The Journal of Supercritical Fluids 229 (2026) 106801

Contents lists available at ScienceDirect

The Journal of Supercritical Fluids

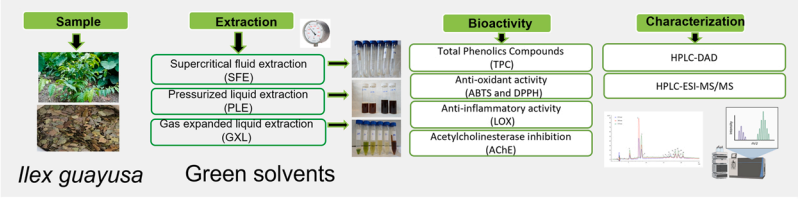
journal homepage: www.elsevier.com/locate/supflu

Towards sustainable extraction of bioactive compounds from guayusa (*Ilex guayusa*): A comparative study on compressed fluids' performance

Minxing Zhang [a,b], Lidia Montero [a], Jose A. Mendiola [a,*], Elena Ibáñez [a]

[a] Laboratory of Foodomics, Institute of Food Science Research, CIAL, CSIC-UAM, Madrid, Spain
[b] College of Agriculture and Forestry Science and Technology, Chongqing Three Gorges Vocational College, Chongqing 404155, China

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Compressed fluid extraction
Neuroprotective phytochemicals
Green solvents
Phenolic acids and flavonoids
Mixture experimental design

ABSTRACT

Ilex guayusa, a traditional Amazonian plant rich in caffeine and polyphenols, has attracted interest for its antioxidant and neuroprotective properties. This study evaluates the potential of green compressed fluid extraction techniques to recover bioactive compounds from *I. guayusa* leaves using environmentally friendly solvents: pressurized liquid extraction (PLE), gas-expanded liquids (GXL), and supercritical fluid extraction (SFE). A mixture design approach combining CO2, ethanol, water, and 2-methyltetrahydrofuran (2-MTHF) in different proportions as solvents was applied to optimize extraction conditions. Among the tested methods, GXL extraction yielded the highest phenolic content and demonstrated superior antioxidant (ABTS and DPPH) and anti-inflammatory (inhibition of lipoxigenase, LOX) activities. Conventional water maceration showed stronger cholinesterase inhibition, suggesting complementary neuroprotective potential. Chemical profiling (HPLC-DAD-ESI-MS/MS) of the optimized extract confirmed the presence of phenolic acids and flavonoids associated with the observed bioactivities. These findings highlight the effectiveness of compressed fluid-based green extraction for obtaining multifunctional phytochemicals from *I. guayusa*, with promising applications in functional foods and nutraceuticals.

1. Introduction

Ilex guayusa is a plant native to the Amazon basins of Ecuador, Colombia and Peru [1]; it belongs to the genus *Ilex* of the family *Aquifoliaceae*. It is traditionally cultivated in Amazonian chakra agroforestry systems, a diversified and sustainable land-use practice managed primarily by Indigenous communities. These chakra systems integrate culturally significant plants for food, medicine, and ritual, preserving both biodiversity and traditional ecological knowledge [2,3]. Within this context, *I. guayusa* has long been used as a medicinal plant by the indigenous Kichwa to help digestion, stimulate appetite, diuresis, clean blood and mouth, headache, treat diabetes and infertility, etc. [1,4,5]

* Corresponding author.
E-mail address: j.mendiola@csic.es (J.A. Mendiola).

https://doi.org/10.1016/j.supflu.2025.106801
Received 15 July 2025; Received in revised form 30 September 2025; Accepted 1 October 2025
Available online 6 October 2025
0896-8446/© 2025 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

but also as ritualistic plant by Jivaro Indians [6]. In a tea-like beverage, it is mainly made by picking and drying leaves [7]. Due to its health benefits and almost absence of toxicity [8], together with a mellow and unbitter taste, it has been widely promoted as a new type of healthy beverage in Europe and the USA in recent years [9–11]. Guayusa is well known to be a caffein-rich source [12,13], but also, this plant, contains other bioactive components with potential antioxidant, anti-inflammatory, antibacterial, and neuroprotective properties [14–18]. However, there is a limited understanding of these bioactive compounds, especially in terms of neuroprotection. Recently, studies have reported that phenolic compounds, mainly chlorogenic acids, in guayusa extract have certain potential for improving people's cognition, mood and motor-cognitive performance [19,20]. Within this context, *I. guayusa* is valued not only for its stimulant properties when consumed as a tea, but also for its broader role in community health, spiritual practices, and as part of local pharmacopoeias. This deep integration of cultivation and cultural significance underscores the importance of exploring *I. guayusa*'s bioactive compounds to support both traditional uses and potential new applications in ethnopharmacology.

Alzheimer's disease (AD) is the most prevalent neurocognitive disorder, characterized by cognitive dysfunction and declining behavioral ability, etc. AD incidence has grown rapidly in recent years, with the global number of reported dementia cases estimated to reach 74.7 million by 2030 and 131.5 million by 2050 [21]. The pathogenesis of AD is complex, involving multiple factors; among them antioxidant, neuroinflammation and cholinergic deficit [22] have been identified as possible target mechanisms and therefore, the search for bioactive compounds addressing these multiple factors has become one of the therapeutic strategies [22]. While several studies have explored the biological activities of plant-derived compounds [23–29], the present work focuses primarily on the optimization and comparative evaluation of compressed fluid-based extraction techniques for *Ilex guayusa*. The inclusion of in vitro assays (antioxidant, anti-inflammatory, and anti-cholinesterase) serves to characterize the functional potential of the extracts obtained under different extraction conditions, rather than to assess their therapeutic efficacy. This approach allows us to correlate extraction parameters with bioactive compound recovery, providing insights into the efficiency and selectivity of green solvent systems.

Green extraction techniques support sustainability by using eco-friendly solvents and energy-efficient processes. Compressed fluid technologies, supercritical fluid extraction (SFE), pressurized liquids (PLE), and gas-expanded liquids (GXL), enable precise control of extraction conditions while minimizing environmental impact [30]. SFE uses non-toxic CO_2 with tunable solvent strength [31,32]. PLE enhances efficiency by operating at conditions above solvent boiling points [31, 33]. GXL combines CO_2 with organic solvents to reduce viscosity and improve yield [34–38]. These methods effectively recover antioxidant and neuroprotective compounds [35,38–40].

In this study, we used four compressed green solvents, including CO_2, water, ethanol and 2-methyltetrahydrofuran (2-MTHF) to extract bioactive compounds from *Ilex guayusa* through different techniques, GXL, SFE and PLE. Recent studies have demonstrated the superior efficiency of 2-MTHF in recovering phenolic compounds and antioxidants from plant matrices, as well as its recyclability and environmental advantages compared to conventional solvents [41,42]. These properties make 2-MTHF, along with well-known green solvents, a suitable candidate for sustainable extraction processes, aligning with the goals of green chemistry and the scope of this study. Cadena-Carrera et al. [43] showed that SFE using CO_2 and ethanol as a cosolvent significantly enhanced antioxidant capacity and phenolic content, while also revealing antifungal activity and identifying key phytochemicals such as caffeine and squalene. More recently, Meneses et al. [44] applied a supercritical antisolvent process to encapsulate guayusa antioxidants, achieving caffeine reduction and improved recovery of chlorogenic acid. While previous studies have explored the use of compressed fluids for extracting phenolic compounds from various plant sources, including *Ilex guayusa,* they have typically focused on isolated techniques or limited bioactivity endpoints. In contrast, the present study aims to provide a comprehensive and comparative evaluation of three green compressed fluid-based extraction technologies (GXL, SFE and PLE) for the recovery of multifunctional bioactive compounds from guayusa leaves. The specific objectives of this work are: (i) to optimize solvent composition using a mixture design approach using CO_2, ethanol, water and 2-MeTHF; (ii) to assess extraction efficiency in terms of yield and total phenolic content of extracts; (iii) to evaluate the antioxidant (DPPH, ABTS), anti-inflammatory (LOX), and anticholinesterase (AChE, BuChE) activities of the extracts; and (iv) to identify key phenolic acids and flavonoids via HPLC-DAD-MS/MS and correlate them with observed bioactivities. Notably, this is the first study to report lipoxygenase (LOX), acetylcholinesterase (AChE), and butyrylcholinesterase (BuChE) inhibition in *Ilex guayusa* extracts obtained through compressed fluid extraction, linking these bioactivities to specific phenolic compounds identified via HPLC-MS and positioning our work as a novel contribution to both sustainable extraction technology and guayusa bioactivity research.Besides the sustainability of the process has been quantified using a Green Chemistry metric. This comprehensive comparison aimed to evaluate the potential of compressed fluids for extracting bioactive compounds from *Ilex guayusa*, providing insights for its future applications in antioxidant, anti-inflammatory, and neuroprotective therapies.

2. Materials and methods

2.1. Plant material

The *Ilex guayusa* samples were kindly donated by Fundación Comercio para el Desarrollo (Fundación COPADE). The samples were dried and minced leaves of *Ilex guayusa*, also containing small pieces of twigs, collected in Archidona, Napo Province, Ecuador. The particle size ranged from 0.2 to 2 mm, which is the typical range used for guayusa tea preparations. The sample was stored in opaque bags at 4 °C for experiments after the first unpacking.

2.2. Chemicals and reagents

2-Methyltetrahydrofuran (2-MTHF), ethanol absolute, and Folin-Ciocalteu's reagent were obtained from VWR chemicals (Fontenay-sous-Bois, France). Gallic acid (GA) was supplied by Alfa Aesar (Thermo Fisher scientific, Karlsruhe, Germany). Sodium carbonate (Na_2CO_3) and trizma hydrochloride (Tris-HCl) were purchased from VWR chemicals (Leuven, Belgium). 2,2'-azino-bis(3-ethilbenzothiazoline-6-sulphonic acid) (ABTS) and fluorescein sodium salt was purchased from Sigma Aldrich (St. Louis, MO, USA). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Sigma Aldrich (97 %, Moscow, Russia). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F), galantamine hydrobromide were purchased from TCI Chemicals (Tokyo, Japan). Lipoxidase (LOX) from Glycine max (soybean) was purchased from Sigma-Aldrich (Gillingham, United Kingdom). Phosphatidylcholine acetylcholinesterase (AChE) Type V1-S from *Electrophorus electricus*, butyrylcholinesterase (BuChE) from equine serum, acetylthiocholine iodide (ACth), butyrylcholine iodide (BCth), linoleic acid (LA), quercetin, potassium persulfate ($K_2S_2O_8$), disodium phosphate (Na_2HPO_4) and monopotassium phosphate (KH_2PO_4) were obtained from Sigma-Aldrich (Madrid, Spain). LC-MS-grade formic acid and LC-MS-grade acetonitrile (ACN) were purchased from VWR Chemicals (Lutterworth, United Kingdom). CO_2 (99.9 % purity) was purchased from Carburos Metálicos (Air Products Group, Madrid, Spain). Ultrapure MiliQ water was obtained from a Milipak® Express 40 system (Merk-Milipore, Darmstadt, Germany).

2.3. Compressed fluids extractions

To optimize the extraction conditions for bioactive compounds from *Ilex guayusa*, a mixture experimental design was applied using Response Surface Methodology (RSM). This design evaluated the influence of different solvent compositions by changing the ratios of CO_2, water, ethanol and 2-MTHF, that means, employing SFE, PLE and GXL, while maintaining constant temperature (50 °C) and pressure (100 bar). Twelve experimental conditions (Experiments 1–12, Table 1) were tested, where the solvent composition varied among supercritical CO_2, ethanol/water (1:1, *v/v*), and 2-MTHF. Each experimental condition, including the central point and all other design points, was performed in triplicate (n = 3). In the mixture design, the central point corresponds to three separate experimental runs (Experiments 7, 11, and 12), each of which was independently replicated three times. To further assess the impact of pressure in SFE, additional experiments (Experiments 13–16, Table 1) were conducted using pure CO_2 at pressures of 100, 200, and 300 bar, while maintaining a constant temperature of 50 °C. For all the extractions extraction time was 50 min and total solvent flow was 4 mL/min. Solvent ratios expressed as volumetric percentage (v%) measured in the pump head (room conditions for liquids, 50 bar, −2 ºC for CO_2). Taking into account that guayusa is typically consumed as tea, extractions with water at 75 °C were considered as benchmark control to compare the efficiency with compressed fluid techniques (M1 and M2, Table 1); for these maceration experiments 24 h were selected to ensure maximal compound diffusion, see 2.4 for details. This experimental setup allowed for a comprehensive comparison of compressed fluid extraction techniques, identifying optimal conditions for obtaining bioactive compounds from *Ilex guayusa* while maintaining a sustainable and environmentally friendly approach.

Extractions were carried out in a homemade extractor, equipped with an oven, CO_2 pump (PU-2080 Plus CO_2; Jasco, Hachioji, Japan) and a manual micro-metering needle valve (Vici-Valco Instruments Co. Inc., Houston, TX, USA). In addition, the liquid solvents were delivered to the system using a PU-2080 pump (Jasco, Hachioji, Japan). The flows (CO_2 and liquid) were combined in a stainless-steel tee before entering the pre-heater loop, as previously described [45]. The experimental procedure is as follows: for each experiment run, weigh 1.0 g of guayusa sample into the stainless-steel extraction cell (hand-tight homemade design, 25 mL). Each cell was packed from bottom to top with glass wool/ sample/ glass wool, to retain coarse plant residues while allowing efficient passage of dissolved compounds, minimizing clogging and ensuring consistent flow. As a result, the final extract was free of visible particulates, and no additional filtration was required. The pressure was set at 100 bar and the temperature at 50 °C. The pressure of 100 bar was chosen based on previous studies showing minimal influence of pressure on gas-expanded liquid (GXL) extraction performance [46]. The solvent consisted of a quaternary mixture of CO_2-ethanol-water-2-MTHF (depending on the experimental conditions, see Table 1) with a constant flow rate of 4 mL/min. The extraction temperature (50 °C) and pressure (100 bar) were selected to ensure operation above the critical point of pure CO_2, while remaining below the boiling points of the liquid co-solvents (ethanol, water, and 2-methyltetrahydrofuran). However, the actual phase state of the mixture depends on its specific composition and phase equilibria. We followed this strategy of mixture experimental design due to the lack of equilibrium data of the quaternary mixture of these solvents. Although phase equilibrium data are available for the CO_2–EtOH–H_2O system, the presence of 2-MTHF likely alters the phase behavior significantly. Under the extraction conditions used, it is probable that multiple phases (liquid, gas and supercritical) coexist in the extraction cell. However, a detailed equilibrium study of the quaternary mixture is beyond the scope of this work. A kinetic study was also performed for all the experimental runs, collecting fractions every 10 min, up to a total extraction time of 50 min. After each extraction, the system was flushed with CO_2 to avoid carryover. The solvent was removed using a gentle N_2 stream at 40 °C to obtain the dried extracts. All the dried extracts were protected from light with aluminum foil and stored at 4 °C until further analysis. The extraction yield (%) was calculated by dividing the weight of extract after drying by the total weight of the material before extraction (Equation 1)

Extraction yield (%) = dry extract mass (g) × 100 / dry initial mass (g) (1) (Equation 1)

The extraction yield, TPC, DPPH radical scavenging ability, ABTS antioxidant activity, AChE inhibitory activity, and LOX inhibitory activity were used as response variables to explore the optimal solvent composition.

Table 1
Experimental conditions for extracting bioactive compounds from *Ilex guayusa* using different compressed fluids techniques.

	#	CO_2 (v%)	Ethanol/water (1:1, *v/v*) (v%)	2-MTHF (v%)	Temp (ºC)	Pressure (bar)
GXL/SFE/ PLE	1	100	0	0	50	100
	2	0	100	0	50	100
	3	0	0	100	50	100
	4	50	50	0	50	100
	5	50	0	50	50	100
	6	0	50	50	50	100
	7	33.33	33.33	33.33	50	100
	8	50	25	25	50	100
	9	25	50	25	50	100
	10	25	25	50	50	100
	11	33.33	33.33	33.33	50	100
	12	33.33	33.33	33.33	50	100
SFE	13	100			50	200
	14	100			50	200
	15	100			50	300
	16	100			50	300
Maceration	M1	Water 100 %			75	1
	M2	Water 100 %			75	1

2.4. Conventional extraction (Maceration)

One gram of sample was weighed and mixed with 40 mL of water. The extraction was carried out at 75 °C for 24 h using a Thermomixer Comfort (Eppendorf SE, Hamburg, Germany). After extraction, the mixture was naturally filtered using filter paper with a pore size of 34–42 μm.The 24-hour extraction at 75 °C was selected to ensure complete solubilization and diffusion of target bioactive compounds from the plant matrix. This duration and temperature combination was based on preliminary trials showing that shorter times resulted in lower yields, while 75 °C provided optimal thermal energy to enhance mass transfer without degrading thermolabile compounds. For a comparison with other extraction techniques, the solvent was also removed by using a stream of nitrogen gas directed over the surface of the liquid. This technique accelerates evaporation by reducing the water vapour pressure on the surface. The use of nitrogen also minimizes oxidation of sensitive compounds during the process. Dried extract was stored in a refrigerator at 4 °C protected from light until use. This procedure was performed in duplicate, namely M1 and M2 in Table 1.

2.5. Total phenolic content (TPC)

TPC was determined using the Folin-Ciocalteu method, following the colorimetric method described by Koşar et al. [47]. Briefly, 600 μL of water was mixed with 10 μL of sample, and then 50 μL of Folin-Ciocalteu reagent was added. After waiting for one minute, 150 μL of 20 % (w/v) Na_2CO_3 was added, followed by the addition of 90 μL of water, and the solutions were finally shaken to mix. The solutions were incubated in the dark at room temperature (22–25 ºC) for 2 h. After the reaction was complete, the absorbance was read at 760 nm (BioTeck Synergy HT microplate reader, Winooski, VT, USA). The TPC results (mean of three replicates) were obtained by plotting a calibration curve for gallic acid

(concentration range 0.031–2 mg/mL) and expressed as gallic acid equivalents (mg GA/g extract).

2.6. In vitro bioactivity assays

2.6.1. Antioxidant capacity assay: DPPH and ABTS

DPPH assay followed the method of Brand-Williams *et al.* [48] with some modifications. First, a working solution (0.06 mM) of DPPH was prepared by diluting it in 100 % ethanol. Then 25 µL of the extracts at different concentrations were added to 975 µL of the DPPH working solution and mixed. A volume of 300 µL was transferred to a 96-well microplate and the initial absorbance (t = 0 h) was read at 516 nm (BioTeck Synergy HT microplate reader, Winooski, VT, USA). The absorbance was then measured again after 4 h of incubation in the dark at room temperature (22–25 ºC). The inhibition rate was calculated to obtain a linear response between 20 % and 80 %. The DPPH results (mean of three replicates) were expressed as the half-maximal inhibitory concentration (IC50 µg/mL).

ABTS assay was determined according to the method proposed by Sánchez-Martínez et al. [49]. Briefly, $ABTS^{+\bullet}$ radicals were prepared in the dark at room temperature (22–25 ºC) by reacting 5 mL of 7 mM ABTS with 88 µL of potassium persulfate stock solution for 16 h. Then, on the day of the test, a diluted ABTS working solution was prepared by dissolving 1 mL of $ABTS^{+\bullet}$ radical solution in 70 mL PBS 5 mmol. Next, 100 µL of extracts dissolved at different concentrations in pure EtOH and 250 µL of ABTS working solution were added to a 96-well microplate. After incubation at room temperature in the dark for 45 min, the absorbance at 734 nm was measured (BioTeck Synergy HT microplate reader, Winooski, VT, USA). The ABTS results (mean of three replicates) were expressed as the half-maximal inhibitory concentration (IC50 µg/mL).

2.6.2. Anti-inflammatory activity assay: LOX

LOX inhibitory assay was determined using a fluorescence assay based on enzymatic kinetics described by Whent *et al.* [50] and Sánchez-Martínez *et* al. [49]. In brief, 100 µL of extracts of different concentrations, 75 µL of fluorescein (2 µM) in 150 mM Tris/HCl buffer (pH 9), 75 µL of LOX (0.0208 U/mL in buffer), and 100 µL of linoleic acid (the concentration was calculated based on the Km value in EtOH) were added to a 96-well plate whose absorbance was measured at 485 nm ($\lambda_{excitation}$) and 530 nm ($\lambda_{emission}$) every minute for 15 min at 25 °C. Quercetin was used as positive control and negative control did not contain sample extract (concentration is 0). The LOX results (mean of three replicates) were expressed as the half-maximal inhibitory concentration (IC50 µg/mL).

2.6.3. Anti-cholinesterase activity assay: AChE and BuChE

Both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibition assays were measured by fluorescent enzyme kinetics based on Ellman's method and on the procedure by Sánchez-Martínez *et al.* [49]. Briefly, 100 µL of extracts of different concentrations, 100 µL of Tris-HCl buffer (pH 8), and 25 µL of AChE or BuChE (0.8 U/mL in buffer) were added to a 96-well plate, and the plate was incubated for 10 min in the dark at room temperature (22–25 ºC). Next, the 96-well plate was placed in a microplate reader with an automatic dispenser (Biotek, Winoski, VT, USA), and the device was set to automatically inject 25 µL of ABD-F (125 µ Mol in buffer) and 50 µL of ACth or BuCth (the concentration was calculated based on the Km value in ultrapure water) into each well. After the automatic addition, the reaction medium was mixed and the absorbance was measured at 389 nm ($\lambda_{excitation}$) and 513 nm ($\lambda_{emission}$) every 10 s for 15 min at 37 °C. Galantamine was used as positive control and negative control did not contain sample extract (concentration is 0). The results (mean of three replicates) were expressed as the half-maximal inhibitory concentration (IC50 µg/mL).

2.7. HPLC-DAD-ESI-MS/MS analysis

The GXL extract (concentration 0.1 mg/mL in ethanol and water (1:1, *v/v*)) was filtered with a 0.22 µm nylon filter before entering the chromatographic separation. A HPLC system Agilent 1200 series (Agilent Technologies, Palo Alto, CA, USA) equipped with an online degasser, binary pump, constant temperature chamber, autosampler and a diode array detector (DAD) was used. For the chromatographic separation, a Phenomenex Gemini NX-C18 column (150 mm × 2 mm, 3 µm) was selected. The mobile phase consisted of (A) water with 0.1 % formic acid and (B) acetonitrile with 0.1 % formic acid. The gradient elution program was as follow: 10 % B at 0 min, 40 % B at 25 min. Flow rate was 0.2 mL/min, while the column temperature was set at 30 ℃. Injection volume was 20 µL. For the detection of the compounds in the DAD, the selected wavelengths were 254 nm, 280 nm and 310 nm.

Mass spectrometry was coupled after the DAD. The mass spectrometer was a Bruker Ion Trap MS Esquire 2000 (Bruker Corp, Bremen, Germany). Using an ESI electrospray ionization source in negative ionization mode, with a nebulizer gas at 25 psi (1.72 bar), a dry gas flow rate of 10 L/min, a capillary temperature of 350 °C, a capillary voltage of 3500 V in an automatic MS/MS fragmentation, and scan range of 50–1200 *m/z*.

2.8. Statistical analysis

Design-Expert 13 software was used for optimization and statistical analysis of mixture experimental designs. The significance of model terms was evaluated by analysis of variance (ANOVA), employing the F-test. A significance level of $p < 0.05$ was used to determine statistical significance. All other statistical analyses were performed using IBM SPSS Statistics 25.0, and Origin 2024 was used for graphics.

2.9. Sustainability assessment using Path2green

To evaluate the environmental sustainability of the optimized compressed fluid extraction process, we applied the Path2Green metric, a semi-quantitative tool based on the updated 12 principles of green extraction [51]. This framework assesses the entire workflow, including solvent selection, energy input, scalability, waste generation, and post-extraction handling. Each principle is scored from −1 to + 1, reflecting its alignment with sustainable practices. The evaluation was performed using the Path2Green mobile application developed by Souza Mesquita et al. [51], which generates a radar chart to visualize strengths and areas for improvement, along with an aggregated sustainability score.

3. Results and discussion

3.1. Solvent mixture optimization

Kinetic studies were conducted as part of the optimization process for the mixture experimental design (Table 1, Experiments 1–12), with the entire extraction process lasting 50 min and extracts collected at 10-minute intervals. According to the definitions proposed by Jessop and Subramaniam [34], the experiments can be classified based on their extraction pressure, temperature, and solvent composition. Experiment 1 using neat CO_2 is SFE, experiments that use only liquid solvents (2, 3, and 6) are PLE, and the others are GXL. Fig. 1**a** illustrates the yield progression across the mixture experimental design (GXL/SFE/PLE) extractions, showing a rapid increase within the first 30 min, corresponding to the constant extraction rate (CER) period, followed by a slower but continuous rise in the final 20 min, corresponding to the falling extraction rate (FER) period. Even if the diffusion-controlled period (DC) is not reached, considering the slow increase in yield that can be expected after the FER, a total extraction time of 50 min was selected. This selection was confirmed by the average yield from the

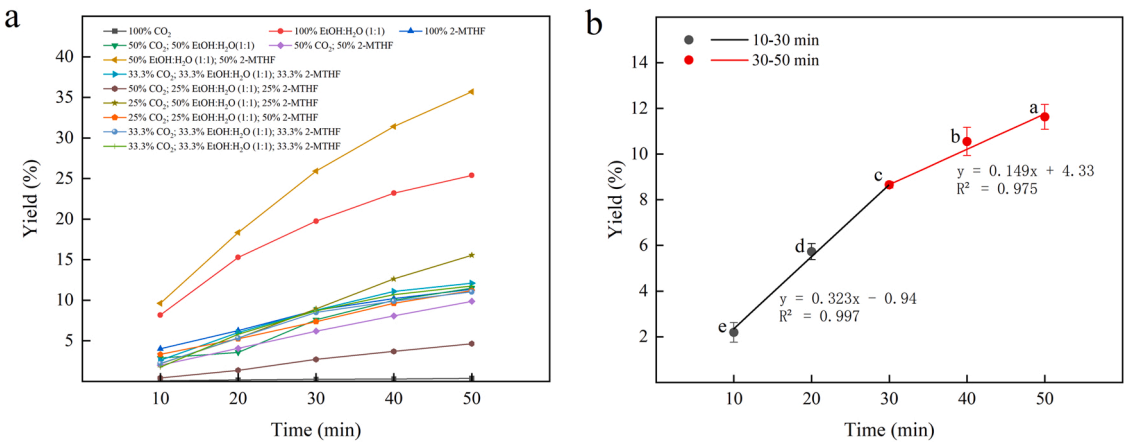


Fig. 1. (a) Kinetic extraction curves showing the yield progression of *Ilex guayusa* extracts under different solvent compositions (CO_2, ethanol/water, 2-MTHF) at 50 °C and 100 bar. (b) Extraction yield kinetics at the central point of the mixture design (33.3 % CO_2, 33.3 % ethanol/water, 33.3 % 2-MTHF), highlighting statistically significant differences ($p < 0.05$) at different time intervals. Results are expressed as mean ± SD (n = 3), with different letters indicating significant differences.

three central point replicates (33.3 % CO_2, 33.3 % ethanol/water (1:1, *v/v*), 33.3 % 2-MTHF) in the mixture design (Fig. 1**b**) that revealed statistically significant differences ($p < 0.05$) in yield at different time points up to 50 mins, although the slope of the curve in the FER period (0.149) was much smaller than the slope of the curve in the FER period (0.323) and therefore, further improvement cannot be expected after this extraction time. Although some extraction curves did not reach a complete plateau within 50 min, the extraction time was limited to 50 min to balance yield maximization with solvent and energy consumption. Extending the extraction time further would have resulted in only minor yield increases, while significantly increasing solvent use and processing time. This decision aligns with the principles of green extraction and sustainable process design. Based on these findings, a total extraction time of 50 min was selected for further analyses. For this purpose, new extractions were performed in which the entire extract was collected as a single fraction over the 50-min period. Since SFE (Table 1, Experiments 13–16) was performed using the same equipment, to maintain experimental consistency, extracts for SFE were also collected at 10-min intervals, with a total extraction duration of 50 min.

Response surface optimization was applied to the mixture experimental design to determine the optimal composition of the quaternary solvent system (CO_2, water, ethanol, and 2-MTHF) for extracting bioactive compounds from *Ilex guayusa*. Each solvent component was tested at varying proportions (0 %, 25 %, 33.3 %, 50 %, and 100 %) to evaluate their effects on key response variables, including extraction yield, TPC, antioxidant capacity (DPPH and ABTS), acetylcholinesterase inhibition (AChE), and lipoxygenase inhibition (LOX). The experimental results are summarized in Table 2.

To assess the significance and interactions of these variables, ANOVA of the quadratic regression models for each response variable was conducted, with results presented in Tables S1–S6 (Supplementary Materials). These analyses highlight the individual (linear) contributions and quadratic interactions of each solvent component on the measured bioactivities. Additionally, Fig. 2 illustrates the response surface plots for each response variable, visually depicting the trends and optimization patterns identified in the quadratic regression models. This approach enabled a comprehensive understanding of the solvent composition's influence on extraction efficiency and bioactivity, guiding the selection of optimal solvent composition for sustainable and effective extraction.

As can be seen in Table 2, solvents' composition significantly influenced yield, TPC, and bioactivity of the obtained *Ilex guayusa* extracts.

3.1.1. Extraction yield

As shown in Table 2, extraction yield for Experiments 1–12 ranged from 0.37 % to 35.69 %. Fig. 2**a** illustrates the response surface for extraction yield, where the highest yields were observed in areas close to Ethanol/water (1:1, *v/v*) and 2-MTHF, while the regions near pure CO_2

Table 2
Results of the mixture experimental design for extracting bioactive compounds from *Ilex guayusa* using compressed fluids. Each extraction was performed in triplicate (n = 3). Values are expressed as mean ± standard deviation (SD).

#	Experimental Factors			Response variables					
	CO_2 (v %)	Ethanol/water (1:1 v/v) (v%)	2-MTHF (v%)	Extraction yield (%)	TPC (mg GA/ g extract)	DPPH (IC50 μg/ mL)	ABTS (IC50 μg/ mL)	AChE (IC50 μg/ mL)	LOX (IC50 μg/ mL)
1	100	0	0	0.37 ± 0.04	n.d	n.d	n.d	417.74 ± 28.44	60.06 ± 6.5
2	0	100	0	25.38 ± 3.04	188.69 ± 0.45	38.14 ± 1.04	4.96 ± 0.05	125.81 ± 6.04	20.07 ± 1.94
3	0	0	100	11.36 ± 1.98	18.33 ± 0.88	194.8 ± 5.51	38.76 ± 0.58	88.81 ± 13.1	111.03 ± 10.91
4	50	50	0	9.50 ± 1.31	202.41 ± 3.81	11.66 ± 0.34	4.68 ± 0.15	110.02 ± 10.78	12.21 ± 0.96
5	50	0	50	9.87 ± 1.17	7.03 ± 0.2	319.63 ± 8.39	83.22 ± 2.42	110.79 ± 12.45	64.31 ± 3.89
6	0	50	50	35.69 ± 3.99	133.69 ± 5.1	21.36 ± 0.83	7.41 ± 0.06	117.5 ± 10.96	14.61 ± 0.36
7*	33.33	33.33	33.33	12.09 ± 1.99	167.92 ± 3.42	21.23 ± 0.05	6.15 ± 0.23	94.67 ± 1.54	25.5 ± 2.15
8	50	25	25	4.64 ± 0.61	109.91 ± 5	38.35 ± 0.96	9.67 ± 0.21	98.51 ± 4.83	12.58 ± 1.81
9	25	50	25	15.55 ± 2.17	198.36 ± 3.21	21.88 ± 1.94	5.83 ± 0.02	93.11 ± 8.15	26.94 ± 2.01
10	25	25	50	11.18 ± 1.82	163.35 ± 12.4	21.31 ± 0.91	6.65 ± 0.19	82.25 ± 6.06	17.26 ± 2.23
11*	33.33	33.33	33.33	11.03 ± 1.47	112 ± 10.01	25.57 ± 0.44	6.64 ± 0.15	84.91 ± 9.38	25.46 ± 2.17
12*	33.33	33.33	33.33	11.75 ± 1.88	168.44 ± 5.13	17.74 ± 1.48	5.99 ± 0.14	78.08 ± 9.68	30.62 ± 0.29

Extraction; v%, volumetric percentage* Central point of mixture experimental design; IC50 values represent the concentration required to inhibit 50 % of activity in antioxidant (DPPH, ABTS), anti-inflammatory (LOX), and neuroprotective (AChE) assays., higher activity means lower IC_{50}. n.d: not determined (maximum level of inhibition below 50 %). "n.d." indicates activity below 50 % inhibition threshold

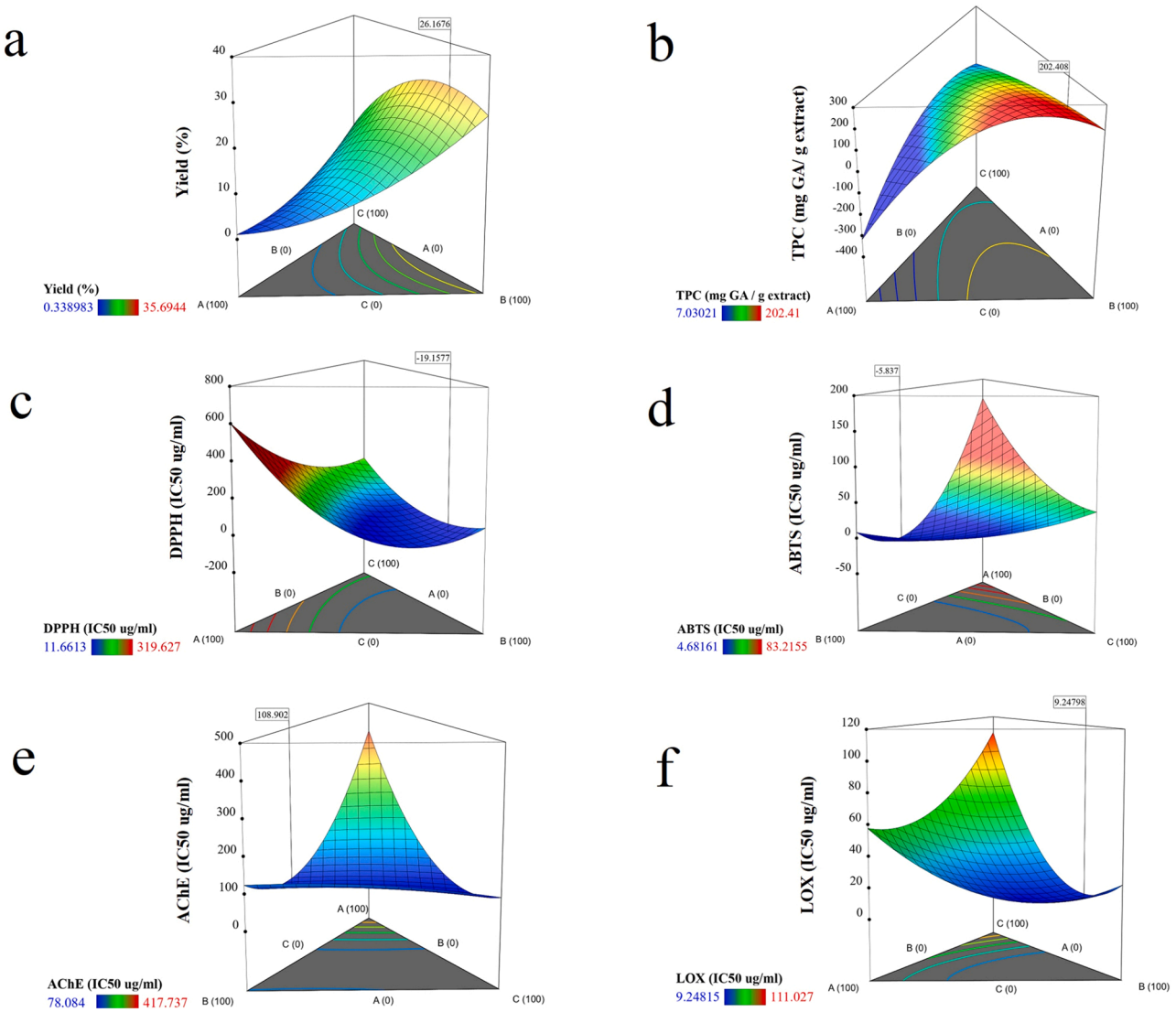


Fig. 2. Estimated response surface plots from the mixture design optimization showing the effect of solvent composition on: (a) Extraction yield (%), (b) Total phenolic content (TPC, mg GA/g extract), (c) DPPH radical scavenging activity (IC50 μg/mL), (d) ABTS antioxidant activity (IC50 μg/mL), (e) Acetylcholinesterase (AChE) inhibition (IC50 μg/mL), (f) Lipoxygenase (LOX) inhibition (IC50 μg/mL). A is the volumetric percentage of CO_2 in the mixture, B is the volumetric percentage Ethanol/water (1:1 v/v) in the mixture and C is the volumetric percentage of 2-methyltetrahydrofuran in the mixture.Each plot visualizes the influence of CO_2, ethanol/water, and 2-MTHF proportions on the respective bioactivity or yield, with blue regions indicating higher activity or yield and red regions indicating lower values. Negative values in the response surfaces are a result of the mathematical modeling and do not have physical meaning. All experimentally measured values were positive.

exhibited the lowest yields. The highest yield (35.69 %) was obtained with 50 % Ethanol/water (1:1, *v/v*) and 50 % 2-MTHF (Experiment 6), whereas the lowest yield (0.37 %) was recorded with 100 % CO_2 (Experiment 1). These results indicate that the ternary solvent system (Ethanol/water (1:1, *v/v*) + 2-MTHF) was the most effective for maximizing extraction yield, followed by the binary solvent system (Ethanol/water (1:1, *v/v*)), which achieved a 25.38 % yield. Analysis of variance (ANOVA) for extraction yield (Table S1) revealed that the quadratic regression model was statistically significant ($F = 9.19$, $p = 0.0036$), with strong contributions from the linear mixture terms ($F = 19.43$, $p = 0.0008$). This indicates that the individual solvent components (CO_2, ethanol/water, and 2-MTHF) had a substantial impact on extraction yield. Interaction terms (named as AB, AC, BC in Table S1) were not statistically significant ($p > 0.05$), suggesting limited synergistic effects between solvents. The model's goodness-of-fit was supported by $R^2 = 0.85$ and adjusted $R^2 = 0.76$, confirming its predictive reliability. Although the lack-of-fit was significant ($F = 18.09$, $p = 0.0079$), the low pure error (mean square = 2.12) indicates that the model still captures the main trends in the data. The regression equation derived from the model quantifies the influence of each solvent and supports the experimental observation that ethanol/water and 2-MTHF combinations yield the highest extraction efficiency. The ranking of solvent effects on yield was as follows: Ethanol/water (1:1, *v/v*)> 2-MTHF > CO_2. These results are consistent with previous reports [40] in which the combination of multi-solvents maximized the extraction yield compared to pure CO_2 extraction.

3.1.2. Total Phenolic Compounds (TPC)

The highest TPC (202.41 mg GA/g extract) was observed using 50 % CO_2, 50 % Ethanol/water (1:1, *v/v*) (Experiment 4) as extraction solvent, followed by 198.36 mg GA/g extract using 25 % CO_2, 50 % EtOH: H_2O (1:1) and 25 % 2-MTHF (Experiment 9). Interestingly, the highest extraction yield (35.69 %) did not correspond to the highest TPC (133.69 mg GA/g extract, experiment 6), indicating that CO_2 combined with an ethanol-water system was more effective for extracting phenolic compounds from *Ilex guayusa*. This phenomenon aligns with findings

from other studies. For example, Dauber et al. [52] demonstrated that the addition of olive leaf extracts (obtained using supercritical CO_2 modified with ethanol) to canola oil resulted in improved oxidative stability of the oil. This enhancement was attributed to the selective extraction of phenolic antioxidants present in olive leaves. Moreover, Modzelewska et al. [53] emphasized that a polarity index between 6 and 9 is optimal for phenolic compound extraction, with 50 % aqueous ethanol mixtures outperforming pure organic solvents in both yield and antioxidant activity. This supports the use of CO_2-ethanol-water mixtures in this study and explains why the highest TPC did not coincide with the highest extraction yield. Besides a dilution effect can be happening by extracting other non-phenolic compounds when increasing the yield.

ANOVA for TPC (Table S2) demonstrated that the quadratic regression model was highly significant ($F = 15.32$, $p = 0.0023$), confirming the strong influence of solvent composition on phenolic compound recovery. The linear mixture terms were particularly impactful ($F = 27.13$, $p = 0.001$), with ethanol/water showing the greatest positive contribution. Among the interaction terms, the AB interaction (CO_2 × ethanol/water) was statistically significant ($F = 11.57$, $p = 0.0145$), suggesting a synergistic effect that enhances phenolic extraction. The model exhibited excellent fit, with $R^2 = 0.93$ and adjusted $R^2 = 0.87$, indicating high predictive reliability. The lack-of-fit test was not significant ($p = 0.70$), and the pure error remained low, further validating model adequacy. The regression equation derived from the model supports the experimental observation that combining CO_2 with ethanol/water improves TPC yield, aligning with previous findings on the polarity-dependent solubility of phenolic compounds. These results highlight the advantages of GXL extraction for phenolic compound recovery. Previous reports [38,40] also indicated that the highest TPC can be achieved by combining pressurized solvents water and ethanol with CO_2 using GXL extraction. Furthermore, Ethanol/water (1:1, *v/v*) (Experiment 2, TPC: 188.69 mg GA/g extract) had significantly higher efficiency than pure 2-MTHF (Experiment 3, TPC: 18.33 mg GA/g extract), suggesting that guayusa phenolics are predominantly highly polar, in agreement with previous studies [43,54,55].

3.1.3. Antioxidant capacity

Antioxidant activity was evaluated using DPPH and ABTS assays, with lower IC50 values indicating stronger activity. As shown in Figs. 2**c** and 2**d**, blue areas correspond to higher antioxidant activity. Negative values in the response surfaces are a result of the mathematical modeling and do not have physical meaning, all experimentally measured values were positive. DPPH IC50 values ranged from 11.66 μg/mL (Experiment 4: 50 % CO_2; 50 % ethanol/water) to 319.63 μg/mL (Experiment 5: 50 % CO_2; 50 % 2-MTHF). Similarly, ABTS IC50 values ranged from 4.68 μg/mL (Experiment 4) to 83.22 μg/mL (Experiment 5). Notably, the antioxidant activity measured by DPPH was lower than that of ABTS, which can be attributed to differences in reaction mechanisms. The DPPH assay primarily detects polar antioxidants, whereas the ABTS assay detects both polar and non-polar antioxidants [39]. Despite these methodological differences, the highest and lowest antioxidant activities correlated with the highest and lowest TPC values.

ANOVA for antioxidant activity, measured via DPPH (Table S3) and ABTS (Table S4) assays, revealed that both models were statistically significant ($p < 0.01$), confirming the strong influence of solvent composition on antioxidant capacity. The linear mixture terms were significant in both cases (DPPH: $F = 10.28$, $p = 0.0115$; ABTS: $F = 7.99$, $p = 0.0203$), indicating that individual solvents (particularly ethanol/water) contributed substantially to radical scavenging activity. Interaction terms such as AB and BC showed moderate effects, with AB approaching significance in both models. The regression models exhibited good fit (DPPH: $R^2 = 0.88$, ABTS: $R^2 = 0.85$), comparable to the TPC model ($R^2 = 0.93$), suggesting a consistent relationship between phenolic content and antioxidant activity. Although the lack-of-fit was significant for both models, the low pure error values support their predictive reliability. These results align with Table S2, reinforcing the strong correlation between TPC and antioxidant capacity, and confirming that ethanol/water-rich solvent systems are optimal for extracting phenolic antioxidants from *Ilex guayusa*. The visual patterns in Figs. 2b, 2c, and 2d differ due to the rotated 3D perspectives used to enhance surface visibility. It is important to note that antioxidant activities are expressed as IC50 values, where lower values indicate higher activity, while TPC is expressed in concentration. Therefore, the higher TPC values correlate with the lower IC50 values for antioxidant activity. This inverse scale explains the apparent discrepancy. Despite visual differences, the underlying data show strong correlation between phenolic content and antioxidant capacity, which can be quantitatively seen in the mathematical models shown in Tables S2-4. A correlation analysis was conducted between TPC and antioxidant activity (DPPH and ABTS), revealing a Pearson correlation coefficient of 0.88 between TPC and DPPH, 0.85 between TPC and ABTS, and 0.99 between DPPH and ABTS. These findings reinforce the strong correlation between phenolic content and antioxidant capacity, aligning with previous reports [15,16,43]. Therefore, we can conclude that the main antioxidant compounds in guayusa are related to phenolic compound family.

3.1.4. Acetylcholinesterase (AChE) Inhibition

For AChE inhibition, lower IC50 values indicate higher inhibitory activity. In Fig. 2**e**, the blue region (high AChE inhibition) is concentrated near Ethanol/water (1:1, *v/v*) and 2-MTHF, whereas the red region (low inhibition) is near pure CO_2. The lowest IC50 (78.08 μg/mL, Experiment 12: 33.3 % CO_2; 33.3 % ethanol/water; 33.3 % 2-MTHF) suggests that a balanced solvent system enhances AChE inhibition. In contrast, the highest IC50 (417.74 μg/mL, Experiment 1: 100 % CO_2) demonstrates the limited efficacy of SFE alone in extracting cholinesterase-inhibiting compounds.

ANOVA results for AChE inhibition (Table S5) confirmed the high significance of the quadratic regression model ($F = 215.81$, $p < 0.0001$), with strong contributions from both linear mixture terms ($F = 262.92$, $p < 0.0001$) and interactions, particularly AB and AC ($p < 0.0001$). The model showed excellent fit ($R^2 = 0.99$; adjusted $R^2 = 0.99$), indicating near-perfect predictive accuracy. Although the BC interaction was not significant ($p = 0.14$), the overall model reliably captured the solvent effects. These findings support the experimental observation that combining ethanol/water and 2-MTHF enhances AChE inhibition in *I. guayusa* extracts. Solvent effects on AChE inhibition followed the order: 2-MTHF > Ethanol/water (1:1, *v/v*)> CO_2. These results highlight the advantage of GXL extraction over SFE and PLE for isolating AChE-inhibitory compounds. Hasni et al. [40] observed the most effective AChE inhibition during the extraction of another plant (*Eucalyptus marginata* Leaves), which was also achieved under GXL conditions (CO_2/ethanol (50:50, v/v)).

3.1.5. Lipoxygenase (LOX) Inhibition

For LOX inhibition, lower IC50 values indicate higher anti-inflammatory activity. As shown in Table 2, the lowest IC50 (9.25 μg/mL) was observed in Experiment 4 (50 % CO_2; 50 % ethanol/water), whereas the highest IC50 (111.03 μg/mL) was found in Experiment 3 (100 % 2-MTHF). These findings confirm that GXL extraction effectively enhances LOX inhibition, aligning with results from antioxidant and AChE assays. ANOVA for LOX inhibition (Table S6) confirmed the significance of the quadratic regression model ($F = 14.24$, $p = 0.0015$), with strong contributions from the linear mixture terms ($F = 21.66$, $p = 0.001$). Among the interaction terms, AB and BC were significant ($p < 0.05$), suggesting synergistic effects between CO_2 and ethanol/water, and between ethanol/water and 2-MTHF. The model showed good fit ($R^2 = 0.91$; adjusted $R^2 = 0.85$), supporting its predictive reliability. Although the lack-of-fit was significant ($F = 30.18$, $p = 0.0093$), the low pure error indicates the model adequately captures the main trends in LOX inhibition. The quadratic model had a high predictive accuracy ($R^2 = 0.91$, adjusted $R^2 = 0.85$). Previous studies have

demonstrated the anti-inflammatory potential of guayusa extracts using various in vitro models. Pardau et al. [14] showed that guayusa tea preparations reduced nitric oxide (NO) production by 10–30 % in LPS-stimulated RAW 264.7 macrophages, indicating moderate anti-inflammatory activity comparable to *Camellia sinensis* teas. Villacís-Chiriboga et al. [15] reported over 65 % inhibition of hyaluronidase activity in vitro, suggesting strong anti-inflammatory potential of aqueous and hydroalcoholic extracts. The GXL extract of Experiment 4 (50 % CO2; 50 % ethanol/water) showed the lower IC50 value (9.25 μg/mL), which could be comparable to previously reported values, but the bioactivity measurement methods are different. Our results align with previous findings [40], where GXL (CO_2/ethanol/water (25:50:25, v/v/v) yielded the most potent LOX inhibition in *Eucalyptus marginata* Leaves. These findings reinforce the relevance of guayusa as a source of anti-inflammatory agents, while highlighting the importance of extraction conditions in modulating bioactivity.

3.1.6. Multiple response optimization

To determine the optimal extraction conditions for maximizing bioactivity while maintaining high extraction efficiency, a multi-response optimization approach was applied. This method simultaneously optimized six response variables, aiming to maximize extraction yield and TPC while minimizing DPPH, ABTS, AChE, and LOX IC50 values, as lower IC50 values indicate higher bioactivity. This approach allows simultaneous optimization of multiple, potentially conflicting responses by identifying a compromise solution that balances the responses to produce a desirability function, which is mathematically combines individual responses into a single objective function. Equal weight was given to all six responses for optimization. The optimal conditions reported reflect the best compromise among all responses, rather than absolute maxima or minima [56].

The optimization analysis identified 6.7 % CO_2, 78.4 % ethanol water (1:1, *v/v*), and 14.9 % 2-MTHF as the most favorable solvent composition for achieving this balance. Under these conditions, the desirability index was 0.934, indicating a high degree of fitting of the model and optimization efficiency. The predicted values derived from the quadratic regression model under these optimized conditions are presented in Table 3, along with the corresponding experimental validation results.

To verify the accuracy and reliability of the model, three independent parallel experiments were conducted under the optimized conditions. The experimental values closely matched the predicted mean values, with all six response variables falling within the 95 % confidence interval (CI) range of the model predictions. For instance, the experimental extraction yield (23.23 ± 3.2 %) was well within the predicted range (19.97–32.37 %), and the experimental TPC (192.52 ± 20.35 mg GA/g extract) was consistent with the predicted mean (202.41 mg GA/g extract). Similarly, the DPPH (13.63 ± 0.45 μg/mL), ABTS (6.16 ± 0.16 μg/mL), AChE (104.65 ± 6.29 μg/mL), and LOX (9.06 ± 0.45 μg/mL) values demonstrated strong alignment with the model predictions.

Table 3
Predicted and experimental values under the optimal conditions (6.7 % CO_2, 78.4 % ethanol/water (1:1, *v/v*), 14.9 % 2-MTHF) using GXL mixture design. The results are expressed as the mean ± SD of three independent experiments.

Response Variables	Predicted Values			Experimental Values
	95 % CI low *	Predicted Mean	95 % CI high*	
Yield (%)	19.9675	26.1671	32.3668	23.23 ± 3.2
TPC (mg GA/ g extract)	166.849	202.409	237.969	192.52 ± 20.35
DPPH (IC_{50} ug/ mL)	−84.4484	−19.1655	46.1174	13.63 ± 0.45
ABTS (IC_{50} ug/ mL)	−23.8536	−5.83863	12.1764	6.16 ± 0.16
AChE (IC_{50} ug/ mL)	95.6144	108.898	122.182	104.65 ± 6.29
LOX (IC_{50} ug/ mL)	−6.83646	9.24703	25.3305	9.06 ± 0.45

* CI: Confidence Interval

These findings confirm that the quadratic regression model developed through the mixture experimental design is highly reliable and effective in predicting extraction outcomes. The optimized conditions not only achieved a strong balance between extraction efficiency and bioactivity but also demonstrated the practical applicability of the GXL extraction method for obtaining bioactive compounds with antioxidant, anti-inflammatory, and neuroprotective properties. This study further reinforces the potential of compressed fluid extraction with green solvents as an efficient and sustainable approach for the recovery of bioactive compounds from *Ilex guayusa*, in agreement with previous findings on green extraction processes [43,54,55].

3.2. Comparison of different extraction technologies

To further compare the optimized GXL extraction conditions obtained from the mixture design with other extraction techniques, additional experiments were conducted using SFE and conventional extraction methods. SFE was employed to assess the effectiveness of a pure, non-polar green solvent (CO_2) in extracting bioactive compounds. Besides maceration experiment with water was used as benchmark control. Results, presented in Table 4, highlight the significant impact of different technologies on the extraction efficiency and bioactive composition of *Ilex guayusa* leaves.

3.2.1. Extraction yield

Extraction yield varied significantly across techniques (Table 4). The highest yield was achieved with maceration (25.88 %) and optimized GXL (23.23 %) with no significant differences between them. The maceration yield aligns with previous findings (26.39 %, 96 h, 18 °C) [57].

SFE yielded the lowest values (0.37 % at 100 bar, 0.86 % at 200 bar, 1.43 % at 300 bar), as pure CO_2, being non-polar, poorly extracts polar phenolic compounds. Yield increased slightly with pressure, consistent with previous reports (3.08 % at 250 bar, 75 °C) [43]. Studies show that adding co-solvents improves extraction: 7 % ethanol increased yield to 6.02 %, and 7 % water boosted it to 32.01 % [43,57], while in our case we studied adding the mixture of ethanol:water in experiment 4 (Table 2) which yielded 9.50 %. The optimized GXL system (23.23 %), using CO_2, ethanol/water, and 2-MTHF, demonstrated how a ternary solvent mixture can bridge polarity gaps, significantly enhancing yield.

3.2.2. Total phenolic content (TPC)

The highest TPC was obtained with optimized GXL (192.52 mg GA/g), followed by maceration (110.1 mg GA/g). TPC was not determined for SFE due to low yield, but prior studies report 0.28–1.18 mg GA/g for pure SFE-CO_2 [43].

The GXL system outperformed all other techniques, even exceeding PLE (9.24–46.33 mg GA/g) and SFE with water as a co-solvent (28.34–43.25 mg GA/g) [57]. This confirms GXL's higher efficiency in extracting phenolic compounds, likely due to the synergistic effect of CO_2 expansion and the polar/non-polar balance of the quaternary system.

3.2.3. Antioxidant activities: DPPH and ABTS

The strongest antioxidant activity was observed under optimized GXL conditions (IC50 DPPH: 13.63 μg/mL; ABTS: 6.16 μg/mL), while maceration showed weaker activity. SFE failed to reach 50 % inhibition, consistent with previous results (DPPH IC50: 4370–8190 μg/mL) [43].

Interestingly, despite maceration yielding the highest extraction efficiency, its antioxidant capacity was lower than GXL, indicating that solvent composition is more critical than yield alone. Compared to prior studies (PLE IC50: 131.23–693.42 μg/mL; SFE + water IC50:

Table 4
Extraction yield, TPC, antioxidant activity (DPPH and ABTS), anti-inflammatory (LOX), and acetylcholinesterase enzyme inhibition (AChE and BuChE) of extracts obtained from *Ilex guayusa* using different extraction techniques.

Experiment	Extraction yield (%)	TPC (mg GA/ g extract)	DPPH (IC50 μg/mL)	ABTS (IC50 μg/mL)	LOX (IC50 μg/mL)	AChE (IC50 μg/mL)	BuChE (IC50 μg/mL)
GXL-Optimized	23.23 ± 3.2a	192.52 ± 20.35	13.63 ± 0.45	6.16 ± 0.16	9.06 ± 0.45c	104.65 ± 6.29c	199.68 ± 5.85a
SFE−200 bar	0.86 ± 0.15b		n.d	n.d	67.45 ± 22.49b	357.19 ± 36.12a	
SFE−300 bar	1.43 ± 0.39b		n.d	n.d	95.26 ± 16.5b	282.64 ± 38.87b	
Maceration	25.88 ± 0.27a	110.1 ± 8.67	23.84 ± 0.98	6.56 ± 0.38	141.74 ± 11.16a	62.35 ± 1.77c	95.99 ± 0.66b
Galantamine						0.98 ± 0.14d	36.19 ± 9.89c
Quercetin					8.48 ± 0.7c		

n.d: not determined (maximum level of inhibition below 50 %). a– d different letters indicate significant differences $p > 0.05$

109.25–167.95 μg/mL) [57], GXL extraction stands out as the most effective for extracting antioxidant compounds, even improving supercritical antisolvent fractionation (SAF) that showed an optimal DPPH IC50 equal to 510 μg/mL and an optimal ABTS IC50 equal to 180 μg/mL [44].

3.2.4. Anti-Inflammatory activity: LOX inhibition

This study is the first to report LOX inhibition for *Ilex guayusa* to date. The strongest inhibition was observed with optimized GXL (IC50: 9.06 μg/mL), comparable to quercetin (IC50: 8.48 μg/mL). Maceration showed the weakest inhibition (IC50: 141.74 μg/mL), while SFE had moderate to low activity. The anti-inflammatory activity of guayusa extracts has been shown to vary significantly depending on the extraction technique. For instance, Pardau et al. [14] used conventional water infusions and observed modest NO inhibition, while Villacís-Chiriboga et al. [15] employed hydroalcoholic extraction and reported stronger hyaluronidase inhibition. Our findings suggest GXL extracts contain compounds with potent LOX inhibitory activity, adding a new layer to guayusa's anti-inflammatory potential.

3.2.5. Anticholinergic activities: AChE and BuChE inhibition

This is also the first study to evaluate AChE and BuChE inhibition in *Ilex guayusa*. Maceration showed the strongest inhibition, followed by optimized GXL, with SFE showing the weakest activity. Notably, guayusa extracts exhibited stronger inhibition of AChE than BuChE, as reflected by lower IC50 values. In Alzheimer's disease (AD), acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) play complementary but distinct roles in cholinergic neurotransmission. While AChE activity declines significantly in advanced AD, BuChE activity increases, particularly in the cortex and hippocampus, shifting the AChE/BuChE ratio and contributing to cholinergic imbalance [28,58]. BuChE has also been implicated in amyloid-β aggregation and tau hyperphosphorylation, key pathological features of AD [59]. Selective BuChE inhibitors have shown promise in reducing neuroinflammation and improving cognitive performance in animal models, making BuChE a relevant therapeutic target, especially in late-stage AD. Therefore, evaluating both enzymes provides a more comprehensive understanding of the neuroprotective potential of guayusa extracts.

Although GXL extracts (AChE IC50: 104.65 μg/mL; BuChE IC50: 199.68 μg/mL) were less potent, of course, than the drug used as positive control galantamine (AChE IC50: 0.98 μg/mL), the activity was still significant, suggesting potential as a natural neuroprotective agent.

The observed LOX and cholinesterase inhibitory activities were strongly influenced by the solvent composition used during extraction. Our mixture design revealed that combinations rich in ethanol/water and moderate in 2-MTHF significantly enhanced these bioactivities, as confirmed by the regression models (Tables S5 and S6). This suggests that solvent polarity and specific interactions play a key role in recovering compounds with neuroprotective and anti-inflammatory potential. Although these activities are reported here for the first time in *Ilex guayusa*, their correlation with extraction parameters was statistically validated and mechanistically supported by the presence of phenolic acids and flavonoids identified in the optimized extract. These compounds are known to contribute to both LOX and cholinesterase inhibition, reinforcing the link between extraction strategy and functional outcomes.

3.3. Chemical characterization

The GXL optimized extract (6.7 % CO_2, 78.4 % ethanol/water (1:1, *v/v*), 14.9 % 2-MTHF) from *Ilex guayusa* was analyzed using HPLC-DAD-ESI-MS/MS in negative ionization mode. The phenolic compounds present in the guayusa extract were tentatively identified based on the obtained UV–vis spectra as well as the MS spectra and fragmentation patterns, combined with existing references. Results are shown in Table 5 and Fig. 3. A total of 16 compounds were detected, with 11 tentatively identified as phenolic compounds. Among the 11 phenolic compounds, 6 belong to phenolic acids and 5 belong to flavonoids.

Three caffeoylquinic acid isomers (CQA) were detected in the optimized GXL extract form *Ilex guayusa*, namely peaks 1, 3 and 4. The $[M–H]^-$ of these compounds was *m/z* 353.7. The characteristic fragmentation information of these three peaks was the same, specifically the main fragments were found at *m/z* 191, 179, 173, and 135. The characteristic fragments corresponded to neutral losses of 162 Da ([M-caffeoil]$^-$), 174 Da ([M-quinic acid]$^-$), 179 Da ([M- caffeic acid]$^-$) and

Table 5
Analysis of bioactive components in optimized GXL *Ilex guayusa* extract by HPLC-DAD-ESI-MS/MS.

Peak ID	RT (min)	$[M-H]^-$	M/MS fragments	λ max (nm)	Proposed compound
1	5.4	353.7	191, 179, 135	324,296sh	Chlorogenic acid isomer
2	7.9	479.2	386, 291, 197	272	Unidentified, caffeine?
3	8.6	354.2	191, 179, 135	326,296sh	Chlorogenic acid isomer
4	8.9	353.7	191, 179, 135	324,296sh	Chlorogenic acid isomer
5	15.3	609.2	301, 271	253, 343	Quercetin rutinoside
6	16.2	609.2	301, 271	256, 343	Quercetin rutinoside
7	16.6	593.3	285, 255, 227	254, 351	Kaempferol-3-O-rhamnoside
8	16.7	464.0	301, 271	254, 351	Quercetin hexoside
9	17.1	464.1	301, 271	254, 351	Quercetin hexoside
10	18.1	623.3	315, 301, 271	326, 296sh	Unidentified
11	18.3	516.3	353, 191, 179, 173	326, 296sh	Dicaffeoylquinic acid isomer
12	18.4	623.3	315, 301, 271	326, 296sh	Unidentified
13	18.9	539.3	497, 377, 335, 317	326, 296sh	Unidentified
14	19.2	515.6	353, 191, 179	326, 296sh	Dicaffeoylquinic acid isomer
15	19.4	563.1	435, 399, 284 254	326, 296sh	Unidentified
16	20.5	515.6	353, 191, 179, 173	326, 296sh	Dicaffeoylquinic acid isomer

sh: shoulder in the uv–vis spectrum

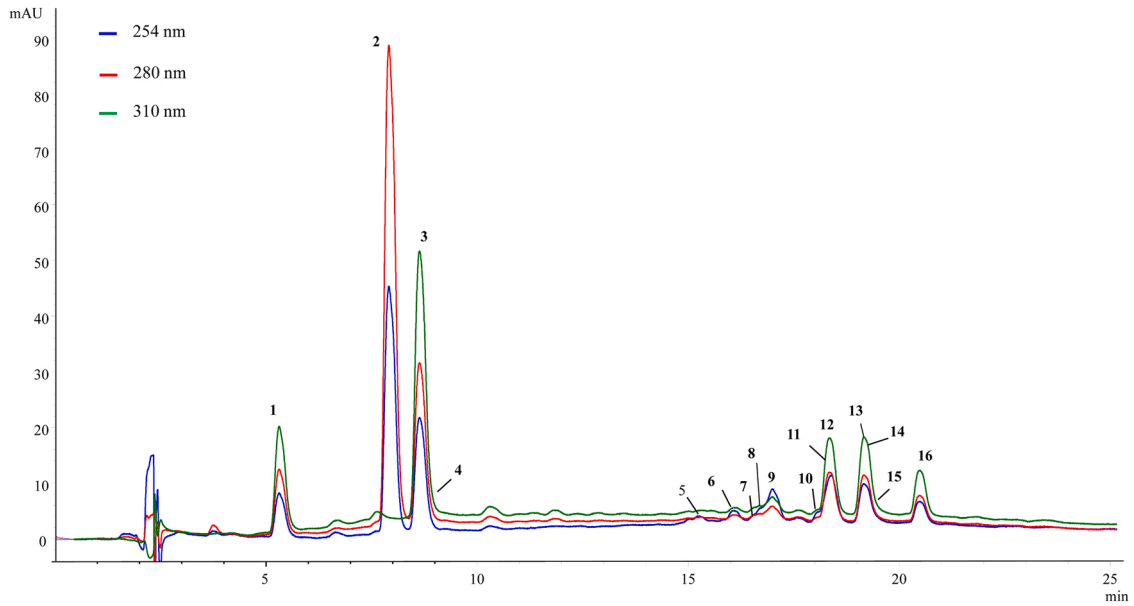


Fig. 3. HPLC-DAD chromatogram of the optimized GXL extract of *Ilex guayusa* (6.7 % CO_2, 78.4 % ethanol/water, 14.9 % 2-MTHF). Peaks correspond to phenolic acids and flavonoids tentatively identified via MS/MS analysis.

44 Da (M-quinic acid -CO_2]$^-$), respectively and it is inferred that these three peaks are likely isomers of caffeoylquinic acid. Based on their retention times and polarity differences, and by comparing with references [14–16,60], it could be suggested that peak 1 belong to neochlorogenic acid, peak 3 to chlorogenic acid, and peak 4 to cryptochlorogenic acid.

Four dicaffeoylquinic acid related compunds (diCQAs) were also detected in the extract, namely peaks 11, 14 and 16. The [M–H]$^-$ for these compounds was observed at *m/z* 515.6. These three peaks share similar characteristic fragmentation spectra formed by the ions at *m/z* 353, 191, 179. These characteristic fragments are generated through neutral losses of 162 Da ([M- caffeoil]$^-$), 324 Da (([M- 2 caffeoil]$^-$), and 136 Da ([M-quinic acid]$^-$), respectively. Comparing with references and retention order [9–11,45], peak 11 could be tentatively identified as 3, 4-diCQA, peak 14 as 3,5-diCQA, and peak 16 as 4,5-diCQA.

Peaks 5, 6, 7, 8 and 9 in Table 5 have been identified as flavonoids [14–16,60]. Specifically, peaks 5 and 6 were tentatively identified as stereoisomers of quercetin rutinoside. The characteristic fragment information of the MS/MS spectra was the same for both peaks (*m/z* 301, 300, 271). Peak 7 is inferred to be kaempferol rhamnoside, with a quasi-molecular ion [M-H]$^-$ at *m/z* 593.3 and MS/MS fragment ions at *m/z* 285, 255, and 227. Peaks 8 and 9 share the same quasi-molecular ion [M-H]$^-$ (*m/z* 464), and identical MS/MS spectral characteristics, suggesting they are isomers of quercetin glycoside.

The identification results indicated that the bioactive components extracted from *Ilex guayusa* in this study mainly include phenolic acids and flavonoids. All the tentatively identified compounds in this work have been proven to have bioactive functions. Chlorogenic acid is a highly functional active compound with benefits for antioxidant, glycemic control, anti-inflammatory, anti-cancer, anti-obesity, neuroprotective activities, etc., and is widely recommended as a pharmaceutical or natural food additive supplement [61,62]. Plants from the same genus, such as *Ilex paraguariensis* (yerba mate) and *Ilex kudingcha*, also contain dicaffeoylquinic acids (diCQAs), compounds that have also been found in our extracts and they have demonstrated potent anti-inflammatory activity through mechanisms such as nitric oxide suppression and NF-κB pathway modulation [63,64]. Wu *et al.* [65] compared various CQAs and diCQAs, pointing out that 5-CQA has the most significant contribution to the anti-inflammatory effect. Kaempferol and its derivatives are promising natural neuroprotective agents and may be new drug candidates for the treatment of neurodegenerative diseases (NDDs) [66]. The neuroprotective effects and antioxidant and anti-inflammatory properties of quercetin have been extensively evaluated [67,68].

Therefore, all the identified compounds provide strong support to be correlated to the *in vitro* assays conducted in this study on guayusa, demonstrating remarkable antioxidant, anti-inflammatory, and neuroprotective potential. These findings provide a scientific basis for the further development and application of guayusa's biological activities.

3.4. Sustainability assessment

To assess the environmental sustainability of our optimized compressed fluid extraction process, we applied the Path2Green metric, a semi-quantitative tool grounded in the 12 principles of green extraction [51]. This framework evaluates key aspects such as solvent origin, energy efficiency, scalability, waste generation, and post-treatment, assigning scores from −1.0 to + 1.0 for each principle. Our optimized GXL method (CO_2/ethanol-water/2-MTHF) achieved a Path2Green score of + 0.450 (see Fig. 4 for pictogram), indicating strong alignment with sustainable extraction practices. This value is comparable to other published green processes using compressed fluids, such as the extraction of bioactives from tangerine leaves using SFE and NADES (score 0.43) [69], and phenolic recovery from orange peel waste via PLE (score 0.54) [70]. In contrast, lipid recovery from insects using SFE scored significantly lower (≈ 0.06–0.02) [71], reflecting higher energy demands. These comparisons validate the environmental performance of the optimized GXL to obtain bioactive compounds from *I. guayusa* and support its classification as a green and scalable alternative for bioactive compound recovery. Nevertheless, there is still room for improvement to be a completely green process.

Although the optimized GXL extraction method demonstrated strong performance in terms of yield, bioactivity, and sustainability, its scalability remains to be validated. Industrial implementation would require further assessment of solvent recovery systems, energy consumption under continuous operation, and integration with downstream processing. These aspects are critical for future applications in functional food and nutraceutical manufacturing.

Fig. 4. Path2Green pictogram illustrating the sustainability profile of the optimized GXL extraction process. The twelve principles of green extraction are visually represented and color-coded based on their individual scores, ranging from −1.0 (red, poor alignment) to + 1.0 (green, excellent alignment). The central score reflects the weighted average of all principles, offering a quick visual summary of the process's overall environmental performance.

4. Conclusions

This work provides a robust and comprehensive evaluation of advanced compressed fluid extraction technologies, namely gas-expanded liquids (GXL), supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE), for the sustainable and efficient recovery of bioactive compounds from *Ilex guayusa* leaves. By taking advantage of green solvents such as CO_2, ethanol/water, and 2-methyltetrahydrofuran (2-MTHF), the study demonstrates how solvent polarity tuning and fluid compressibility can be strategically combined to enhance extraction performance while minimizing environmental impact.

Among the tested approaches, GXL extraction using a ternary solvent system (6.7 % CO_2, 78.4 % ethanol/water, 14.9 % 2-MTHF) clearly outperformed both SFE and PLE in terms of extraction yield, total phenolic content, and bioactivity. The optimized GXL extract exhibited exceptional antioxidant capacity (DPPH and ABTS assays) and potent lipoxygenase (LOX) inhibition, positioning this method as a superior alternative for obtaining multifunctional phytochemicals with potential anti-inflammatory applications.

Notably, this study is the first to report LOX, AChE, and BChE inhibitory activities in *I. guayusa* extracts obtained via compressed fluids, opening new routes for the development of natural ingredients targeting oxidative stress and neuroinflammation. The chemical profiling of the optimized extract by HPLC-DAD-ESI-MS/MS confirmed the presence of key phenolic acids and flavonoids, which are likely responsible for the observed bioactivities. To support the sustainability claim, the Path2Green metric has been applied, which confirmed the environmental relevance of the optimized GXL method and supports its classification as a green technology.

In conclusion, these findings underscore the remarkable potential of compressed fluid-based green extraction technologies, particularly GXL, as scalable, tunable, and environmentally conscious platforms for the recovery of high-value bioactives from plant matrices. This work contributes valuable insights to the field of sustainable process engineering and supports the integration of compressed fluids into the design of next-generation functional ingredients for food, nutraceutical, cosmetic and health-related applications.

CRediT authorship contribution statement

Minxing Zhang: Writing – original draft, Investigation. **Elena Ibáñez:** Writing – review & editing, Project administration, Funding acquisition. **Lidia Montero:** Writing – original draft, Investigation. **Mendiola Jose Antonio:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Funding sources

PID2020–113050RB-I00 funded by MCIN/AEI/10.13039/501100011033 (Agencia Estatal de Investigación, Spain), and by Project AECID 2024/PRYC/000153 (Ministerio de Asuntos Exteriores, Spain). China Scholarship Council (No. 202308500056). The authors want to thank the Bioanalytical Techniques Unit of CIAL Center (CSIC-UAM) for their participation in the physical-chemical analyses. The authors also acknowledge the kind donation of guayusa samples from COPADE (Comercio para el Desarrollo).

Supporting information

ANOVA tables for quadratic regression models (Tables S1-S6) are included in Supplementary Information file

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This work was supported by grant PID2020–113050RB-I00 funded by MCIN/AEI/10.13039/501100011033 (Agencia Estatal de Investigación, Spain), and by Project AECID 2024/PRYC/000153 (Ministerio de Asuntos Exteriores, Spain). The authors gratefully acknowledge the kind donation of *Ilex guayusa* samples by Fundación Comercio para el Desarrollo (COPADE). Minxing Zhang gratefully acknowledges the financial support from the China Scholarship Council (No. 202308500056).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.supflu.2025.106801.

Data Availability

Data will be made available on request.

References

[1] J.F. Dueñas, C. Jarrett, I. Cummins, E. Logan–Hines, Amazonian guayusa (*ilex guayusa* Loes.): a historical and ethnobotanical overview, Econ. Bot. 70 (2016) 85–91, https://doi.org/10.1007/s12231-016-9334-2.
[2] R. Porro, R.P. Miller, M.R. Tito, J.A. Donovan, J.L. Vivan, R. Trancoso, R.F. Van Kanten, J.E. Grijalva, B.L. Ramirez, A.L. Gonçalves, Agroforestry in the Amazon region: a pathway for balancing conservation and development, in: P.K.R. Nair, D. Garrity (Eds.), Agrofor. - Future Glob. Land Use, Springer Netherlands, Dordrecht, 2012, pp. 391–428, https://doi.org/10.1007/978-94-007-4676-3_20.
[3] B. Torres, O.J. Maza, P. Aguirre, L. Hinojosa, S. Günter, Contribution of traditional agroforestry to climate change adaptation in the Ecuadorian Amazon: the chakra system. in: Handb. Clim. Change Adapt, Springer, Berlin, Heidelberg, 2014, pp. 1–19, https://doi.org/10.1007/978-3-642-40455-9_102-1.
[4] E.B. Russo, Headache treatments by native peoples of the Ecuadorian Amazon: a preliminary cross-disciplinary assessment, J. Ethnopharmacol. 36 (1992) 193–206, https://doi.org/10.1016/0378-8741(92)90044-R.
[5] L. Sequeda-Castañeda, G. Costa, C. Celis, F. Gamboa, S. Gutierrez, P. Luengas, *ilex guayusa* (Aquifoliaceae): Amazon and andean native plant, Pharmacologyonline 3 (2016) 93–202.
[6] W.H. Lewis, E.J. Kennelly, G.N. Bass, H.J. Wedner, M.P. Elvin-Lewis, D.F. W, Ritualistic use of the holly *ilex guayusa* by amazonian jivaro Indians,

J. Ethnopharmacol. 33 (1991) 25–30, https://doi.org/10.1016/0378-8741(91)90156-8.
[7] J. Cuestas-Caza, L. Toledo, F. Rodríguez, Transcultural bioeconomy governance in a plurinational state: sumak kawsay and bio-based production in two kichwa territories of Ecuador, For. Policy Econ. 163 (2024) 103227, https://doi.org/10.1016/j.forpol.2024.103227.
[8] R.W. Bussmann, G. Malca, A. Glenn, D. Sharon, B. Nilsen, B. Parris, D. Dubose, D. Ruiz, J. Saleda, M. Martinez, L. Carillo, K. Walker, A. Kuhlman, A. Townesmith, Toxicity of medicinal plants used in traditional Medicine in Northern Peru, J. Ethnopharmacol. 137 (2011) 121–140, https://doi.org/10.1016/j.jep.2011.04.071.
[9] A. García-Ruiz, N. Baenas, A.M. Benítez-González, C.M. Stinco, A.J. Meléndez-Martínez, D.A. Moreno, J. Ruales, Guayusa (*ilex guayusa* L.) new tea: phenolic and carotenoid composition and antioxidant capacity, J. Sci. Food Agric. 97 (2017) 3929–3936, https://doi.org/10.1002/jsfa.8255.
[10] G. Wise, A. Negrin, A critical review of the composition and history of safe use of guayusa: a stimulant and antioxidant novel food, Crit. Rev. Food Sci. Nutr. 60 (2020) 2393–2404, https://doi.org/10.1080/10408398.2019.1643286.
[11] X. Yao, F. Zhang, R.T. Corlett, Utilization of the hollies (*ilex l.* spp.): a review, Forests 13 (2022) 94, https://doi.org/10.3390/f13010094.
[12] A. Negrin, C. Long, T.J. Motley, E.J. Kennelly, LC-MS metabolomics and chemotaxonomy of caffeine-containing holly (ilex) species and related taxa in the aquifoliaceae, J. Agric. Food Chem. 67 (2019) 5687–5699, https://doi.org/10.1021/acs.jafc.8b07168.
[13] S.B. Luna-Fox, J.H. Uvidia-Armijo, J.L. Rivera-Barreto, Kinetic study of solid-liquid extraction of caffeine in *ilex guayusa* loes, Rev. Fac. Agron. 41 (2024), https://doi.org/10.47280/RevFacAgron(LUZ).v41.n3.08.
[14] M.D. Pardau, A.S.P. Pereira, Z. Apostolides, J.C. Serem, M.J.Jean Bester, Antioxidant and anti-inflammatory properties of *ilex guayusa* tea preparations: a comparison to camellia sinensis teas, Food Funct. 8 (2017) 4601–4610, https://doi.org/10.1039/C7FO01067B.
[15] J. Villacís-Chiriboga, A. García-Ruiz, N. Baenas, D.A. Moreno, A.J. Meléndez-Martínez, C.M. Stinco, L. Jerves-Andrade, F. León-Tamariz, J. Ortíz-Ulloa, J. Ruales, Changes in phytochemical composition, bioactivity and in vitro digestibility of guayusa leaves (Ilex guayusa Loes.) in different ripening stages, J. Sci. Food Agric. 98 (2018) 1927–1934, https://doi.org/10.1002/jsfa.8675.
[16] H. Kelebek, H.K. Sasmaz, O. Aksay, S. Selli, O. Kahraman, C. Fields, Exploring the impact of infusion parameters and in vitro digestion on the phenolic profile and antioxidant capacity of guayusa (ilex guayusa loes.) tea using liquid chromatography, diode array detection, and electrospray ionization tandem mass spectrometry, Foods 13 (2024), https://doi.org/10.3390/foods13050694.
[17] F. Gamboa, C.-C. Muñoz, G. Numpaque, L.G. Sequeda-Castañeda, S.J. Gutierrez, N. Tellez, Antimicrobial activity of piper marginatum jacq and *ilex guayusa* loes on microorganisms associated with periodontal disease, Int. J. Microbiol 2018 (2018), https://doi.org/10.1155/2018/4147383.
[18] G. Chianese, S.D. Golin-Pacheco, O. Taglialatela-Scafati, J.A. Collado, E. Munoz, G. Appendino, F. Pollastro, Bioactive triterpenoids from the caffeine-rich plants guayusa and maté, Food Res. Int. 115 (2019) 504–510, https://doi.org/10.1016/j.foodres.2018.10.005.
[19] M.B. La Monica, B. Raub, E.J. Ziegenfuss, S. Hartshorn, J. Grdic, A. Gustat, J. Sandrock, T.N. Ziegenfuss, Acute effects of naturally occurring guayusa tea and nordic lion's mane extracts on cognitive performance, Nutrients 15 (2023), https://doi.org/10.3390/nu15245018.
[20] N.J. Helwig, L.E. Schwager, A.C. Berry, A.C. Zucker, J.S. Venenga, S.C. Sterbenz, N. D.M. Jenkins, Acute, dose–response effects of guayusa leaf extract on mood, cognitive and motor-cognitive performance, and blood pressure, heart rate, and ventricular repolarization, J. Int. Soc. Sports Nutr. 21 (2024), https://doi.org/10.1080/15502783.2024.2379424.
[21] S. Gavrilova, A. Alvarez, Cerebrolysin in the therapy of mild cognitive impairment and dementia due to Alzheimer's disease: 30 years of clinical use, Med. Res. Rev. 41 (2020) 2775–2803, https://doi.org/10.1002/med.21722.
[22] E. Alfadly, P. Elzahhar, A. Tramarin, S. Elkazaz, H. Shaltout, M. Abu-Serie, J. Bzdilova Janockova, O. Soukup, D. Ghareeb, A. Elyazbi, R. Rafeh, N. Bakkar, F. Kobeissy, I. Iriepa, I. Moraleda, M. Saudi, M. Bartolini, A. Belal, Tackling neuroinflammation and cholinergic deficit in alzheimer's disease: multi-target inhibitors of cholinesterases, cyclooxygenase-2 and 15-lipoxygenase, Eur. J. Med. Chem. 167 (2019), https://doi.org/10.1016/j.ejmech.2019.02.012.
[23] Q. Huang, C. Zhang, S. Qu, S. Dong, Q. Ma, Y. Hao, Z. Liu, S. Wang, H. Zhao, Y. Shi, Chinese herbal extracts exert neuroprotective effect in alzheimer's disease mouse through the dopaminergic synapse/apoptosis signaling pathway, Front. Pharm. 13 (2022), https://doi.org/10.3389/fphar.2022.817213.
[24] K. van Leyen, K. Arai, G. Jin, V. Kenyon, B. Gerstner, P.A. Rosenberg, T.R. Holman, E.H. Lo, Novel lipoxygenase inhibitors as neuroprotective reagents, J. Neurosci. Res. 86 (2008) 904–909, https://doi.org/10.1002/jnr.21543.
[25] C. Cakir-Aktas, E. Bodur, M. Yemisci, Ozkan, K. van Leyen, H. Karatas, 12/15-lipoxygenase inhibition attenuates neuroinflammation by suppressing inflammasomes, Front. Cell. Neurosci. 17 (2023), https://doi.org/10.3389/fncel.2023.1277268.
[26] J.S. Birks, Cholinesterase inhibitors for Alzheimer's disease, Cochrane Database Syst. Rev. (2006), https://doi.org/10.1002/14651858.CD005593.
[27] Y. Ismail, Y. Haitham, M. Walid, H. Mohamed, Y. El-Satar, Efficacy of acetylcholinesterase inhibitors on reducing hippocampal atrophy rate: a systematic review and meta-analysis, BMC Neurol. 25 (2025), https://doi.org/10.1186/s12883-024-03933-4.
[28] T. Sun, T. Zhen, C. Harakandi, L. Wang, H. Guo, Y. Chen, H. Sun, New insights into butyrylcholinesterase: pharmaceutical applications, selective inhibitors and multitarget-directed ligands, Eur. J. Med. Chem. 275 (2024) 116569, https://doi.org/10.1016/j.ejmech.2024.116569.
[29] T. Santos, T. Gomes, B. Pinto, A. Camara, A. Paes, Naturally occurring acetylcholinesterase inhibitors and their potential use for Alzheimer's disease therapy, Front. Pharm. 9 (2018) 1192, https://doi.org/10.3389/fphar.2018.01192.
[30] P. Poletto, G. Alvarez-Rivera, T.M.S. Torres, J.A. Mendiola, E. Ibañez, A. Cifuentes, Compressed fluids and phytochemical profiling tools to obtain and characterize antiviral and anti-inflammatory compounds from natural sources, TrAC Trends Anal. Chem. 129 (2020), https://doi.org/10.1016/j.trac.2020.115942.
[31] J.A. Mendiola, M. Herrero, A. Cifuentes, E. Ibañez, Use of compressed fluids for sample preparation: food applications, J. Chromatogr. A 1152 (2007) 234–246, https://doi.org/10.1016/j.chroma.2007.02.046.
[32] M. Herrero, J.A. Mendiola, A. Cifuentes, E. Ibáñez, Supercritical fluid extraction: recent advances and applications, J. Chromatogr. A 1217 (2010) 2495–2511, https://doi.org/10.1016/j.chroma.2009.12.019.
[33] M. Herrero, M. Castro-Puyana, J.A. Mendiola, E. Ibañez, Compressed fluids for the extraction of bioactive compounds, TrAC Trends Anal. Chem. 43 (2013) 67–83, https://doi.org/10.1016/j.trac.2012.12.008.
[34] P.G. Jessop, B. Subramaniam, Gas-Expanded liquids, Chem. Rev. 107 (2007) 2666–2694, https://doi.org/10.1021/cr040199o.
[35] J.D. Sánchez-Martínez, G. Alvarez-Rivera, R. Gallego, M.B. Fagundes, A. Valdés, J. A. Mendiola, E. Ibañez, A. Cifuentes, Neuroprotective potential of terpenoid-rich extracts from Orange juice by-products obtained by pressurized liquid extraction, Food Chem. X 13 (2022) 100242, https://doi.org/10.1016/j.fochx.2022.100242.
[36] M. Herrero, J.A. Mendiola, E. Ibáñez, Gas expanded liquids and switchable solvents, Curr. Opin. Green. Sustain. Chem. 5 (2017) 24–30, https://doi.org/10.1016/j.cogsc.2017.03.008.
[37] G. Alvarez-Rivera, M. Bueno, D. Ballesteros-Vivas, J.A. Mendiola, E. Ibañez, Pressurized liquid extraction, Liq. Phase Extr. (2019) 375–398, https://doi.org/10.1016/B978-0-12-816911-7.00013-X.
[38] N. Abderrezag, Z.J.S. Montenegro, O. Louaer, A.-H. Meniai, A. Cifuentes, E. Ibáñez, J.A. Mendiola, One-step sustainable extraction of silymarin compounds of wild Algerian milk thistle (*silybum marianum*) seeds using gas expanded liquids, J. Chromatogr. A 1675 (2022), https://doi.org/10.1016/j.chroma.2022.463147.
[39] G. Sorita, W.D. Caicedo Chacon, M. Strieder, C. Rodriguez García, A. Fritz, S. Verruck, G. Ayala Valencia, J. Mendiola, Biorefining Brazilian Green propolis: an eco-friendly approach based on a sequential high-pressure extraction for recovering high-added-value compounds, Molecules 30 (2025) 189, https://doi.org/10.3390/molecules30010189.
[40] S. Hasni, H. Riguene, J. Mendiola, E. Ibáñez, L. Montero, G. Domínguez-Rodríguez, H. Ghazghazi, R. Ghayth, R. Salem, Optimization of a pressurized extraction process based on a ternary solvent system for the recovery of neuroprotective compounds from *eucalyptus marginata* leaves, Int. J. Mol. Sci. 26 (2024), https://doi.org/10.3390/ijms26010094.
[41] C. Rizzo, S. Amata, A. Palumbo Piccionello, A. Mineo, G. Mannina, An environmentally sustainable extraction protocol for polyhydroxyalkanoates from mixed culture biomass, J. Environ. Chem. Eng. 13 (2025), https://doi.org/10.1016/j.jece.2025.117691.
[42] Ü. Erdoğan, D. Onder, S. Onder, M. Tonguç, R.E. Ince, Green solvent 2-methyltetrahydrofuran (2-MeTHF) improves recovery of bioactive molecules from oilseeds and prevents lipid peroxidation in oils, Food Chem. 478 (2025), https://doi.org/10.1016/j.foodchem.2025.143659.
[43] S. Cadena-Carrera, D.P. Tramontin, A. Bella Cruz, R.C. Bella Cruz, J.M. Müller, H. Hense, Biological activity of extracts from guayusa leaves (*ilex guayusa* Loes.) obtained by supercritical CO2 and ethanol as cosolvent, J. Supercrit. Fluids 152 (2019), https://doi.org/10.1016/j.supflu.2019.104543.
[44] M.Á. Meneses, J. Guzmán, J. Cabrera, J. Magallanes, E. Valarezo, M.D.C. Guamán-Balcázar, Green processing of *ilex guayusa*: antioxidant concentration and caffeine reduction using encapsulation by supercritical antisolvent process, Molecules 29 (2024), https://doi.org/10.3390/molecules29225309.
[45] N. Abderrezag, F.S. Bragagnolo, O. Louaer, A.-H. Meniai, A. Cifuentes, E. Ibáñez, J. A. Mendiola, Optimization of supercritical fluid extraction of bioactive compounds from *ammodaucus leucotrichus* fruits by using multivariate response surface methodology, J. Supercrit. Fluids (2024) 106211, https://doi.org/10.1016/j.supflu.2024.106211.
[46] J.A. Mendiola, Green processes in foodomics. Gas-expanded liquids extraction of bioactives, Compr. Food (2020) 744–753, https://doi.org/10.1016/B978-0-08-100596-5.22927-2.
[47] M. Koşar, H.J.D. Dorman, R. Hiltunen, Effect of an acid treatment on the phytochemical and antioxidant characteristics of extracts from selected lamiaceae species, Food Chem. 91 (2005) 525–533, https://doi.org/10.1016/j.foodchem.2004.06.029.
[48] W. Brand-Williams, M.E. Cuvelier, C. Berset, Use of a free radical method to evaluate antioxidant activity, LWT Food Sci. Technol. 28 (1995) 25–30, https://doi.org/10.1016/S0023-6438(95)80008-5.
[49] J.D. Sánchez-Martínez, M. Bueno, G. Alvarez-Rivera, J. Tudela, E. Ibañez, A. Cifuentes, *in vitro* neuroprotective potential of terpenes from industrial Orange juice by-products, Food Funct. 12 (2021) 302–314, https://doi.org/10.1039/D0FO02809F.
[50] M. Whent, T. Ping, W. Kenworthy, L. Yu, High-Throughput assay for detection of soybean Lipoxygenase-1, J. Agric. Food Chem. 58 (2010) 12602–12607, https://doi.org/10.1021/jf1028784.
[51] L.M. de, S. Mesquita, L.S. Contieri, F.A. e Silva, R. Henrique Bagini, F. S. Bragagnolo, M.M. Strieder, F.H.B. Sosa, N. Schaeffer, M.G. Freire, S.P. M. Ventura, J.A.P. Coutinho, M.A. Rostagno, Path2Green: introducing 12 Green

extraction principles and a novel metric for assessing sustainability in biomass valorization, Green. Chem. 26 (2024) 10087–10106, https://doi.org/10.1039/D4GC02512A.
[52] C. Dauber, T. Carreras, L. González, A. Gámbaro, A. Valdés, E. Ibañez, I. Vieitez, Characterization and incorporation of extracts from olive leaves obtained through maceration and supercritical extraction in canola oil: oxidative stability evaluation, LWT 160 (2022) 113274, https://doi.org/10.1016/j.lwt.2022.113274.
[53] A. Modzelewska, M. Jackowski, A. Trusek, Extraction of valuable compounds from spent hops, including xanthohumol—the influence of the solvent's polarity index, Food Bioprocess Technol. (2025), https://doi.org/10.1007/s11947-025-03977-5.
[54] I. Chóez-Guaranda, R. Viteri-Espinoza, A. Barragán-Lucas, M. Quijano-Avilés, P. Manzano, Effect of solvent-solvent partition on antioxidant activity and GC-MS profile of *ilex guayusa* loes. Leaves extract and fractions, Nat. Prod. Res. 36 (2022) 1570–1574, https://doi.org/10.1080/14786419.2021.1882455.
[55] C.F.C. Tavera, J.C.C. Villate, M.J.H. Roa, O.E.R. Aguirre, P. Borrego-Muñoz, L.M. P. Ospina, High polarity extracts obtained from *ilex guayusa* loes. Leaves, exhibit antioxidant capacity, Vitae 29 (2022), https://doi.org/10.17533/udea.vitae.v29n1a347297.
[56] N.R. Costa, Z.L. Pereira, Multiple response optimization: a global criterion-based method, J. Chemom. 24 (2010) 333–342, https://doi.org/10.1002/cem.1312.
[57] S. Cadena-Carrera, D.P. Tramontin, R. Jacques, E. Scapin, J.M. Müller, H. Hense, Green-based methods to obtain bioactive compounds from *ilex guayusa* loes. Using polar solvent, Nat. Prod. Res. 37 (2023) 3103–3108, https://doi.org/10.1080/14786419.2022.2140802.
[58] G. Mushtaq, N.H. Greig, J.A. Khan, M.A. Kamal, Status of acetylcholinesterase and butyrylcholinesterase in Alzheimer's disease and type 2 diabetes mellitus, CNS Neurol. Disord. Drug Targets 13 (2014) 1432–1439, https://doi.org/10.2174/1871527313666141023141545.
[59] S. Zhou, G. Huang, The biological activities of butyrylcholinesterase inhibitors, Biomed. Pharm. 146 (2022) 112556, https://doi.org/10.1016/j.biopha.2021.112556.
[60] L. Bravo, L. Goya, E. Lecumberri, LC/MS characterization of phenolic constituents of mate (*ilex paraguariensis*, St. Hil.) and its antioxidant activity compared to commonly consumed beverages, Food Res. Int. 40 (2007) 393–405, https://doi.org/10.1016/j.foodres.2006.10.016.
[61] R. Kumar, A. Sharma, M.S. Iqbal, J.K. Srivastava, Therapeutic promises of chlorogenic acid with special emphasis on its anti-obesity property, Curr. Mol. Pharm. (2019), https://doi.org/10.2174/1874467212666190716145210.
[62] L. Huijie, Z. Tian, Y. Cui, Z. Liu, X. Ma, Chlorogenic acid: a comprehensive review of the dietary sources, processing effects, bioavailability, beneficial properties, mechanisms of action, and future directions, Compr. Rev. Food Sci. Food Saf. 19 (2020), https://doi.org/10.1111/1541-4337.12620.
[63] S. Puangpraphant, M. Berhow, K. Vermillion, G. Potts, E. Mejia, Dicaffeoylquinic acids in yerba mate (Ilex paraguariensis St. Hilaire) inhibit NF-ΚB nucleus translocation in macrophages and induce apoptosis by activating caspases-8 and -3 in human colon cancer cells, Mol. Nutr. Food Res. 55 (2011) 1509–1522, https://doi.org/10.1002/mnfr.201100128.
[64] W. Peng, M. Xie, G. Chen, Z. Dai, B. Hu, Y. Sun, Anti-inflammatory effects of dicaffeoylquinic acids from *ilex kudingcha* on lipopolysaccharide-treated RAW264.7 macrophages and potential mechanisms, Food Chem. Toxicol. 126 (2019), https://doi.org/10.1016/j.fct.2019.01.011.
[65] T.-Y. Wu, C.-C. Chen, J.-Y. Lin, Anti-inflammatory in vitro activities of eleven selected caffeic acid derivatives based on a combination of pro−/anti-inflammatory cytokine secretions and principal component analysis – a comprehensive evaluation, Food Chem. 458 (2024) 140201, https://doi.org/10.1016/j.foodchem.2024.140201.
[66] S. Jin, L. Zhang, L. Wang, Kaempferol, a potential neuroprotective agent in neurodegenerative diseases: from chemistry to Medicine, biomed. Pharmacother, Biomedecine Pharm. 165 (2023) 115215, https://doi.org/10.1016/j.biopha.2023.115215.
[67] S.-C. Cheng, W.-C. Huang, J.-H. Pang, Y.-H. Wu, C. Cheng, Quercetin inhibits the production of IL-1β-Induced inflammatory cytokines and chemokines in ARPE-19 cells via the MAPK and NF-κB signaling pathways, Int. J. Mol. Sci. 20 (2019) 2957, https://doi.org/10.3390/ijms20122957.
[68] Y.-Y. Wang, C.-Y. Chang, S.-Y. Lin, J.-D. Wang, C.-C. Wu, W.-Y. Chen, Y.-H. Kuan, S.-L. Liao, W.-Y. Wang, C.-J. Chen, Quercetin protects against cerebral ischemia/reperfusion and oxygen glucose deprivation/reoxygenation neurotoxicity, J. Nutr. Biochem 83 (2020) 108436, https://doi.org/10.1016/j.jnutbio.2020.108436.
[69] G.D. Sorita, C. Rodríguez-García, M.M. Strieder, G. Domínguez-Rodríguez, L. Montero, F.S. Bragagnolo, L. Palomeque, F. Parada-Alfonso, E. Ibáñez, An innovative approach for the recovery of nobiletin, hesperidin, and other phytochemicals from tangerine leaves (*citrus reticulata*) by-products using a solvent mixture based on natural compounds, Food Chem. 486 (2025) 144628, https://doi.org/10.1016/j.foodchem.2025.144628.
[70] M. Plaza, R. Gallardo-Martínez, M.L. Marina, Two-step pressurized liquid extraction: sustainable recovery of phenolic antioxidants and high-purity limonene from Orange peel waste, Sustain. Chem. Pharm. 46 (2025) 102117, https://doi.org/10.1016/j.scp.2025.102117.
[71] L.C. dos Santos, C. Villeligoux, E.A. Rebelatto, M. Lanza, S. Ferreira, E. Destandau, A. Cifuentes, E. Ibáñez, Lipid extraction from *galleria mellonella* larvae: introducing the use of eco-friendly solvents and emerging technologies, J. Insects Food Feed 1 (2025) 1–17, https://doi.org/10.1163/23524588-bja10280.