiber Determination

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Crude fiber content was determined using the ANKOM semi-automated fiber analysis system following the sequential acid-base digestion method (AOAC Official Method 978.10, modified). Approximately 0.5 g of dried, finely ground sample (particle size <1 mm) were accurately weighed (W_2) and placed in heat-sealed filter bags (ANKOM F57, pre-weighed as W_1). Sealed bags were subjected to sequential digestion with 2.0 L of sulfuric acid solution (H_2SO_4, 0.255 N) followed by 2.0 L of sodium hydroxide solution (NaOH, 0.313 N) in the ANKOM fiber analyzer, with each digestion phase maintained at boiling temperature for approximately 40 minutes under continuous agitation.

Following digestion, bags were removed, rinsed thoroughly with hot distilled water (3 × 500 mL), then immersed in acetone for 10 minutes to remove residual fats and resins. Bags were dried in a forced-air oven at 105°C for 4 hours until constant mass, cooled in a desiccator, and weighed (W_3). Subsequently, bags containing dried fiber residue were transferred to pre-tared porcelain crucibles and ashed in a muffle furnace at 550°C for 3 hours. Crucibles were cooled in a desiccator and weighed (W_4).

Crude fiber percentage was calculated as:

$$\text{Crude Fiber (\%)} = \frac{(W^3 - W^4)}{W^2} x\ 100$$

where $W_3 - W_4$ represents the mass of organic fiber residue remaining after acid-base digestion.

2.4.8. Crude Protein Determination

Crude protein content was determined by the Kjeldahl method (AOAC Official Method 2001.11) using automated Kjeldahl digestion and distillation apparatus. Approximately 0.5 g of dried, finely ground sample were accurately weighed into Kjeldahl digestion tubes. Digestion catalyst mixture (2.0 g anhydrous potassium sulfate + 0.2 g copper sulfate pentahydrate) and 15 mL of concentrated sulfuric acid (95–98%, analytical grade) were added to each tube. Samples were digested at 420°C for approximately 2 hours until the digest became clear and colorless, indicating complete conversion of organic nitrogen to ammonium sulfate.

After cooling to room temperature, digests were quantitatively transferred to the distillation apparatus. Distillation was performed by adding 50 mL of sodium hydroxide solution (40% w/v) to liberate ammonia, which was steam-distilled into 50 mL of boric acid solution (2% w/v) containing mixed pH indicators (methyl red and bromocresol green). Distillation proceeded for 6 minutes, collecting approximately 150 mL of distillate. The ammonia trapped in boric acid was titrated with standardized hydrochloric acid solution (0.1 N) to a gray endpoint, with the volume of titrant recorded.

Total nitrogen content was calculated from the titration volume, and crude protein content was estimated using the nitrogen-to-protein conversion factor of 6.25 (assuming average nitrogen content of 16% in plant proteins):

$$\text{Crude Protein (\%)} = (\%\ \text{Nitrogen}) \times 6.25$$

2.4.9. Crude Fat Determination

Crude fat (total lipid) content was determined by an automated Soxhlet system (Gerhardt SOXTHERM SOX414) using n-hexane as the extraction solvent. Approximately 15 g of dried, ground sample were weighed and placed in cellulose extraction thimbles (Whatman, 33 × 80 mm).

Thimbles were placed in Soxhlet extraction apparatus connected to pre-weighed, dried round-bottom flasks (W_1) containing 250 mL of n-hexane (analytical grade, 95% purity). Extraction was performed under reflux conditions for 4 hours at a condensation rate of 5–6 drops per second, ensuring continuous extraction of lipophilic compounds.

Following extraction, thimbles were removed, and the solvent was recovered using a rotary evaporator (BÜCHI Rotavapor R-210) operated at 50°C and 60 rpm under reduced pressure until complete solvent removal. Flasks containing extracted fat were dried in an oven at 105°C for 1 hour to remove residual moisture and solvent, cooled in a desiccator, and weighed (W_2).

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Crude fat content was calculated as:

$$\text{Crude Fat (\%)} = \frac{(W_2 - W_1)}{\text{Sample mass}} \text{ x } 100$$

2.4.10. Total Phenolic Content Determination

Total phenolic content was determined using the Folin-Ciocalteu spectrophotometric method adapted from Singleton and Rossi [33]. Aqueous extracts were prepared by mixing 1.0 g of finely ground sample with 10 mL of distilled water, homogenizing for 30 minutes at room temperature, and centrifuging at 3,000 rpm for 10 minutes. The supernatant (200 µL) was mixed with 1.0 mL of diluted Folin-Ciocalteu reagent (1:10 v/v with distilled water) and allowed to react for 5 minutes. Subsequently, 800 µL of sodium carbonate solution (7.5% w/v) were added to neutralize the acidic conditions and develop the blue chromophore. The reaction mixture was incubated in darkness at room temperature for 2 hours, and absorbance was measured at 765 nm using a UV-Vis spectrophotometer (Thermo Scientific Evolution 201) against a reagent blank. Total phenolic content was quantified using a calibration curve prepared with gallic acid standards (0–500 µg/mL) and expressed as mg gallic acid equivalents per gram of dry sample (mg GAE/g). For the purposes of this study, results are reported as dimensionless units representing relative phenolic abundance based on the analytical response.

2.4.11. Tannin Content Determination

Tannin content was determined using a modified Folin-Ciocalteu method incorporating polyvinylpolypyrrolidone (PVPP) precipitation to differentiate condensed tannins from other phenolic compounds. Two parallel extracts were prepared: (1) total phenolics extract (as described in 2.5.10), and (2) non-tannin phenolics extract, prepared by treating the aqueous extract with 100 mg of PVPP, which selectively binds and precipitates tannins. After 15 minutes of mixing and centrifugation, the supernatant containing non-tannin phenolics was analyzed using the Folin-Ciocalteu method.

Tannin content was calculated by difference:

$$\text{Tannins} = \text{Total phenolics} - \text{non-tannin phenolics}$$

Results are expressed in dimensionless units representing relative tannin abundance based on analytical response.

2.4.12. Gelatinization Temperature Determination

Gelatinization temperature was determined by a simplified heating method. One gram of finely ground sample was mixed with 10 mL of distilled water in a glass beaker. The mixture was heated gradually on a temperature-controlled hot plate (ISOLAB) with continuous magnetic stirring at 200 rpm. Temperature was monitored using a calibrated thermometer (±0.5°C precision) immersed in the mixture. The gelatinization temperature was recorded as the temperature at which the mixture exhibited a noticeable increase in viscosity, indicated by resistance to stirring and visible thickening, signaling the onset of starch gelatinization. This parameter provides preliminary information on potential thermal processing requirements for residue utilization.

2.4.13. Soluble Solids Content (°Brix) Determination

Soluble solids content was measured using a digital refractometer (KEM RA-620) calibrated with distilled water (0.0 °Brix) and operated at ambient temperature (20–25°C). For solid samples, aqueous extracts were prepared by mixing 3.0 g of sample with 45 mL of distilled water (1:15 w/v), homogenizing with orbital shaking for 30 minutes, and centrifuging at 3,000 rpm for 5 minutes. A drop of clarified supernatant was placed on the prism surface, and the measurement was recorded after automatic temperature compensation. Results are expressed as degrees Brix (°Bx), representing

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the mass percentage of dissolved solids in the aqueous extract. The prism was cleaned with distilled water and dried with lint-free tissue between measurements.

2.4.14. Determination of caffeine by HPLC

Statistical analyses were performed to describe and evaluate the variability of the physicochemical parameters obtained from the guayusa residue fractions. Data are presented as mean ± standard deviation (SD) together with minimum, median, and maximum values to characterize the distribution of each parameter.

The normality of the data distribution was assessed using the Shapiro–Wilk test. Comparative analysis between the fine and coarse residue fractions was performed using an independent samples Student's t-test when normality assumptions were satisfied. Statistical significance was established at $p < 0.05$.

All statistical analyses were conducted using Jamovi statistical software (version 2.x).

2.5. Statistical Analysis

We report data as mean ± standard deviation (SD), supplemented with minimum, median, and maximum values for each parameter. Before comparing groups, we tested for normality using the Shapiro-Wilk test. For parameters with normal distributions, we ran independent-samples t-tests to compare fine and coarse residues; for non-normal data, we used Mann-Whitney U tests instead. We set the threshold for statistical significance at $p < 0.05$. All calculations and tests were performed in Jamovi (version 2.3, The Jamovi Project, Sydney, Australia).

To explore relationships among chemical variables, we computed Pearson correlation coefficients and visualized them with a correlation matrix. We also conducted Principal Component Analysis (PCA) to identify patterns in the multivariate dataset. Before running PCA, we checked sampling adequacy with the Kaiser-Meyer-Olkin (KMO) measure; values above 0.5 were considered acceptable for this exploratory analysis. We retained components with eigenvalues >1 and report the percentage of variance explained by each.

In addition, a Principal Component Analysis (PCA) with Varimax rotation was performed to explore the relationships among physicochemical variables and identify the main factors explaining variability among the samples.

3. Results

Within the Kallari Association, guayusa (Ilex guayusa) cultivation operates as part of smallholder agroforestry systems characteristic of Ecuador's Amazonian lowlands. Participating families integrate 50–100 guayusa trees per household into multi-strata polycultures alongside cacao, vanilla, and native fruit species. Trees enter productive harvest approximately two years after establishment, yielding 1.5–3.0 kg of fresh leaves per plant annually. Families conduct manual harvests three to four times per year, timing cycles to match regional rainfall patterns and plant phenology.

Harvested leaves are sun-dried or dried in cooperative facilities at 45–50 °C before being sold as raw material for herbal infusions and functional beverages. Farmgate prices for dried guayusa range from USD 2.50 to 4.00 per kilogram, fluctuating with leaf quality (color uniformity, particle size, absence of stem fragments) and seasonal market demand.

3.1. Quantification of Processing by Granulometry

We run nine replicate sieving trials, each starting with 160.0 g of dried, milled guayusa. Particle separation into coarse (>425 µm), intermediate (300–425 µm), and fine (<300 µm) fractions showed a consistent pattern (Table 1, Figure 1). The coarse fraction dominated, accounting for 108.00 ± 1.50 g that's 67.50 ± 0.94% of the total. The intermediate fraction was much smaller: 19.11 ± 0.78 g (11.94 ± 0.49%). Fine particles made up 32.89 ± 1.45 g, or 20.56 ± 0.91%.

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Variability across the nine trials was low. The coarse fraction had a coefficient of variation (CV) of just 1.4%, indicating very tight control. The intermediate and fine fractions showed slightly more scatter (CV 4.1% and 4.4%, respectively), but these values are still well within normal analytical limits. Essentially, about two-thirds of the material passes quality standards for export, while roughly one-third—intermediate plus fine—gets rejected for being too coarse or too fine, discolored, or containing stem bits.

Table 1. Granulometric distribution of processing residues.

Fraction	Size range	Mean mass (g)	SD (g)	CV (%)	Mean (%)
Coarse	>425 µm	108.00	1.41	1.31	67.50
Intermediate	300–425 µm	19.11	0.78	4.09	11.94
Fine	<300 µm	32.89	1.45	4.42	20.56

The coarse fraction represented the largest proportion of material, accounting for 67.5% of the total mass, while the intermediate and fine fractions represented 11.94% and 20.56%, respectively.

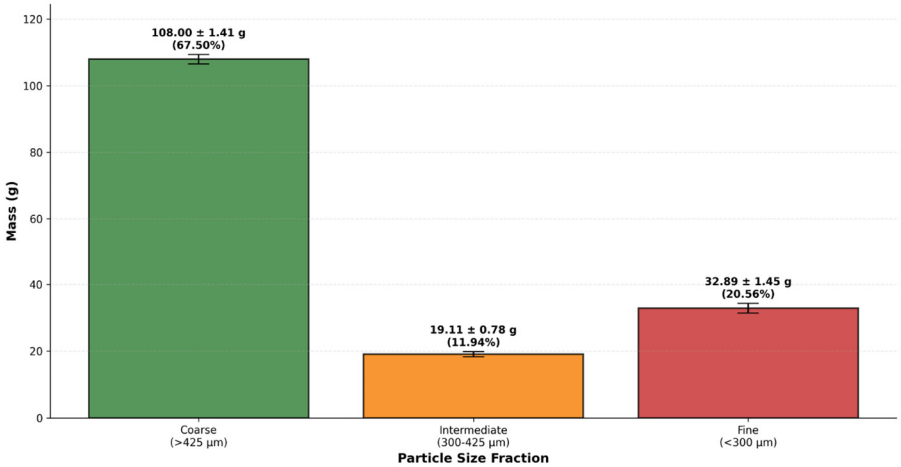

Figure 1. *Granulometric distribution of Guayusa Processing.*

3.2. Comparative Physicochemical Characterization

We analyzed 20 replicates each of intermediate-fine (<300 µm) and coarse (>425 µm) residues to determine whether particle size influenced chemical composition. Among the **twelve physicochemical parameters evaluated**, no statistically significant differences were observed between fractions (**$p > 0.05$**, Table 2). However, **crude fat content showed a marginal trend toward higher values in the fine fraction** ($p = 0.058$), approaching but not reaching the conventional significance threshold.

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Table 2. Physicochemical properties of fine and coarse guayusa residues (n = 20 per fraction).

Parameter	Coarse fraction	Fine fraction	*t*	*p*-value
Density (g cm^{-3})	0.255 ± 0.020	0.268 ± 0.017	−1.542	0.141
Conductivity (μS cm^{-1})	3.53 ± 0.38	3.66 ± 0.37	−0.805	0.431
pH	5.83 ± 0.02	5.85 ± 0.03	−1.223	0.237
Moisture (%)	6.54 ± 0.60	6.22 ± 0.88	0.943	0.358
Ash (%)	7.76 ± 0.19	7.63 ± 0.14	1.726	0.101
Organic matter (%)	92.24 ± 0.19	92.37 ± 0.14	−1.726	0.101
Crude fiber (%)	0.263 ± 0.029	0.250 ± 0.030	0.960	0.350
Protein (%)	1.60 ± 0.02	1.62 ± 0.03	−1.710	0.105
Crude Fat (%)	6.63 ± 0.14	6.78 ± 0.18	−2.022	0.058
Total phenolics	15.98 ± 1.76	15.70 ± 1.51	0.382	0.707
Tannins	9.95 ± 1.11	9.41 ± 1.13	1.064	0.302
Soluble solids (°Brix)	3.37 ± 0.39	3.41 ± 0.43	−0.221	0.828

Values are mean ± SD. t-values and p-values from independent-samples t-tests. No parameter reached statistical significance at $p < 0.05$.

Mean values for density, pH, moisture, ash, organic matter, fiber, protein, phenolics, tannins, and soluble solids were essentially the same across both fractions. Boxplot distributions (Figure 2) confirmed substantial overlap for all variables, reinforcing the statistical findings. Fat content showed a slight trend toward higher values in fine particles (mean 6.78% vs. 6.63%, $p = 0.058$), possibly due to enrichment of membrane lipids or waxes in finer cellular fragments, but this difference remained statistically non-significant.

Overall, these results indicate that granulometric separation does not substantially alter the chemical composition of guayusa residues. Both fractions retain similar levels of bioactive compounds, moisture, minerals, and macronutrients, supporting their interchangeable use in downstream valorization processes.

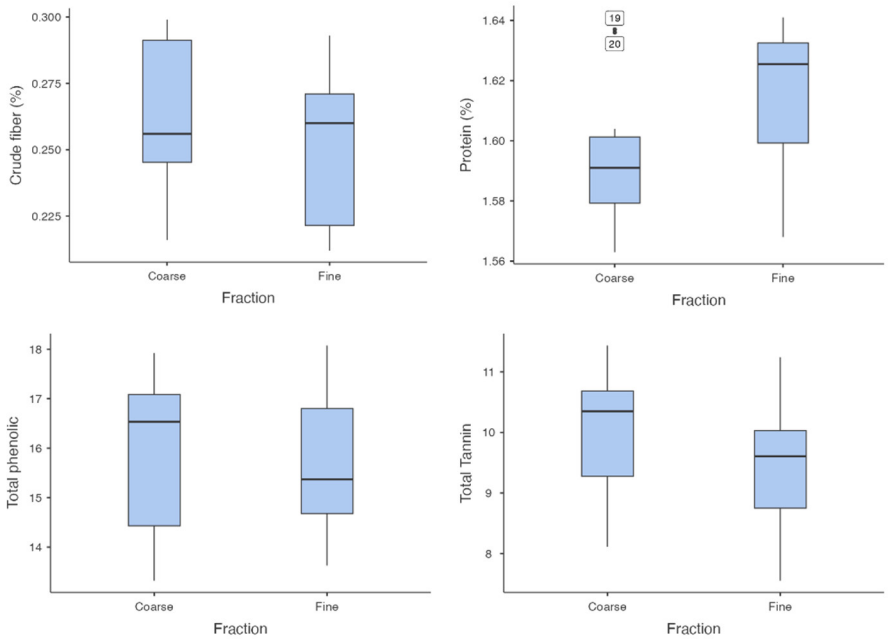

Figure 2. Boxplot comparison of selected physicochemical parameters between coarse (C) and fine (F) fractions. showing distributions for fat, protein, fiber, phenolics, tannins.

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3.3 Correlation Analysis of Chemical Components

We examined pairwise relationships among chemical variables using Pearson correlation coefficients (Figure 3). The correlation matrix revealed several noteworthy patterns. A statistically significant negative correlation emerged between protein content and tannin concentration ($r = -0.644$, $p = 0.002$), indicating that samples with higher protein tended to have lower tannins. This inverse relationship likely reflects the well-documented affinity of tannins for proteins tannins bind and precipitate proteins through hydrogen bonding, so their co-occurrence in extractable form is limited [19, 34]. From a biological perspective, this phenomenon explains why tannin-rich plant tissues often exhibit reduced protein digestibility.

Crude fiber showed a moderate positive correlation with tannin content ($r = 0.457$, $p = 0.043$), suggesting that tannins are associated with structural plant components. This makes sense: in leaf tissues, condensed tannins often accumulate in cell walls and vacuoles adjacent to fibrous matrices, serving dual roles as chemical defense compounds and structural reinforcement [35]. The co-localization of tannins with cellulose and lignin has been observed in many woody plant species, including other Ilex taxa.

Total phenolic compounds also showed a moderate positive correlation with tannins ($r = 0.523$, $p < 0.05$), as expected since tannins represent a major subclass of phenolic compounds. However, the correlation coefficient below 1.0 indicates that guayusa residues contain substantial quantities of non-tannin phenolics—likely chlorogenic acids, flavonoid glycosides, and hydroxycinnamic acid derivatives that vary independently of tannin concentrations [15, 20]

Other variable pairs fat vs. protein ($r = -0.234$), fat vs. phenolics ($r = -0.089$), protein vs. fiber ($r = -0.156$)—showed weak, non-significant correlations ($p > 0.05$), indicating that these components vary relatively independently within the analyzed samples. This compositional independence suggests that residue fractions may exhibit diverse chemical profiles suitable for different valorization pathways.

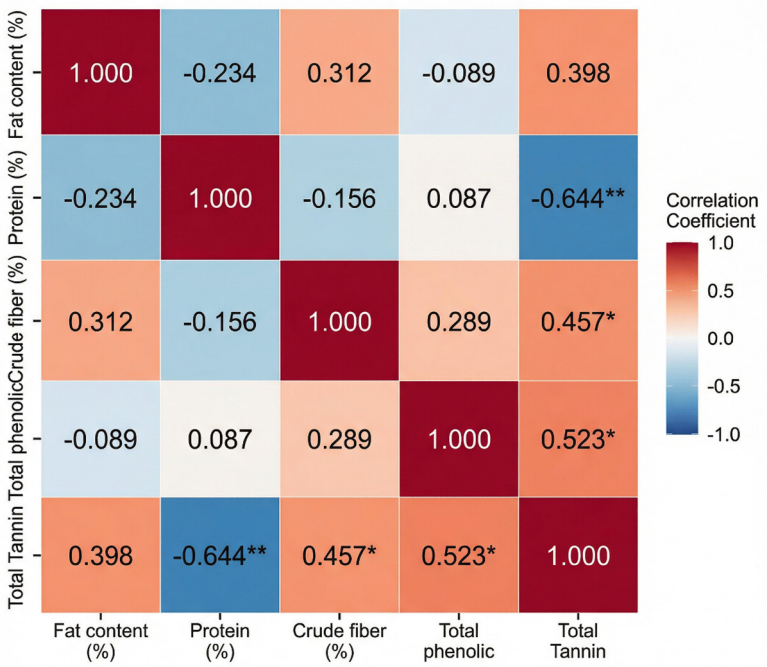

Figure 3. Correlation matrix (Pearson r) for chemical composition variables. Significant correlations ($p < 0.05$) are marked with asterisks.

3.4. Multivariate Analysis: Principal Component Analysis

To explore the underlying structure of the chemical dataset, we performed Principal Component Analysis (PCA). The Kaiser-Meyer-Olkin measure of sampling adequacy was 0.546, indicating

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moderate (though not ideal) suitability for factor analysis. We retained two principal components with eigenvalues >1, which together explained 62.8% of the total variance (Table 3).

Table 3. Eigenvalues and variance explained by principal components.

Component	Eigenvalue	Variance (%)	Cumulative (%)
PC1	2.030	40.60	40.60
PC2	1.111	22.21	62.80
PC3	0.964	19.28	82.10

The first component (PC1) accounted for 40.6% of variance and was strongly associated with fat content (loading 0.998), tannins (0.847), and crude fiber (0.706), while protein showed a strong negative loading (−0.849). This suggests that PC1 captures a "structural-phenolic axis"—samples high in fat, fiber, and tannins tend to be low in protein, consistent with the negative protein-tannin correlation discussed above.

The second component (PC2) explained 22.2% of variance and was dominated by total phenolic compounds (loading 0.908). This indicates that phenolic content varies somewhat independently of the tannin-fiber-fat cluster, likely reflecting different classes of polyphenols (chlorogenic acids, flavonoids) that don't always co-occur with condensed tannins.

Table 4. Rotated component matrix.

Variable	PC1	PC2
Fat content	0.998	—
Protein	−0.849	0.279
Crude fiber	0.706	0.502
Total phenolics	−0.304	0.908
Total tannins	0.847	0.283

The PCA biplot (Figure 4) reveals the multivariate relationships among chemical components. The first principal component (PC1, horizontal axis) represents a 'structural-phenolic axis' where fat content, crude fiber, and tannins cluster together (loading toward positive PC1), while protein loads negatively. This inverse relationship between protein and tannins ($r = -0.644$, $p = 0.002$) reflects the well-documented affinity of tannins for proteins, resulting in precipitation complexes that reduce soluble protein availability. The co-occurrence of fat, fiber, and tannins along PC1 suggests that condensed tannins accumulate preferentially in lipid-rich cell membranes and cell wall matrices in guayusa leaves.

The second component (PC2, vertical axis) captures an independent dimension of variation dominated by total phenolic compounds (loading 0.908). The near-orthogonal separation between PC1 tannins and PC2 phenolics indicates that these two phenolic fractions vary independently, likely reflecting distinct chemical classes: PC1 represents condensed proanthocyanidins bound to structural components, while PC2 captures soluble hydroxycinnamic acids (e.g., chlorogenic acid derivatives) and flavonoid glycosides that do not co-localize with the tannin-fiber-fat complex. This compositional heterogeneity suggests that guayusa residues may serve dual valorization pathways: tannin-rich structural fractions for antimicrobial coatings or natural preservatives, and phenolic-rich soluble fractions for antioxidant extracts or functional food ingredients.

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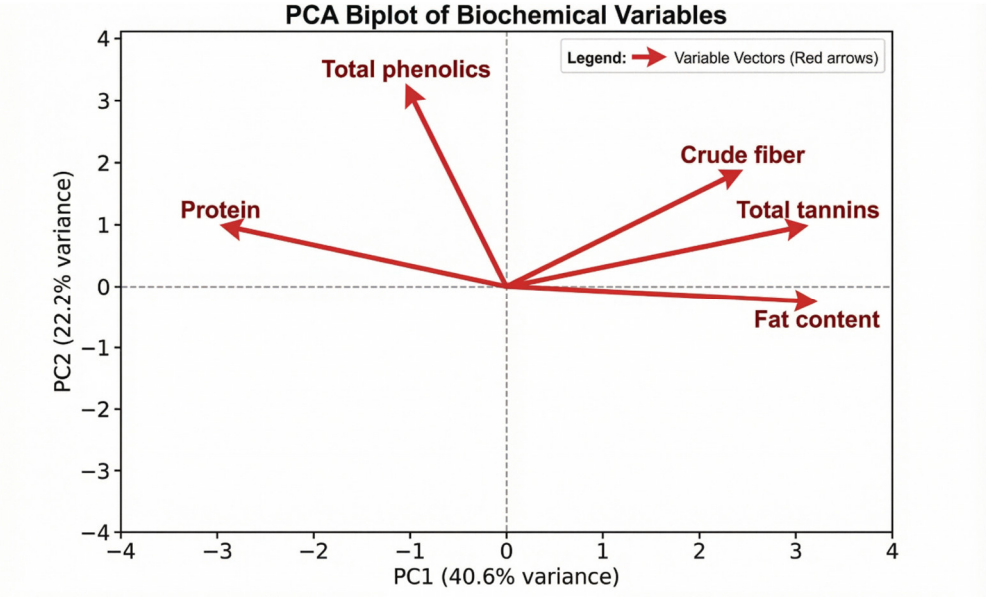

Figure 4. *Principal Component Analysis.*

3.5. Caffeine Content

We quantified caffeine in two representative residue samples using reversed-phase HPLC with UV detection at 272 nm. Chromatographic peaks appeared at retention times around 7.5 min, matching the calibration standards. Sample 1 (fine fraction) contained 20.91 mg/g caffeine (2.09% w/w), while Sample 2 (coarse fraction) contained 18.88 mg/g (1.89% w/w). The difference between samples—approximately 10.7%—likely reflects biological variability among source leaves or slight batch-to-batch variation during processing.

Table 5. Caffeine concentration in guayusa processing residues.

Sample	Caffeine (mg g^{-1})	Caffeine (% w/w)
Fine fraction	20.91	2.09
Coarse fraction	18.88	1.89

These concentrations represent 55–70% retention relative to published values for whole dried guayusa leaves (typically 2.5–3.5% caffeine) [16,20]. Given that caffeine is water-soluble and some extraction may occur during humid processing and handling, retaining nearly two-thirds of the caffeine in rejected residues is notable. At ~2% caffeine, these residues could serve as natural caffeine sources for functional beverages, energy bars, or nutraceutical extracts applications where consumers increasingly prefer plant-derived over synthetic caffein.

4. Discussion

This study provides the first comprehensive physicochemical characterization of guayusa processing residues generated during industrial leaf processing at Kallari cooperative in the Ecuadorian Amazon. Our work addresses a critical knowledge gap in the emerging guayusa value chain by analyzing the chemical composition, bioactive compound retention, and compositional relationships in materials that result from particle size classification during commercial processing.

Three principal findings from our analyses. First, granulometric separation generated three distinct particle-size fractions: coarse material (>425 μm) comprising 67.5% of processed biomass, intermediate particles (300–425 μm) representing 11.9%, and fine powder (<300 μm) accounting for 20.6%. While export markets preferentially demand finer powder fractions suitable for tea bag applications, our data demonstrate that particle size alone does not determine valorization potential. Second, comparative physicochemical analysis of fine and coarse fractions revealed identical

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chemical composition across all measured parameters ($p > 0.05$). This compositional uniformity indicates that coarse particles often considered lower-value material for certain applications retain the same nutritional and bioactive profile as fine powder, challenging assumptions that particle size correlates with chemical quality. Third, guayusa processing fractions retained substantial bioactive compounds: caffeine concentrations averaged 1.89–2.09%, total phenolic content measured 15.7–16.0 units, and tannins averaged 9.4–10.0 units, demonstrating that industrial handling did not substantially degrade these compounds.

The retention of bioactive compounds in processed guayusa fractions substantially exceeds patterns documented for other botanical commodities. Wise and Negrin [19] reported caffeine concentrations of 2.9–3.5% in whole dried guayusa leaves, while Cadena-Carrera, Tramontin [17] documented ranges of 2.4–3.0%. Our values of 1.89–2.09% represent 55–70% retention, which compares favorably to spent coffee grounds that typically retain only 15–25% of original caffeine after brewing [28].

The comparison with yerba mate (Ilex paraguariensis), the most closely related commercial Ilex species, provides additional context. Heck and De Mejia [36] characterized yerba mate processing waste and reported caffeine content of 1.2–1.6% in rejected leaf fractions, approximately 25–35% lower than our guayusa values. Additionally, moisture content in guayusa fractions (6.2–6.5%) was lower than typical mate waste (8–10%), conferring better microbiological stability during storage. These differences may reflect species-specific leaf morphology, differential drying protocols, or inherent differences in secondary metabolite biosynthesis between the two Ilex taxa.

Our correlation analysis revealed patterns consistent with fundamental plant biochemistry. The strong negative correlation between protein and tannin content ($r = -0.644$, $p = 0.002$) aligns with research documenting tannin-protein interactions. Hagerman and Butler [35] demonstrated that proanthocyanidins form stable complexes with proline-rich proteins through multiple hydrogen bonds, effectively precipitating proteins from solution. Siebert, Carrasco [37] showed that this interaction depends on tannin molecular weight, degree of galloylation, and protein conformation.

The moderate positive correlation between crude fiber and tannin content ($r = 0.457$, $p = 0.043$) suggests co-localization of phenolic compounds with structural cell wall components. Barbehenn and Constabel [38] documented that condensed tannins in woody plant leaves frequently accumulate in cell walls and vacuoles adjacent to fibrous matrices, serving dual functions as chemical defense and structural reinforcement. In guayusa, this association may explain why tannin extraction efficiency varies with milling intensity finer particles with disrupted cell walls would theoretically release bound tannins more readily than coarse fragments with intact structural matrices.

Principal Component Analysis provided additional insight into compositional patterns. The first principal component (40.6% variance) showed a "structural-phenolic axis" where fat content, crude fiber, and tannins clustered together while protein loaded negatively, reinforcing the inverse protein-tannin relationship observed in correlation analysis. The second component (22.2% variance) was dominated by total phenolic compounds, indicating that phenolic content varies somewhat independently of the tannin-fiber-fat complex. This separation likely reflects different polyphenol classes: condensed proanthocyanidins (tannins) physically bound to cell walls and membranes (loading on PC1), versus soluble hydroxycinnamic acids such as chlorogenic acid derivatives and flavonoid glycosides (loading on PC2) that do not co-localize with structural components. Together, these two components explained 62.8% of total variance, suggesting that most compositional variation in guayusa fractions can be described by these dual patterns.

From a circular economy perspective, our findings demonstrate that guayusa processing generates fractions with uniform bioactive content regardless of particle size, simplifying potential valorization strategies. For indigenous producer cooperatives like Kallari, this translates to practical opportunities: coarse fractions unsuitable for premium tea bags could supply functional food ingredient markets (guayusa flour for baked goods, natural caffeine sources for energy products), cosmetic applications (antioxidant serums, cellulite creams leveraging caffeine's lipolytic properties), or natural preservative formulations exploiting antimicrobial tannins. The compositional equivalence

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between fine and coarse fractions means that market segmentation can be driven by physical properties (particle size, flow characteristics, dissolution kinetics) rather than chemical composition, allowing cooperatives to match fractions to applications based on technical requirements rather than nutritional profiles.

Several methodological limitations warrant acknowledgment. Our analysis focused on aggregate measurements of total phenolics, tannins, and caffeine rather than identifying individual compounds through chromatographic separation. While this approach suffices for initial feasibility assessment of valorization potential, detailed HPLC-PDA-MS/MS profiling of specific chlorogenic acid isomers, proanthocyanidin composition, and minor bioactives would strengthen future work. Additionally, only two samples were analyzed for caffeine, limiting statistical inference about between-fraction differences; future studies should expand to 15–20 replicates per fraction.

Despite these limitations, our study makes several contributions. While multiple studies have examined whole-leaf composition, none have specifically analyzed materials generated during industrial particle-size classification. The rigorous statistical validation replicated measurements (n = 20 per fraction for most parameters), formal hypothesis testing, multivariate analysis establishes reliable baseline data with low coefficients of variation (1.3–4.4%) and perfect mass balance closure (100% recovery). By centering our investigation on Kallari, a smallholder indigenous cooperative managing traditional chakra agroforestry, we provide data directly applicable to Amazonian socio-ecological contexts where economic development must align with biodiversity conservation, cultural preservation, and community empowerment.

5. Conclusions

This study provides the first comprehensive physicochemical characterization of processing residues from Ilex guayusa in the Ecuadorian Amazon, demonstrating that these materials retain significant nutritional and bioactive compounds despite industrial processing. Fine and coarse fractions exhibited similar properties, with high organic matter content (>92%), moderate phenolic compounds (15.7-16.0), and favorable preservation characteristics (6.2-6.5% moisture). The quantification revealed that coarse residues constitute 67.5% of processing waste, representing a substantial opportunity for valorization. The retention of bioactive compounds, combined with favorable physicochemical properties, positions guayusa residues as promising candidates for gastronomic innovation, functional food development, and bioenergy applications. Integration of these valorization pathways into the Kallari cooperative model exemplifies circular bioeconomy principles adapted to Amazonian indigenous contexts, potentially enhancing economic returns while maintaining cultural and ecological sustainability within traditional chakra systems. Future research should focus on pilot-scale product development, comprehensive bioactivity assays, and sensory evaluation to translate these findings into commercially viable applications that benefit indigenous producer communities.

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