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ORIGINAL RESEARCH ARTICLE

Extraction of bioactive phenolic compounds from yerba mate leaves using ultrasound: HPLC profiling and statistical analysis of variables

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ABSTRACT

Extraction is critical step in extracting bioactive phytochemicals from plant sources and separating them. In this study, a Box-Benken (BBD) design was used, with 15 experiments conducted to optimize the ultrasonic extraction process. Three independent variables (ethanol concentration, temperature, and time) were examined to assess their effect on total extract yield (TYE) as the main response. The optimization aimed to maximize the extraction of active compounds from the resilient matrix of yerba mate (Ilex paraguariensis) leaves. Then, used high-performance liquid chromatography (HPLC) to determine levels of gallic acid, caffeine, quercetin, tannic acid, chlorogenic acid, and other compounds from yerba Mate leaf extract. This method was used to find out how temperature (25–60°C), ultrasonic time (20–40 min), and ethanol content (40–80%) affected the characteristics of yerba mate leaf extracts. The results indicated that the ideal temperature was 50°C, the optimal ultrasonic duration was 30 minutes, and the best extraction ratio was Ethanol/Water (v/v) 50:50, which produced the largest concentration of biologically active compounds without thermal degradation into alternative substances. Importantly, The Analytical Greenness, AGREE, metric was also used to measure the environmental impact, achieving a high score of 0.85. This demonstrates that the created process is not only accurate but also environmentally friendly and long-lasting, as it requires minimal energy and reagents.

Keywords: HPLC; yerba mate; green extraction; experimental design; bioactive compounds

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1. Introduction

Studies have shown that drinking Mate tea (*Ilex paraguariensis*) greatly increases the amount of antioxidants in the body. It contains many caffeoylquinic acid derivatives, which may have health benefits. The investigation of Mate's capacity to neutralize reactive oxygen species (ROS) has been associated with peroxidase-like activity. This peroxidase-like activity is closely associated with the polyphenol concentration in Mate; an increase in polyphenol concentration corresponds to an enhancement in peroxidase-like activity. From biologically perspective, this means that polyphenols work like the body's 293 natural antioxidant enzymes and may be strong boosters of these systems[1]. Chlorogenic acid is the chemical most likely to be responsible for this action. Mate extract is a strong antioxidant that protects the liver and heart from ROS-related oxidative stress. During postischemic reperfusion, when blood flow returns to the heart and tissue after a heart attack, the heart is vulnerable to oxidative stress because of the production of ROS[2]. Giving Mate extract to the heart protects the cardiac tissue and lowers lipid

oxidation. Recent research indicates that nitrosative stress, characterized by the interaction of superoxides with nitrous oxide (NO) to create peroxynitrite (ONOO), results in protein nitration or nitrosylation, lipid peroxidation, DNA damage, and cellular apoptosis. When tested on bovine serum albumin, mate tea stopped 95% of protein nitration. This made it better than both green tea and red wine[3,4]. Mate was also examined for its ability to protect cells from damage caused by peroxynitrite, which is linked to stroke, cardiac ischemia, and a lack of blood flow. Compared to green and red wine, Mate tea was the best for inhibiting cellular damage. Mate also slows down the breakdown of ATP, ADP, and AMP (nucleotides), which can assist the circulatory system in staying in balance[5,6]. It has been shown that hyperglycemia leads to diabetic problems due to dicarbonyls implicated in advanced glycation end product (AGE) production[7,8] Oxidation has been associated with glycation, and Mate extracts exhibit a dose-dependent suppression of dicarbonyl activity[8,9]. The most important chemical extraction methods for phenols and flavonoids from yerba mate, according to recent research, are solvent extraction and organic solvent extraction[10]. This is the standard laboratory method for determining chemical content and relies on the principle of polarity and optimal solvent selection. Studies have shown that ethanol at a concentration of 70% or 80% is the most efficient for extracting chlorogenic acid and flavonoids such as rutin[11]. Ethanol is considered a relatively safe substance and is highly effective at penetrating the pores of the yerba mate plant. Hot water extraction is another method, mimicking human consumption but used in research to determine soluble components. Extraction at 90°C for 10 minutes yields the highest concentration of antioxidants in the aqueous extract. Phenolic acids are primarily extracted using this method[11,12]. Ultrasonic Assisted Extraction (UAE) is an advanced technique (Green Extraction) for extracting the active ingredients from recalcitrant plant matrix[13]. It uses high frequencies to break down plant tissues, reducing extraction time from hours to minutes. Its advantages include increasing the amount of extracted flavonoids by up to 20% compared to traditional methods, and preserving the compounds from thermal degradation because it is performed at relatively low temperatures[14]. This study primarily utilizes Ultrasonic-Assisted Extraction (UAE), a technique chosen for its superior advantages over other methods such as Supercritical Fluid Extraction (SFE). While SFE is widely used, UAE offers greater scalability and significantly lower operating costs. Furthermore, based on the AGREE scale, UAE has proven to be a more sustainable and environmentally friendly option. Therefore, this research aims to optimize extraction parameters using Response Surface Methodology (RSM) to achieve the highest possible extraction efficiency for the target compounds[14,15].

2. Materials and methods

2.1. Plant material

This study used commercially prepared dried leaves of the yerba mate plant (*Ilex paraguariensis*). Samples were purchased from local markets in Baghdad, Iraq, under the brand name *'Kharta Khadra'*, a commercial product of Argentinian origin according to the manufacturer's labeling. Upon purchase, the samples were kept in their original, tightly sealed containers and stored in a cool, dark, and dry environment to ensure their chemical stability. The production date was recorded to guarantee the freshness of the plant material before extraction procedures began.

2.2. Extraction of botanical substances

Extraction procedure, 6.5 g of dry ground plant was weighed, 50 mL ethanol - water was added, and the mixture was put in a 100 mL Erlenmeyer conical flask. For 30 minutes at 50°C, the ultrasonic bath (UST 5.7150 Siel, Gabrovo, Bulgaria) ran at a frequency of 35 kHz and a maximum input power of 0.240 kW. After incubation, all the extracts were filtered and kept at 4°C without any preservatives until they were ready to be analyzed.

2.3. Assessment for phenolic compounds

One milliliter of crude ethanolic extract was mixed with 1 mL of 5% ferric chloride, and the color range from deep green to black shows that phenolic acids are present[9].

2.4. Assessment of flavonoid compounds

One milliliter of crude ethanolic extract into a test tube with 2 mL of 1% potassium hydroxide and watched the color change. A yellow color means that flavonoids are present[16].

2.5. Assessment of Total Phenolic Content (TPC)

The Folin–Ciocalteu spectrophotometric quantification method was employed to find the total phenolic content (TPC) after extraction[17]. In a glass vial, 0.1 mL of the extract and 0.1 mL of the Folin–Ciocalteu reagent were combined. After 3 minutes in the dark, 2 mL of a 2% (w/v) aqueous sodium carbonate solution was added to the mixture and returned to the dark for another 30 minutes. After that, the solution's absorbance was measured at 765 nm using a UV-2600 spectrophotometer (Shimadzu, Kyoto, Japan). As indicated in **Figure 1**, the suitable solvent was employed to undertake spectrophotometric studies on gallic acid, **Figure 1** show that y is the absorbance and x is the concentration (µg/mL).

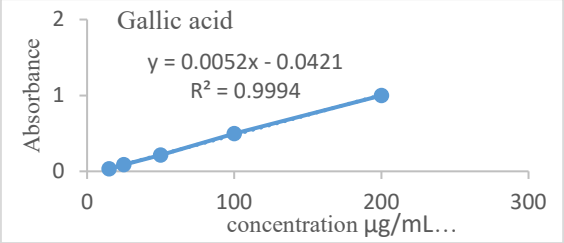

Figure 1. Standard curve of gallic acid using Folin Ciocalteu.

2.6. Assessment of Total Flavonoid Content (TFC)

The aluminum chloride colorimetric method was used to determine the amount of flavonoid in the sample[18]. The first step was to mix 0.15 mL of $NaNO_2$ (5%, w/v) with 0.5 mL of the sample solution. After mixing for five minutes, 0.15 mL of $AlCl_3$ (10%, w/v) was added. After five more minutes, they added 1 mL of NaOH (1 M). Then, ultrapure water was added to the mixture to bring the volume to 5 mL, and the reaction mixture was allowed to sit at room temperature for 20 minutes after all the elements were added. After that, a UV-vis Spectrophotometer (UV-2600, Shimadzu, Kyoto, Japan) was utilized to find the absorbance right away at 410 nm. The TFC was found using quercetin as a reference and the regression equation in **Figure 2**. If y is the absorbance, then x is the amount in µg/mL.

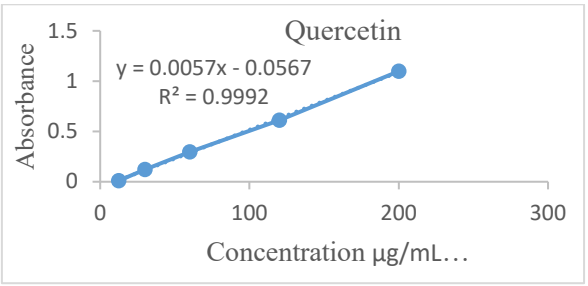

Figure 2. Standard curve for the determination of flavonoids using quercetin.

2.7. Analysis by HPLC

The SYKAM HPLC system (Germany) with a UV detector was used for the chromatographic analysis. The separation was performed with an isocratic mobile phase composed of Acetonitrile (A) and 0.5% aqueous formic acid (B) in a 30:70 (v/v) ratio. A C18-ODS column (5 µm, 4.6 × 250 mm) was utilized for

the process and kept at 30°C. The flow rate was set to 1.0 mL/min, and the injection volume was 20 µL. The detection occurred at 280 nm. We chose this wavelength because it corresponds to the absorption maximum (**λ** max) for many phenolic compounds under this study, such as benzoic acid derivatives (gallic acid), hydroxycinnamic acids (chlorogenic acid), and some flavonoids. This lets us measure them all at once with great sensitivity. To ensure the reliability of the results, the method was validated for linearity, showing correlation coefficients (R^2) of 0.9994 for gallic acid and 0.9992 for quercetin. Preliminary qualitative chemical tests were performed as a screening step to confirm the presence of phenols and flavonoids before proceeding to the more precise HPLC quantification. . To ensure injection repeatability, each sample and standard was injected in triplicate (n=3)[19].

2.8. Analysis of Statistics

Each experiment analyzed three times, and the findings were reported as mean ± standard deviation (SD). We did a variance analysis (ANOVA) and thought the differences were important when the p-value was less than 0.05 then used the Design-Expert version 13 software (Stat-Ease Inc., Minneapolis, MN, USA) for the RSM design and statistical analysis.

3. Results and discussion

3.1. The effect of the Ethanol Ratio on the total yield of Extracted (TYE)

The impact of ethanol ratio on TYF was examined using various ethanol/water (v/v) ratios (40:60, 50:50, 60:40, 70:30, and 80:20, respectively). On the other hand, the ultrasonic time (30 minutes) and extraction temperature (50 °C) were the same for all extractions. **Figure 3** the results showed that extraction yield does not follow a continuous linear increase with decreasing ethanol concentration. Instead, the data indicate a peak yield of 3.12 mg at an ethanol: water ratio of 40:60 (v/v) (Experiment 8), followed by a marked decrease in yield at a 50:50 ratio. This confirms a non-linear relationship, where maximum extraction efficiency depends on achieving the optimal polarity of the solvent, which allows for maximum solubility of the active compounds, rather than simply increasing the ratio of solvent to water. And the equation below was used to find the Extract yield:

$\text{yield}\left(\frac{mg}{g}\right) = \frac{W1}{W0}$, Where W_1 is the weight of the dried extract (mg) and W_0 is the initial weight of the yerba mate powder (g)

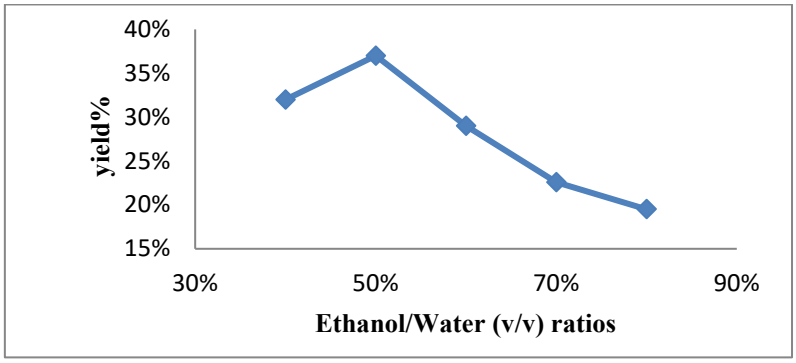

Figure 3. The effect of Ethanol Ratio on extraction rate.

3.2. Effect of Ultrasonic time on total yield of Extracted (TYE)

The extraction period (20–40 min) affects the TYE. **Figure 4** shows that the TYE improved significantly when the extraction time was first increased (20, 25, 30, 35, or 40 minutes). The extraction yield increased somewhat with a longer extraction period. This could be because longer extraction times led

to more dissolved contaminants and some flavonoid degradation[20]. So, 30 minutes was thought to be the best extraction time.

3.3. Effect of Temperature on the total yield of Extracted (TYE)

Temperature has a significant effect on the yield of the extraction. Consequently, to determine the optimal extraction temperature, ultrasonic-assisted extraction operations were conducted at 25, 30, 35, 40, 45, 50, 55, and 60 °C, respectively, with the results illustrated in **Figure 5**. The TYE rose a lot as the extraction temperature increased from 25 to 60 °C. But when the extraction temperature was higher than 60 °C, the TYF went down. One possible explanation for this is that the high temperature damaged the structure of the flavonoids[21]. The high extraction efficiency can be attributed to the acoustic cavitation mechanism induced by ultrasonic waves. As these waves pass through the solvent, they create microscopic bubbles that burst forcefully near the plant surface. This phenomenon generates micro-jet shear forces that rupture the tough plant matrix of yerba mate leaves. As a result, the surface area for substance exchange increases, allowing the solvent to penetrate deeply into the cellular structures and facilitating the rapid release of bioactive compounds. So, 50 °C was chosen as the best temperature for extraction.

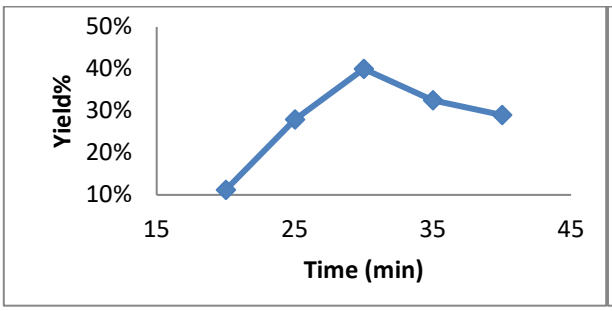

Figure 4. The effect of ultrasonic time on extraction rate.

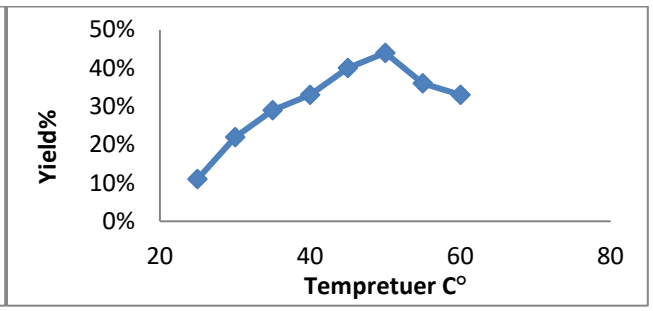

Figure 5. The effect of temperature on extraction rate.

3.4. HPLC analysis of Phenolic-flavonoid compounds in Mate tea extract

The method was validated for reliability and quantification accuracy. Linearity was assessed using calibration curves for the major standards, which showed high correlation coefficients (R^2): 0.9994 for Gallic acid and 0.9992 for Quercetin. Sensitivity: The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were calculated to confirm the method's ability to detect low concentrations of phenolics, and the HPLC method's sensitivity was evaluated by determining these values. For Gallic acid, the LOD and LOQ were 0.15 and 0.45 µg/mL, respectively. For Quercetin, the values were 0.12 and 0.36 µg/mL, indicating high sensitivity for the quantification of phenolic compounds in Yerba Mate extracts. The method demonstrated high precision, with a Relative Standard Deviation (RSD) of < 2%. The recovery of bioactive compounds, such as Chlorogenic acid (concentration: 955.971 mg/L at t_R = 18.577 min) and Rutin (concentration: 198.563 mg/L at t_R = 3.957 min), confirmed the extraction efficiency. Chromatograms of standard phenolic chemical compounds are often discovered in medicinal plant samples that were extracted under the best conditions using High Performance Liquid Chromatography (HPLC). These HPLC fingerprints of standard phenolic-flavonoid components may serve as benchmarks for comparing phenolic-flavonoid compounds in Mate tea extract under optimal conditions[22,23]. When mixed, the Chromatographic fingerprint of these 10 phenolic compounds showed the same elution sequence. The combined Chromatogram looked like a synchronized collection of the individual HPLC profiles, with the individual peak times being very similar to those mentioned earlier (**Figure 6** and **Table 1**). The activities of flavonoid compounds may be attributed to the presence of gallic acid (3.030 min), luteolin (3.753 min), rutin (3.957 min), kaempferol (5.080 min), caffeic acid (7.467 min), quercetin (10.647 min), coumaric acid (13.857 min), apigenin (16.440 min), ferulic acid (16.770 min), and chlorogenic acid (18.577 min). Nonetheless, preclinical investigations are crucial for clinical application and for evaluating the pharmacognostical, phytochemical, toxicological, and biological

characteristics of herbal medications. The qualitative and quantitative examination of phenolic compounds present in any plant part, conducted through systematic scientific methodology, and their comparison with standard phenolic compounds, are crucial for establishing their efficacy[24,25].

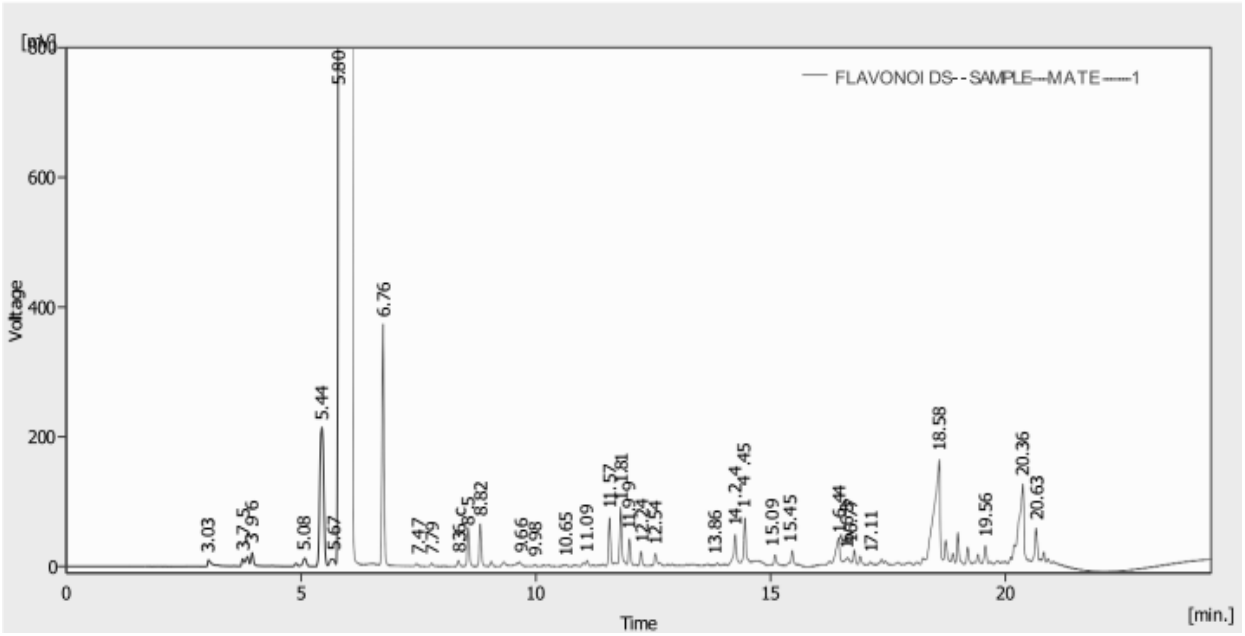

Figure 6. HPLC profile of Phenolic-flavonoid compounds in Mate tea extract at optimum conditions.

Table 1. Retention times of Phenolic-flavonoid compounds peaks in Mate tea extract at optimum conditions.

	Reten. Time (min) for standard	Reten. Time (min) Mate tea extract	Relative Retention Times. RRT	Amount (mg/L)	Height(mV)	W 0.5(min)	Compound Name
1	3.513	3.530	1.005	2.050 1	0.4	0.07	GALLIC
2	3.850	3.753	0.975	10.288	0.3	0.05	LUTEOLIN
3	4.030	3.957	0.982.	198.56	0.7	0.05	RUTIN
4	5.240	5.080	0.969	32.001	0.5	0.09	KAEMPFEROL
5	7.413	7.467	1.007	53.870	0.2	0.05	CAFFEIC
6	10.694	10.647	0.995	51.901	0.1	0.05	QUERCETIN
7	13.617	13.857	1.017	5.509 1	0.1	0.05	COUMARIC
8	16.540	16.440	0.994	6.145 1	0.5	0.03	APIGENIN
9	16.810	16.770	0.997	16.891	0.3	0.02	FERULIC
10	18.453	18.577	1.006	955.97	6.5	0.14	CHLOROGENIC

Most phenolic-flavonoid compounds demonstrate antipyretic, analgesic, anti-inflammatory, anti-arthritic, antioxidant, and immunomodulatory characteristics[26]. Quercetin is a glycoside flavonol that is part of the flavonoid class. It has several pharmacological effects on the central nervous system (CNS), including neuroprotective and antidepressant effects. Research has demonstrated that rutin is prevalent in numerous plants and is crucial for the antidepressant properties of Hypericum perforatum, Hypericum connatum, Schinus molle, and daylily flowers[26,27]. Kaempferol is a natural flavonoid that fights cancer, inflammation, and germs. Apples, tea, and broccoli are just a few of the fruits and vegetables that have it[28,29]. It has been used in Chinese medicine for a long time. It may help preserve the liver, heart, and brain and improve cancer treatments[29]. The FDA has given quercetin the go-ahead as a dietary supplement. It is a natural antioxidant

flavonoid found in the fruits of Aesculus indica, Codonopsis, Chrysanthemum, and Prunella vulgaris[30]. Numerous studies have demonstrated that quercetin inhibits phosphoinositide 3-kinase (PI3K), NF-κB, and other kinases critical for intercellular signaling. Quercetin is well-known for its medicinal effects on malignant tumors, cardiovascular diseases, and cerebrovascular conditions[31,32]. Chlorogenic acid (CGA) is a polyphenol found in many plants, such as green coffee beans, black tea, fruits, vegetables, olive oil, and spices[33,34]. It is a powerful antioxidant known to have several health benefits, including anti-inflammatory, neuroprotective, and possibly metabolic effects that help keep blood sugar and cholesterol levels in check[34,35]. CGA can also protect the nervous system from diseases that cause nerve cells to die, and it may also assist in protecting the heart and blood vessels[34]. CGA also has characteristics that fight germs, viruses, and tumors[36]. CGA is one of the things that make functional foods and drinks good for you. Chlorogenic acid is a polyphenolic chemical of caffeic acid and quinic acid[37]. It is unstable when it gets hot and breaks down into quinic and caffeic acid[38]. This was obtained through HPLC examination of the material after raising the temperature during extraction. The peak at 18.577 minutes shows chlorogenic acid is thermal degradation into caffeic and quinic acid. This was demonstrated by elevating the concentration of caffeic acid at 7.433 minutes and reducing the concentration of chlorogenic acid. Also, heat speeds up Ferulic acid's breakdown, making it less effective as an antioxidant. As seen in **Table 2** and **Figure 7**, this happened when the temperature was raised, and the peak at 16.770 min disappeared. This makes the extract less effective in terms of biology[39-41]. Thermal Stability was observed at 50 °C, with the extraction maintained at 50°C, preserving the integrity of the compounds, whereas temperatures above 60°C led to the degradation of Ferulic acid (previously at t_R = 16.770 min) and the breakdown of Chlorogenic acid.

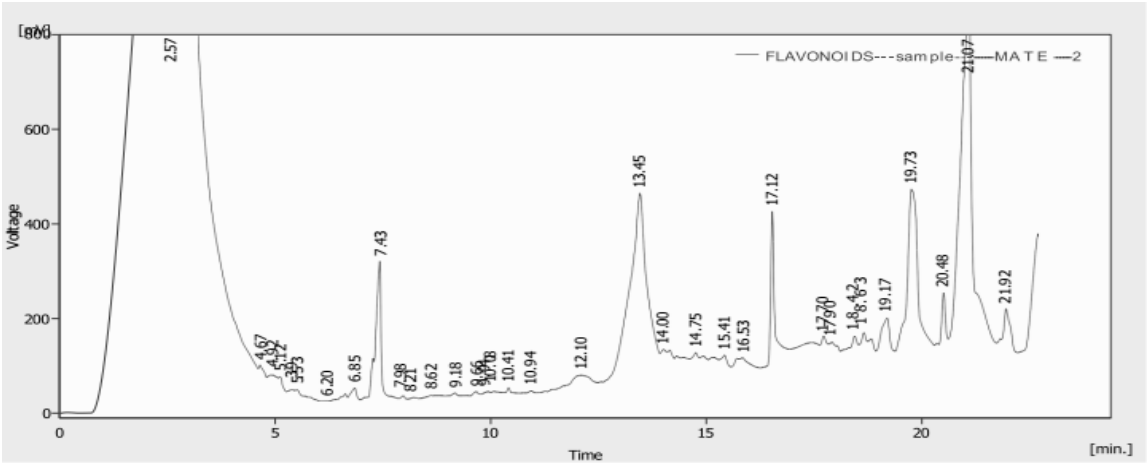

Figure 7. HPLC profile of Phenolic-flavonoid compounds in Mate tea extract when the extraction temperature is increased.

Table 2. Retention times of Phenolic-flavonoid compounds in Mate tea extract when the extraction temperature is increased.

	Reten. Time (min) for standard	Reten. Time (min)	Relative Retention Times. RRT	Amount (mg/L)	Height (mV)	W 0.5 (min)	compound
1	3.513	3.667	1.043	1.086 5	0.4	0.07	GALLIC
2	3.850	3.917	1.017	9.932 3	0.4	0.25	LUTEOLIN
3	4.030	3.957	0.982	171.60	0.7	0.10	RUTIN
4	5.240	5.090	0.971	26.419	0.3	0.11	KAEMPFEROL
5	7.413	7.433	1.002	124.37	3.9	0.10	CAFFEIC
6	10.694	10.477	0.979	47.184	0.2	0.33	QUERCETIN
7	13.617	13.450	0.987	4.800 1	5.6	0.17	COUMARIC
8	16.540	16.527	0.999	5.009 1	0.2	0.13	APIGENIN

	Reten. Time (min) for standard	Reten. Time (min)	Relative Retention Times. RRT	Amount (mg/L)	Height (mV)	W 0.5 (min)	compound
9	16.810	17.120	1.018	21.234	0.7	0.09	CHLOROGENIC

The Relative Retention Times (RRT) values for all compounds in **Tables 1** and **2** were determined to be within the permissible range (0.97 – 1.04). The fact that the RRT values are very close to 1.000 shows that the phenolic compounds in the Mate tea extract were correctly identified relative to their standards. The small changes in absolute retention times are due to slight variations in chromatographic parameters, such as temperature or pressure. These changes do not affect the accuracy of the qualitative analysis.

3.5. Comparative Sustainability Analysis

To assess the environmental sustainability of the developed UAE ultrasound extraction technology, the AGREE prep scale was used to compare it with other traditional and modern extraction methods (**Figure 8**).

3.6. Ultrasound Extraction Method (UAE) - [Score: 0.85]

This process achieved an excellent overall score of 0.85, making it an environmentally friendly alternative to conventional extraction methods. This high level of "greenness" is primarily attributed to the careful selection of a bio- and non-toxic solvent system consisting of an ethanol-water mixture. This reduces the environmental impact compared to chlorinated or petroleum-based solvents, in accordance with Principle 11 of Green Chemistry. Furthermore, the use of ultrasound energy enabled rapid extraction (within 30 minutes) at a moderate temperature (50°C), reducing energy consumption according to Principle 9 and increasing sample yield according to Principle 8. The low liquid-to-solid ratio (7.69 ml/g) also demonstrated the process's effectiveness in minimizing reagent waste, in accordance with Principle 7[42].

Second: Comparative Analysis with Other Methods When comparing these results with other methods, the variation in sustainability criteria becomes clear:

Microwave Extraction (MAE) - [Score: 0.62]: Despite its speed, it scored lower due to the high energy consumption required for heating and the risks associated with high pressure inside the vessels, necessitating more complex safety protocols[43].

Maceration - [Score: 0.58]: Although it saves mechanical energy, it received a low rating due to its short processing time (up to 24 hours) and its consumption of large quantities of solvents that become chemical waste[44].

The Soxhlet method - [Score: 0.42]: was recorded as the least sustainable method due to its need for continuous heating for long periods and its reliance on recycling huge quantities of solvents, which increases its carbon footprint and operational risks[45].

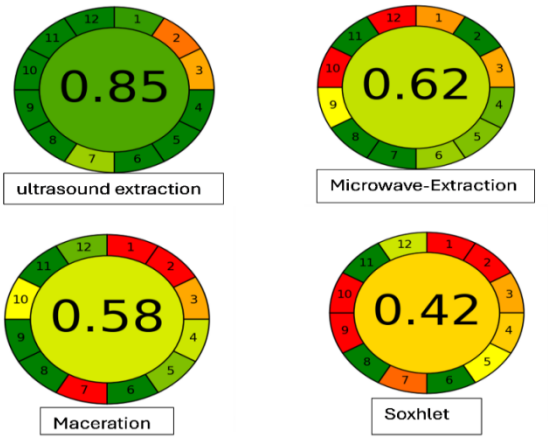

Figure 8. Environmental efficiency assessment (AGREE tool) to compare different extraction methods (ultrasonic, microwave, Maceration, and Soxhlet).

3.7. Model validation

The coefficient values for modified R and projected R^2 were used to see how well the model worked. Analysis of variance (ANOVA) was used to assess the validity of each experimental series and the model. Changed the way phenolic chemicals were taken out of dried Mate tea leaves by changing the ethanol: water ratio, the temperature, and extraction time. The predictive equation from the surface response methodology was used to determine the optimal conditions. The values, ranging from 0.42 mg/g to 3.12 mg/g, represent the total mass of the extract normalized to 1 gram of dry matter. These results indicate that the yield is highly dependent on the ethanol concentration and temperature, which affect the solubility of the total solids, as shown in **Table 3**.

Table 3. Box-Behnken experimental design based on independent variables and experimental and predicted results of the extracted Yield (mg/g).

Experiment	Ethanol/water(v/v)	Temperature C°	Time _min	Extracted yield mg/g
1	50:50	25	20	0.42
2	50:50	25	40	0.65
3	50:50	60	20	0.87
4	50:50	60	40	1.89
5	70:30	42.5	20	0.98
6	70:30	42.5	40	2.21
7	40:60	42.5	20	1.23
8	40:60	42.5	40	3.12
9	70:30	25	30	0.96
10	40:60	25	30	1.11
11	70:30	60	30	0.99
12	40:60	60	30	1.43
13	50:50	42.5	30	1.32
14	50:50	42.5	30	0.88
15	50:50	42.5	30	0.94

3.8. Analysis of Variance (ANOVA)

The ANOVA table shows that the total regression model is marginal significance, which means that the factors analyzed explain a large part of the variance in the answer. To assess the predictive accuracy of the model, an experimental vs. predicted plot was used, which showed a data distribution reflecting a adjusted R^2 value of 0.63. To determine the magnitude of the prediction error, the root mean squared error (RMSE) was calculated, yielding a value of 0.413, representing the average deviation between the observed results and the model's predictions. The low statistical power of the model is attributed to the small sample size (n = 15), a standard and design constraint inherent in the Box-Behnken design used in this exploratory study. Consequently, the model is used as a tool for identifying influencing factors (such as ethanol concentration) rather than as a model for high-precision numerical optimization. The Diagnostic plots showed that the residuals approximately follow a normal distribution (as indicated by Histogram and Normal of Residuals) and are randomly scattered around zero in the Residuals vs Fitted plot. This supports the validity of the regression assumptions and confirms the adequacy of the fitted model. 3D Surface and Contour Plots. The 3D surface and contour plots visualize the combined effects of the factors on the response. For instance,

increasing temperature enhanced the response at certain ethanol levels, while this effect diminished at other concentrations. Similarly, extraction time exhibited interactions with both ethanol percentage and temperature, showing varying influences depending on the factor levels. As shown in **Table 4**, **Figure 9&10**.

Table 4. ANOVA of the response of extracted Mate tea model.

	Sum _sq	**df**	**F**	**PR(>F)**
Ethanol_ pct	1.217959	1	7.124077	0.044397
Temperature	0.8286	1	4.846642	0.078944
Time	0.219943	1	1.28649	0.308129
I(Ethanol_ pct ** 2)	1.159537	1	6.782358	0.047999
I(Temperature** 2)	0.669541	1	3.916275	0.104717
I(Time** 2)	0.418503	1	2.447903	0.178453
Ethanol_ pct: Temperature	0.062411	1	0.365051	0.572079
Ethanol_ pct: Time	0.028737	1	0.168091	0.698789
Temperature: Time	0.156025	1	0.91262	0.383292
Residual	0.854819	5		
R-squared	0.869136			
Adj. R-squared	0.633582			
F-statistic	3.689746			
Prob (F-statistic)	0.082187			
AIC	19.59441			
BIC	26.67492			
No. Observations	15			

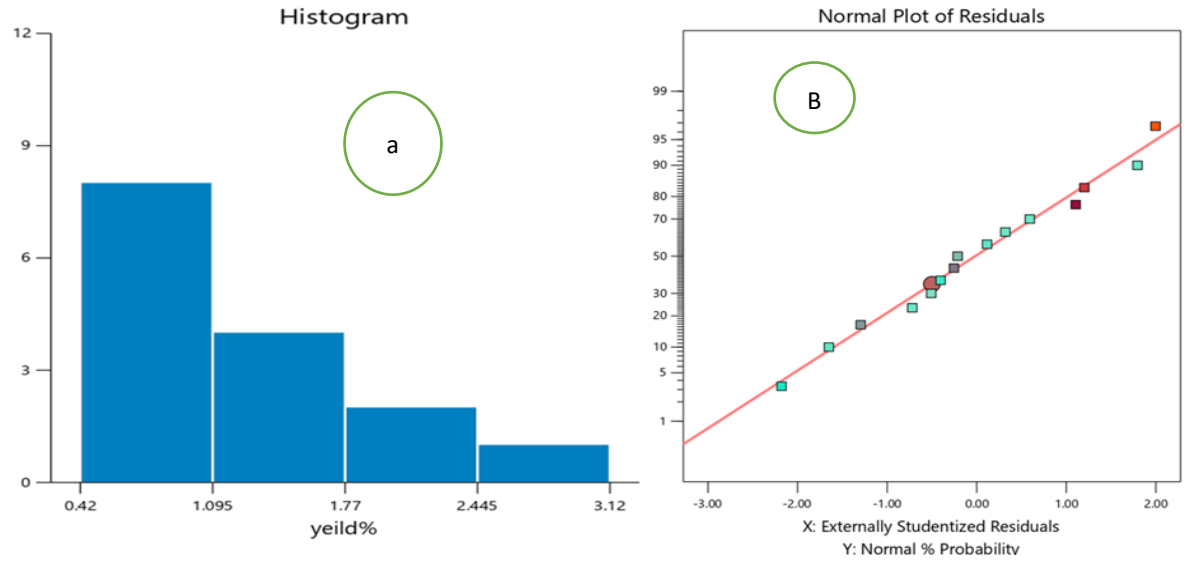

Figure 9. Show the Model Diagnostics Plots a) Histogram of Residuals b) Normal of Residuals.

The histogram (**Figure 9A**) illustrates the distribution of residuals, or the frequency of yield values. The distribution shows a right-skewed slant, with most results concentrated in the range of 0.42% to 1.77%. This indicates that the statistical model used is robust and capable of properly funding the data, and that most experiments produced yields within the expected range, with only a few cases reaching the extreme values

(3.12%).The points in (**Figure 9B)** are distributed approximately straight lines along the reference line. This indicates that errors (Residuals) follow the normal distribution, which confirms the validity of the statistical assumptions of the model and its ability to predict the results accurately without bias.

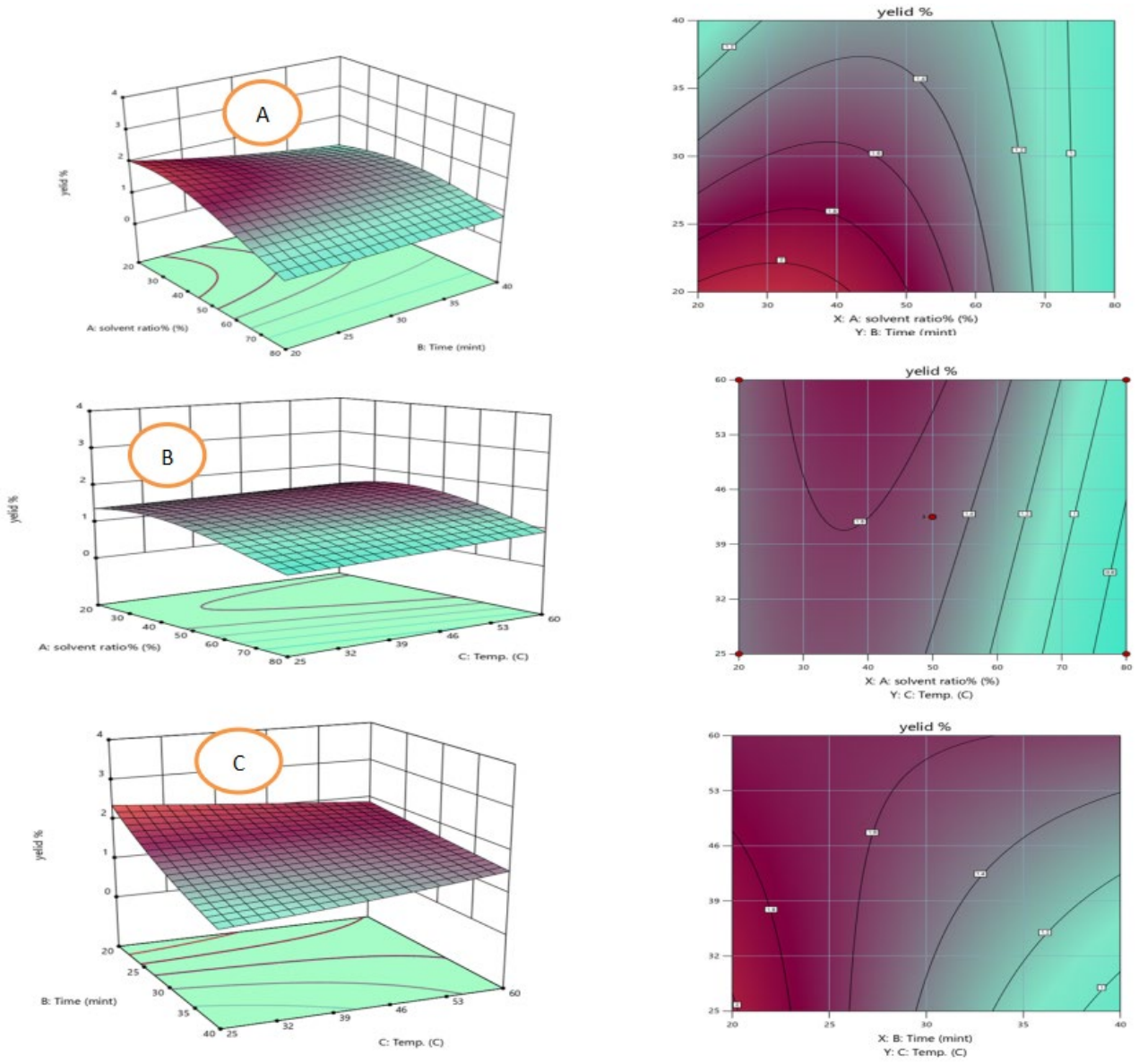

Figure 10. Response surface graphs (left) and contour graphs (right) show how extraction time and temperature affect a 50:50 v/v ethanol/water ratio: (a) ethanol/water ratio and temperature at a 30-minute extraction time; (b) ethanol/water ratio and extraction time at a temperature of 50°C. (c) duration and temperature at a constant 50:50 ethanol/water ratio.

3.9. Analysis of Factors Effect of Figure 10A[46]

1. The Shape of The Response Surface (3D Response Surface) Shows the Presence of Significant Effects of Both The Solvent Ratio (Solvent Ratio) And Time (Time) On The Extraction/Yield Ratio (Yield %). We note through the drawing that the highest value of the crop was recorded at the minimum values of the solvent (20%), where the crop reached its peak (higher than 2%).

2. Effect of solvent ratio: A strong inverse relationship is observed between the percentage of solvent and the crop; the higher the percentage of the solvent from 20% to 80%. The observed inverse relationship between the dielectric constant of the solvent and the yield of bioactive compounds can be explained by the physicochemical properties of the solvent system. Pure water has a high dielectric constant and is only effective with highly polar solutes. Increasing the ethanol content decreases the dielectric constant of the

mixture, thus reducing the polarity of the medium. This modification enhances the solubility of the moderately polar flavonoids and phenolic compounds present in the yerba mate matrix. Consequently, extraction efficiency improves as the dielectric constant of the solvent system decreases to match the polarity of the target compounds.

3. Effect of Time: For the time factor, the curve shows a less severe response compared to the solvent. However, the yield tends to rise at short time intervals (about 20-25 minutes) under low solvent ratios. This indicates that the process reaches Equilibrium quickly, and that increasing the time after that (up to 40 minutes) does not lead to a substantial improvement in the results, but may cause a slight decrease as a result of an increased likelihood of decomposition of the extracted compounds or saturation of the solvent.

4. Interaction between factors (interaction effect): By the curvature of the response surface and the contour lines (Contour Plot) at the base, there is a mutual interaction between the time and the ratio of the solvent. The area with a dark purple color represents "Optimal Conditions", which is when the minimum values of both variables overlap in this studied range.

3.10. Analysis of Factors Effect of Figure 10B[47]

1. Analysis of the relationship between temperature and solvent ratio:The response surface curve (3D Surface Plot) shows a curve pattern (Curvilinear pattern) that shows a strong overlap between the two variables. It is noted that an increase in temperature from 25 °C towards 60 °C leads to an initial improvement in the yield (Yield %), but this improvement reaches a certain peak (Optimum) and then begins to stabilize or decrease slightly at very high temperatures, especially with the increase in the percentage of solvent.

2. Scientific explanation: Temperature effect Increasing the temperature enhances the extraction efficiency by reducing the viscosity of the solvent and increasing the diffusion coefficient, which facilitates the solvent to penetrate the solid and extract the target compounds. However, exceeding the ideal limit may lead to thermal degradation of the heat-sensitive components, which explains the retreat of the curve at the super end of the (C) axis. Effect of solvent ratio: The graph shows that productivity is at its highest levels at low to medium solvent ratios (20%-40%). A significant increase in the solvent (up to 80%) leads to a decrease in the yield, which can be attributed to the “dilution effect” or reaching a state of polar imbalance between the solvent and the solute.

3. Interaction effect: Elliptical Contour Lines (Elliptical Contour Lines) in the base of the drawing indicate the presence of a significant interaction between temperature and solvent ratio. This means that the effect of temperature on the crop is not constant, but depends largely on the proportion of solvent used in the process

3.11. Analysis of Factors Effect of Figure 10C[48]

1. Effect temperature ncreasing heat reduces the viscosity of the solvent and increases the movement of molecules (Kinetic energy), which enhances the diffusion rate within the hard pores of the plant matter. This leads to greater thawing of target vehicles and improved mass transfer.

2. Solvent Ratio Effect: The first figure (oblique violet curve) shows that the highest productivity is achieved at the lowest solvent (about 20%).This may indicate that the target substance dissolves better in a specific mixture (such as a mixture of ethanol and water). Significantly increasing the percentage of organic solvent may reduce the polarity of the medium to the extent that it does not allow the effective extraction of the required ingredients, or it may cause the precipitation of certain proteins or sugars that hinder the extraction process.

3. Time Effect: In the second figure, the effect of time (axis B) appears as a positive factor but with limits. Extraction increases over time at first, then the surface begins to level (Plateau). The extraction process follows the principle of Fick's Law of Diffusion, where the extraction is initially rapid due to the difference in high concentration, and then slows down as the system approaches the equilibrium between the solid and the solvent.

4. Conclusion

This study has established that ultrasonic extraction (UAE) is an effective and sustainable method for extracting bioactive polyphenols from yerba mate leaves. Response surface modeling (RSM) showed that the best conditions for extraction were 50°C for 30 minutes with a 50:50 v/v ethanol-water mixture. Model validation demonstrated a robust correlation between predicted values and experimental data, thereby affirming the precision of the optimization process and the dependability and reproducibility of the methodology. Also, high-performance liquid chromatography (HPLC) demonstrated that the procedure effectively separated and quantified key polyphenols, including gallic acid, caffeine, and quercetin. It also showed how temperature affects the quality of the extract. From a sustainability perspective, the greening assessment using the AGREE prep scale awarded the developed protocol a high score of 0.85 (while traditional methods such as Soxhlet and leaching scored significantly lower at 0.42 and 0.58, respectively), indicating a minimal environmental impact. This result is attributed to energy efficiency, waste reduction, and the use of safe bio solvents. In conclusion, this study presents a proven, robust, and environmentally sustainable analytical methodology suitable for extracting high-quality plant compounds in both academic and industrial applications, opening new avenues for developing natural products in ways that protect both the environment and operator health.

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Conflict of interest

The authors declare no conflict of interest.

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