RECyT
Year 26 / Nº 42 / 2024 / 18–27
DOI: https://doi.org/10.36995/j.recyt.2024.42.002

Successive concentrations of phenolic compounds of 'Yerba Mate' (*Ilex paraguariensis*) and their contribution to antioxidant capacity

Concentración sucesivas de compuestos fenólicos de la Yerba Mate (*Ilex paraguariensis*) y su contribución a la capacidad antioxidante

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Received: 18/05/2024; Accepted: 01/08/2024

Abstract

Four successive concentrations of yerba mate extract were made at 30, 50 and 70 °C, adding new yerba mate at the beginning of each concentration. The content of total phenolic compounds (TPC) was evaluated with the Folin-Ciocalteu method and the antioxidant capacity (AOC) with the DPPH (2.2-diphenyl-1-picrylhydrazyl) free radical assay.

The results of this work showed that TPC increased significantly with increasing temperature, while AOC decreased ($p<0.05$). The TPC extracted was highest at 70 °C, obtaining a value of 38.06 ± 1.56 g EAG/L, and at 30 °C 25.50 ± 0.14 g EAG/L was obtained. The AOC showed a decreasing trend with increasing temperature. Values ranging from 94.39 ± 0.65 g EAA/L at 30 °C to 52.53 ± 0.09 g EAA/L at 70 °C were obtained. For the four times concentrated extracts, the values were (39.33 ± 6.71) % (TPC) and (28.48 ± 0.99) % (AOC) higher than those of the non-concentrated extracts. We conclude that the successive extraction technique provides, by increasing the number of extractions, an effective way to produce extracts rich in polyphenols and high antioxidant capacity using moderate temperatures during the extraction process, avoiding thermal degradation of the extract.

Keywords: *Ilex paraguariensis*; Phenolic compounds; Antioxidant capacity; Extracts; Concentration successive.

Resumen

Se realizaron cuatro concentraciones sucesivas de extractos de yerba mate a 30, 50 y 70 °C, agregando yerba mate nueva al inicio de cada concentración. Se evaluó el contenido de compuestos fenólicos totales (CFT) con el método de Folin-Ciocalteu y la capacidad antioxidante (CAO) con el ensayo del radical libre DPPH (2.2-diphenyl-1-picrylhydrazyl). Nuestros resultados mostraron que el CFT se incrementó significativamente con el aumento de la temperatura, mientras que la CAO disminuyó ($p<0.05$). El CFT extraído fue máximo a 70 °C, obteniéndose un valor de 38.06 ± 1.56 g EAG/L, y para 30 °C se obtuvo 25.50 ± 0.14 g EAG/L. La CAO mostró una tendencia decreciente con el aumento de la temperatura, obteniéndose valores desde 94.39 ± 0.65 g EAA/L para 30 °C, hasta 52.53 ± 0.09 g EAA/L para 70 °C. En los extractos concentrados cuatro veces los valores fueron (39.33 ± 6.71) % (CFT) y (28.48 ± 0.99) % (CAO), superiores que en los extractos sin concentrar. Concluimos que la técnica de extracciones sucesivas proporciona, al aumentar el número de extracciones, una forma eficaz de producir extractos ricos en polifenoles y alta capacidad antioxidante usando temperaturas moderadas durante el proceso de extracción, evitando la degradación térmica del extracto.

Palabras claves: *Ilex paraguariensis*; Compuestos fenólicos; Capacidad antioxidante; Extractos; Concentraciones sucesivas.

Introduction

Phenolic compounds are the most abundant functional secondary metabolites in yerba mate, mainly caffeic acid derivatives (3.5-dicaffeoylquinic, 4.5-dicaffeoylquinic, 3.4-dicaffeoylquinic and chlorogenic acids) and flavonoids (rutin, quercetin, kaempferol and luteolin) [1,2]. Some of the biological properties of yerba mate aqueous extract are attributed to the presence of these compounds: antioxidant properties [1,3–5], anti-cancer effects [6] and protection against lipid peroxidation (LDL) [7]. Research has shown that consumption of yerba mate extracts protects against

free radical reactivity and this antioxidant capacity is mainly attributed to polyphenolic compounds [8]. Antioxidant capacity has been evaluated with different variables such as the technique used to obtain the extract [9,10], concentration of solids in the extract [11], temperature [12] and types of solvents [13]. Different studies on chemical and biological systems have reported the antioxidant capacity using methods based on the evaluation of their free radical scavenging capacity (DPPH and ABTS) [14–16] methods measuring metal reduction capacity (FRAP) [14–17], others measuring oxygen radical absorbance capacity (ORAC) [18] and others quantifying the products generated during lipid peroxidation (TBARS) [19–20]. Due to these and other known biological functionalities, the aqueous extract of yerba mate has started to be used as a potential natural source of bioactive compounds with high antioxidant capacity for food, cosmetic and phytomedicine formulations [1,21,22]. The technological processes that lead to the production of these products involve the extraction of components from the leaves, which guarantee the efficient removal of the bioactive components. The solid-liquid extraction of the bioactive component attached to yerba mate leaves is a complex process involving various phenomena (solubility, washing, diffusion), where the type of solvent, solid matrix and temperature influence the yield of the extract [23–25]. These extracts are then used to develop new products, being subjected to decolorization and drying processes, losing part of their bioactive components [26–29], making it necessary to have extracts with a high concentration of soluble solids to ensure the quality of the final product. The aim of this study was to obtain a concentrated aqueous extract of yerba mate, using successive extractions, evaluating the influence of temperature and number of extraction stages on the content of total phenolic compounds (TPC) and antioxidant capacity (AOC) in the concentrated extracts obtained.

Materials and Methods

Plant material and preparation of extracts

Commercial yerba mate with sticks of local origin was purchased in supermarkets located in Posadas, province of Misiones, Argentina. To perform the extractions, a thermostatic system was used to control the temperature by means of a SCHOTT GERATE bath, model CT1150, with a differential temperature control system. The reactor was a 2000 ml beaker closed at the top with aluminum foil to prevent evaporation of the liquid and with a flat bottom that remains submerged in the water. Stirring was carried out by means of a 60 mm diameter, three-bladed propeller stirrer with a rotation speed of 7.5 rpm. The reactor temperature was controlled by an electronic thermometer, with an accuracy of 0.1 °C. The water used as extraction solvent was purified using an ultrafiltration system (Romi 100, Hidrolit, Argentina). All other solvents used were of analytical grade. The sequence of four solid-liquid extractions was carried out using water as solvent with a solid/liquid ratio of 1/8 weight/volume at temperatures of 30, 50 and 70 °C (Figure 1). The first extraction was carried out with 1.6 liters of water and 200 g of yerba mate; this suspension was subjected to constant agitation in a water bath at the working temperature for 40 minutes, sufficient time to obtain the maximum extraction of solids under these conditions [23]; the extract C1 was obtained by filtering the suspension of water and yerba mate. C1 was used to perform a new extraction, adding only yerba mate (YMN) to maintain the yerba/water ratio 1/8; in this way a second filtrate (C2) was obtained with a higher concentration of soluble solids than the previous one. The procedure was repeated successively to obtain extracts C3 and C4, thus reaching the maximum concentration of soluble solids allowed by the technique.

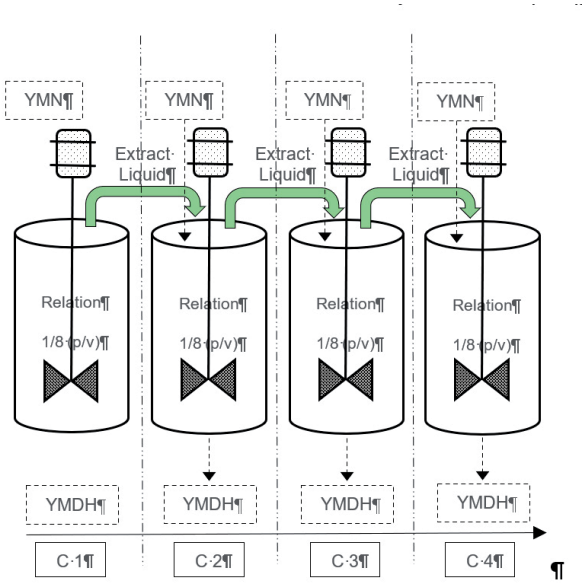

Figure 1: Bach extraction process at three test temperatures (30, 50 and 70 °C). YMN: new yerba mate; YMDH: wet discarded yerba mate; C: concentration.

Total phenolic content (TPC)

Reagents

The reagents used for total polyphenols content analysis were Folin Ciocalteu (Biopack, Argentina), anhydrous gallic acid (Biopack, Argentina), anhydrous sodium carbonate (Emsure, Germany) from Merck (Darmstadt, Germany).

Determination of total phenolic content (TPC)

The TPC was determined by the Folin-Ciocalteu method (ISO 14502-1-2004). Each sample extract was diluted with water at a ratio of 1:500. One milliliter of

the diluted sample extract was transferred into separate tubes containing 5 mL of Folin-Ciocalteu reagent diluted in water (10% v/v). Then, 4 mL of a sodium carbonate solution (7.5 % w/v) was added. The tubes were allowed to stand at room temperature for 60 min before measuring the absorbance at 765 nm using distilled water as blank. The concentration of polyphenols in the samples was derived from a gallic acid standard curve in the range 0 and 50 µg /mL (R^2=0.99). The determinations were performed in triplicate and the total polyphenol content concentration was expressed as gallic acid equivalent per liter (g GAE/L).

Antioxidant capacity (AOC)

Reagents

Methanol (Merck, Argentina), 2.2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, USA) and ascorbic acid (Sigma Ultra, Argentina) were used. All reagents used were of analytical grade.

Determination of AOC by DPPH assay

The AOC of the extracts were determined as a measurement of radical scavenging using the DPPH radical. For this, 100 µL of an aqueous dilution of the extracts was mixed in duplicate with 3.0 mL of a DPPH work solution in absolute methanol. The mixture was incubated for 120 minutes in the dark at room temperature, and the absorbance was then measured at 517 nm against absolute methanol. For the blank probe, the 100 µL of yerba maté extracts were replaced with 100 µL of absolute methanol.

Mathematical models were used to evaluate the effect of extraction temperature on the free radical scavenging capacity of yerba mate extracts obtained through successive concentrations.

Relationship between TPC and AOC

The analysis of the AOC with the DPPH radical involves high economic values and a long assay time. The relationship between TPC and AOC was determined in order to find a quicker and less costly way. Correlations between the variables were established by regression analysis.

Statistical analysis

Results are expressed as the mean and standard deviation (SD) of three replicates. Significance of differences between sample means was determined by analysis of variance (ANOVA) followed by Tukey's test. The influence of temperature on the AOC of yerba mate extracts was analyzed by linear regression. The adjustment of the experimental data of AOC by linear regression was evaluated with the mean relative percentage (E%) and Durbin Watson statistic, which is defined by Eqs. 1 and 2.

$$EP\% = \frac{\sum_{i=1}^{n} \frac{(c_{cal} - c_{\exp})n}{c_n}}{n} x100 \quad (1)$$

$$DW = \frac{\sum_{t=2}^{T}(e_i - e_{i-1})^2}{\sum_{t}^{T} e_i{}^2} \quad (2)$$

where c_{cal} is the experimental value, c_{exp} is the predicted value, and n is the number of experimental data, e_t is the residual associated with the observation over time t; T is the number of observations.

Linear and non-linear correlation from regression analysis was performed to verify the relationship between AOC and TPC. Differences were considered statistically significant when $p<0.05$. All statistical analyses were performed using Statgraphics Centurion XVII, version 17.2.00 and Graph Pad Prism, version 5.04 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

Results and Discussion

Total phenolic content (TPC) in the concentrated yerba mate extract

The TPC values for the different extracts obtained in the successive extractions, expressed in g EAG/L, are shown in Figure 1. Of the extracts prepared with the successive batch extraction system, the fourth extract obtained by concentration (C4), showed the maximum amount of TPC content (38.06 g EAG/L) at the highest temperature (70 °C), of the experimental study.

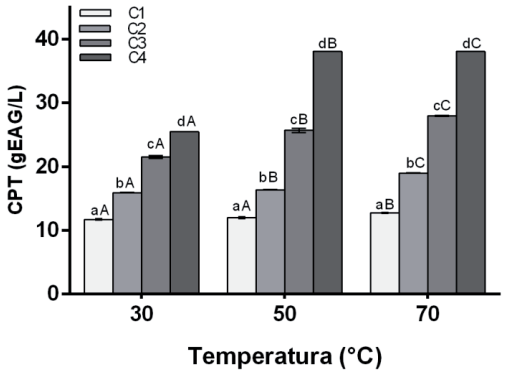

Figure 2: Total phenolic content (TPC) (g EAG/L) of yerba mate extracts obtained at different successive extractions and temperatures. The bars represent the mean ± standard deviation of three independent experiments performed in triplicate. Values with different letters (a-d; extraction number (C1, C2, C3 and C4) and (A-C; Temperature) indicate significant differences ($p < 0.05$).

Figure 2 shows the progressive increase in TPC content values obtained with the successive extraction method. The results obtained showed a high concentration of phenolic

compounds in the different yerba mate extracts studied; however, it can only be compared with work carried out with a single extraction (C1), due to the lack of previous research related to successive extractions.

Arancibia *et al.* (2024) [30] found similar values, using different single-stage extraction methods. The mean total polyphenol content values are shown in table 1, where the results and extraction parameters of the study that reported similar values are included for comparison.

Table 1: Comparison of mean TPC values from this work and other authors.

Reference	T (°C)	YM/W (g /L)	t (min)	Extraction method	Solvent	TPC (mg EAG/ L)
[30]	90	150	5	ES	Water	8500
				EAM		9200
				US		11300
				EUS+AM		11000
This work	30-50 / 70	125	40	EAMe	Water	11876* / 12739

*Mean TPC value for temperatures of 30 and 50 °C ($p>0.05$) for C1. Simple extraction (ES); Extraction assisted by magnetic stirring (EAM); Extraction assisted by ultrasound and magnetic stirring (EUS + AM); Ultrasound (US); Extraction assisted by mechanical stirring (EAMe).

Table 1 shows that extraction with EAMe is similar to US and EUS + AM for C1, working under conditions of lower temperature and concentration with longer extraction time. The differences in the different working conditions and raw materials used to obtain plant compound extracts show differences in the results of the amounts of the components evaluated. In yerba mate the chemical composition varies, the types and amount of phenolic compounds present will differ depending on maturity, place of production, agricultural practices, as well as other environmental factors, manufacturing, and infusion preparation [31,32]. Previous research reported elevated levels of phenolic compounds in extracts obtained from *Ilex paraguariensis* [33– 35], but comparison of results is difficult due to the different ways of expressing them. Also, other authors have reported lower contents of total phenolic compounds in yerba mate extracts [36–38].

The influence of the number of concentrations and temperature on the content of total phenolic compounds (TPC) of yerba mate extracts was studied. ANOVA indicated that there is a difference in total polyphenol content concentration with temperature and number of extractions ($p<0.05$). It was observed that the first extract (C1), obtained by pressing the suspension of leaves, sticks and water, did not show significant differences in the temperature range of 30 and 50 °C; however, at 70 °C the difference in total polyphenol content concentration was slightly higher ($p<0.05$). López *et al.* (2020) [34] in a study of aqueous extraction in the thick leaf fraction of yerba mate, evaluated the kinetics of batch concentration of total polyphenol content in a single extraction until equilibrium was reached. The study showed that there was no significant difference in total polyphenol content concentration in the range of 40 to 70 °C after 60 minutes. These differences with this study can be attributed to the times employed, the heterogeneity of the yerba sample of the present investigation, which includes industrially processed leaves and sticks under prolonged parking and the successive concentration technique. Also, the harvesting, drying and storage conditions of the yerba mate leaves used as samples for the extracts may affect the total polyphenol content obtained experimentally [39,40].

Temperature has a positive effect on the extraction of phenolic compounds from plant sources [41, 42]. Gerke *et al.* (2018) [24] analyzed the extraction kinetics with one-step total polyphenol content shaking in commercial yerba mate samples and suggested the use of higher temperature as an optimization parameter to accelerate the extraction rate. The increase in total polyphenol content concentration can be attributed to the effect of temperature on the permeability of the cell wall that facilitates the diffusive and release mechanism of these compounds, due to the weakening of interactions with their associated compounds [28] and to the improved contact of the solvent with the plant material [34], therefore, the total polyphenol content concentration is controlled by an intraparticle phenomenon at equilibrium [24].

In these experiments, it was observed that the final extract (C4) obtained at the highest extraction temperature (70 °C) increases its concentration, tripling the quantified value of total polyphenol content in the starting extract (C1). Pagliosa *et al.* (2010), [43] found high total polyphenol content values in aqueous and methanolic extracts at 85 °C, confirming that the use of elevated temperatures favors the extraction mechanisms. Moreover, according to these results, the higher polyphenol content of the extracts resulted in a higher antioxidant capacity, when evaluating a single extraction temperature.

Other studies on *Ilex paraguariensis* extracts tested the influence of temperature on extraction parameters of soluble solids [23], caffeine [44,45] and chlorogenic acid [46,47].

Boaventura *et al.* (2013) [10] evaluated the effects of freezing concentration of the aqueous extract of yerba mate leaves on the content of bioactive components and the antioxidant capacity of the concentrated fluid and the ice obtained. The concentrated fluid showed increasing values of phenolic compounds at all stages of freezing concentration. In this study, the total polyphenol content increases with successive concentrations of the extracts, even using a different technique to the type of concentration used by these authors. However, it is relevant to consider the synergistic effect of temperature with increasing concentration of yerba mate in the solvent at each stage.

A higher proportion of solids leads to the formation of creamy deposits, due to the interaction of proteins and colloidal glycosides (saponins) [48,28]. These solid

deposits contribute to the development of turbidity in the concentrated extract, which can affect subsequent filtration, purification, and stabilization processes [28,49]. At the same time, extraction at elevated temperatures may generate different phenolic profiles or have a negative impact on thermosensitive components [50]. Provided that the process is carried out in a temperature range that does not lead to total polyphenol content degradation, batch extraction with agitation and elevated temperature is an effective technique for total polyphenol content concentration in plant matrices such as *Ilex paraguariensis*.

Antioxidant capacity (AOC)

The effect of antioxidants was evaluated through their antiradical capacity or antioxidant activity. The decrease in DPPH concentration is an index to estimate the radical scavenging capacity of plant extracts [51]. The results of the AOC measured by the DPPH method of yerba mate extracts obtained from one to four extractions, at three different temperatures, are given in table 2.

Table 2: AOC by DPPH assay (mg/100 mL) of yerba mate extracts obtained using different extraction concentrations and temperature.

Number of concentrations	Temperature (°C)		
	30	50	70
C1	2840 ± 38^{Aa}	1961 ± 35^{Ba}	1465 ± 36^{Ca}
C2	3290 ± 32^{Ab}	3081 ± 64^{Bb}	2630 ± 40^{Cb}
C3	$7356 \pm 79A^{c}$	6363 ± 25^{Bc}	5306 ± 49^{Cc}
C4	9634 ± 65^{Ad}	6984 ± 12^{Bd}	5253 ± 19^{Cc}

Values are means ± SD of duplicate samples. Mean values with different lower-case letters in the same column and different upper-case letters in the same row are significantly different ($p<0.05$).

There is a statistically significant relationship between antioxidant capacity, temperature, and number of concentrations for a confidence level of 95.0%. The AOC measured by the DPPH method showed that all yerba mate extracts could inhibit DPPH free radicals. Generally, the first concentrations showed a higher capacity to inhibit DPPH radical oxidation for all temperatures, but this increases with the number of concentrations and decreases with increasing temperature.

During extraction at higher temperature, the amount of some antioxidant compounds or their structures might have been modified, decreasing the AOC of the whole sample. Katarzyna Janda *et al.* [52] examined the antioxidant capacity of yerba mate extracts from different countries and preparation methods at temperatures of 25 °C and 85 °C, observing that for yerba mate of different origins the AOC is lower the higher the temperature of the water used in the extract, thus coinciding with these results.

The adjustment of the experimental data of AOC by linear regression shows that the extraction temperature has a significant effect on the antioxidant capacity, finding a good fit to linear equations, as can be seen in their values of correlation coefficient and percentage error. In the analysis of residuals, no outliers are detected, and the Durbin Watson test indicates that the residuals are independent ($p>0.05$). The results are shown in the table 3.

Table 3: Variation of AOC as a function of temperature for each concentrated extract.

Extract	A	b	R^2	Percentage error	DW	P- P-value of DW
C1	3807.91	34.41	0.99	4.41	1.77	0.15
C2	3824.51	16.52	0.97	1.82	1.61	0.09
C3	8904.32	51.20	0.99	0.61	1.92	0.21
C4	12767.71	109.50	0.99	2.91	1.69	0.12

* DW: Durbin-Watson statistic p >0.05: no indication of serial autocorrelation in the residuals at 95.0% confidence level.

Correlation between AOC and TPC

In order to evaluate whether there is a correlation between AOC and TPC in yerba mate extracts, simple mathematical models were used. The experimental data fitted the presented models adequately. The antioxidant capacity, under the working conditions, can be described by the following relationships.

$$AOC_{30\,°C} = (-4039 \pm 957) + (523.2 \pm 49.1) * TPC_{30\,°C} \ (R^2=0.95) \quad (3)$$

$$AOC_{50\,°C} = (-1280 \pm 367) + (274.8 \pm 16.1) * TPC_{50\,°C} \ (R^2=0.98) \quad (4)$$

$$AOC_{70\,°C} = (-4588 \pm 1415) + (553.1 \pm 125.3) * TPC + (-7.7 \pm 2.5) * TPC^2_{70\,°C} \ (R2=0.96) \quad (5)$$

The values of the correlation coefficients (R^2-30 and 50 °C) show that a linearity was found between both methods for the yerba mate extracts, while for 70 °C a coefficient of determination (R^2-70 °C) was found that showed a better fit to a quadratic equation. No outliers were detected in the residual analysis and the Durbin Watson test indicated that there is no serial autocorrelation between the residuals ($p>0.05$). The relationship between antioxidant capacity and total polyphenol content depends on each antioxidant source [53] therefore, for diverse types of samples and different extraction conditions, a new fit of the experimental data to the mathematical models must be performed. Previous research reported that there is a correlation between the total polyphenol content and the antioxidant capacity of different plant extracts [51,54,17]. Studies by other author indicated that a parallel increase is found between total polyphenol content and antioxidant capacity [55,56]. This increase in antioxidant capacity may be due to the increase in total polyphenol content. Bravo *et al*. (2007), [14] pointed out that the antioxidant capacity of mate is mainly related to the presence of TPC and reported good correlations.

Furthermore, Boaventura *et al.* (2013) [10] found a significant correlation ($p<0.05$) between TPC and AOC

measured by two assays, FRAP and DPPH (R^2=0.99 and R^2=-0.84), in yerba mate extracts. In several studies, significant positive correlations have been observed between total phenolic compound and antioxidant capacity from other matrices [57–59]. However, Atoui *et al.* (2005) [60] found no correlations and attributed this to compounds other than polyphenols as responsible for the antioxidant capacity. Parejo *et al.* (2000) [61] reported that the antioxidant properties of individual compounds within a group can vary markedly, so that the same levels of polyphenols do not necessarily correspond to the same antioxidant responses; the DPPH assay is not always related to the TPC. This may be because the AOC of phenolic compounds depends on their chemical structure, as well as the number and orientation of hydroxyl groups attached to their aromatic rings [62] and is not only the result of the polyphenol content, but also a combination of other compounds, such as peptides, organic acids, caffeine, saponins and others [63]. Regarding the effect of temperature on AOC in these experiments, as the temperature increased, the AOC decreased; the most concentrated extract obtained at the highest temperature was the richest in total phenolic compounds and had the lowest antioxidant capacity.

These results suggest that the AOC of the extracts can be attributed to the presence of non-phenolic compounds. In addition, individual phenolic compounds may have different antioxidant activities; there may be antagonistic or synergistic interactions between phenolic compounds and other compounds such as carbohydrates, proteins, and others [64]. Boaventura *et al*. (2013) [10] showed increasing amounts of TPC and AOC at all stages of concentration by freezing. Other authors also observed increased TPC and AOC from aqueous extracts of yerba mate leaves and bark after membrane concentration [65-67].

All these investigations were carried out with extractions at a single temperature, coinciding with this study, if we compare the results considering each extract separately at each of the temperatures tested. In another type of food with high polyphenol content, they reported that as the temperature of the treatments increased, the antioxidant capacity decreased, due to the fact that these compounds are sensitive to high temperatures [68]. How AOC is affected by increasing temperature is important in view of its practical desirability [50]. Despite considerable differences in the research methods used to demonstrate the AOC of yerba mate, the results obtained in all studies clearly indicate its high antioxidant capacity. The results of this study are consistent with the literature data.

Conclusions

The results show that successive extractions and increasing the extraction temperature increase the polyphenol content, while the availability of these as antioxidants decreases with increasing temperature. The most efficient conditions for the extraction process are to carry out four successive concentrations at 30º C.

The technique of successive extractions allows optimization of the extraction conditions to produce an extract rich in polyphenols and high antioxidant capacity using moderate temperatures during the extraction process, avoiding thermal degradation of the extract. Therefore, the design of an industrial successive batch extraction system would offer the operational possibility of adapting the quality and composition of the extract through precise control of the temperature conditions, solid proportion, and discontinuous operation, which ensures the stability of the extract concentrate.

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