

Optimization and Validation of a Microscale Method for the Quantification of Total Phenols in Plant Extracts

Optimización y Validación de un Método a Microescala para la Cuantificación de Fenoles Totales en extractos vegetales

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ABSTRACT

Phenolic compounds are found in plants and possess a high antioxidant capacity. Many methodologies have been reported for total phenol quantification; however, the effect of small modifications in the various variables involved in the procedure is often not evaluated. Hence, this work proposes to optimize the various variables of the analysis, perform validation, and evaluate its application in plant extracts. Water was chosen as the dissolution medium, and the reaction volume was reduced to 200 μ L. The volumes of the Folin-Ciocalteu reagent (33.5 μ L) and 20 % sodium carbonate (86.5 μ L), as well as the reaction time (46.5 min), were optimized. Under these conditions, calibration curves were created, and linearity, precision, accuracy, detection limit, and quantification limit were established. The robustness of the method and the effect of the matrix were evaluated, revealing that the method is robust, although there is matrix interference. It was concluded that quantification should be performed using a standard addition curve.

KEY WORDS: Total Phenols; Folin-Ciocalteu; Microplate Optimization; Microplate Validation.

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RESUMEN

Los compuestos fenólicos se encuentran en las plantas y poseen una alta capacidad antioxidante. Se han reportado diversas metodologías para la Cuantificación de Fenoles Totales, sin embargo, es común que no se evalúe el efecto que tienen las pequeñas modificaciones de las diversas variables involucradas en el procedimiento. Por este motivo, en este trabajo se propuso optimizar las diversas variables del análisis, realizar la validación y evaluar su aplicación en extractos de plantas. Se seleccionó el agua como medio de disolución y se redujo el volumen de reacción hasta 200 μ L. Se optimizó el volumen del reactivo de Folin-Ciocalteu (33.5 μ L), el volumen de carbonato de sodio al 20 % (86.5 μ L) y el tiempo de reacción (46.5 min). Bajo estas condiciones se realizaron curvas de calibración y se estableció la linealidad, la precisión y la exactitud, el Límite de Detección y Límite de Cuantificación. Se evaluó la robustez del método y el efecto de la matriz, determinando que el método es robusto, pero existe interferencia de la matriz. Se concluyó que la cuantificación se debe realizar por una curva de adición estándar.

PALABRAS CLAVE: Fenoles Totales; Folin-Ciocalteu; Optimización en Microplaca; Validación en Microplaca.

Introduction

An antioxidant is a compound that, at low concentrations, prevents or reduces oxidation. This action holds significant biomedical importance, as its utility has been demonstrated in the treatment of major diseases such as cancer, Alzheimer's, Parkinson's, diabetes, and rheumatoid arthritis, among others, additionally, antioxidants exhibit various properties such as neuro-, nephron-, hepato-, and cardio-protective, and are even used in anti-aging treatments (Liu *et al.*, 2018).

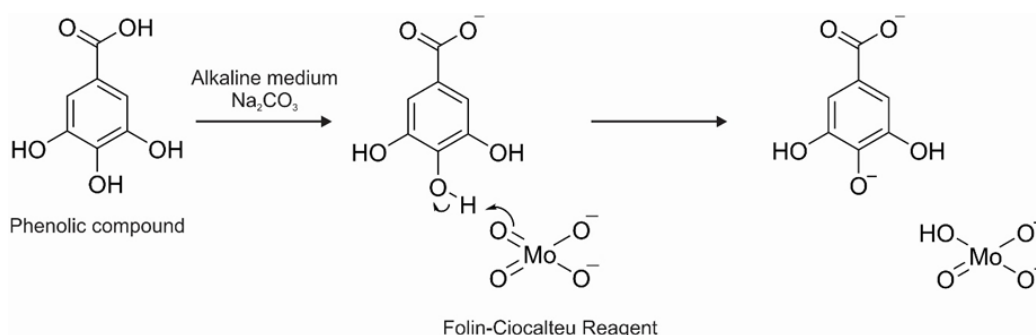
These compounds are abundantly found in the plant kingdom, primarily as phenolic compounds, which are usually consumed through diet. Consequently, many studies have concentrated on the daily consumption of plant products to identify, quantify, and confirm the antioxidant activity of phenolic compounds (Ponnampalam *et al.*, 2022). The biosynthetic pathway of phenolic compounds is shared among bacteria, fungi, plants, and animals, involving both the polyketide and shikimic acid pathways. Phenolic or polyphenolic compounds are classified based on the type and number of phenolic rings they contain and how these rings are linked. In general, they fall into four main categories: phenolic acids, flavonoids, stilbenes, and lignans (Pandey & Rizvi, 2009). Polyphenolic compounds can exert their antioxidant activity through various

mechanisms, including free radical scavenging, singlet oxygen quenching, transition metal chelation, and enzyme inhibition, among others (Mucha *et al.*, 2021).

Although the quantification of total phenols is one of the parameters used to describe the antioxidant capacity of plant material, a high total phenol content does not necessarily indicate strong antioxidant activity. To confirm antioxidant potential, additional assays are required that involve different mechanisms, such as scavenging of the 2,2-diphenyl-1-picrylhydrazyl or N,N-dimethyl-p-phenylenediamine radicals, removal of reactive oxygen or nitrogen species, or assays like 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)/Trolox[®] equivalent antioxidant capacity, ferric reducing antioxidant power, and cupric reducing antioxidant capacity, among others (Apak *et al.*, 2016).

The quantification of total phenols in plant extracts using the Folin-Ciocalteu method is based on the ability of phenolic compounds present in the sample (standard or extract) to reduce the Folin-Ciocalteu's reagent (a mixture of phosphotungstic and phosphomolybdic acids) under alkaline conditions (Scheme 1). This reaction produces a broad blue band that absorbs between 750 and 765 nm (Pérez *et al.*, 2023), with the absorbance being proportional to the phenol content.

To quantify total phenols in a plant extract, a calibration curve is first constructed using an external standard with a phenolic compound such as pyrocatechol, gallic acid, quercetin, or Trolox, among others, then, several dilutions of the plant extract (e.g., 1:2, 1:4) are prepared, each undergoing the same procedure. The obtained absorbance values are interpolated into the external standard curve, and the results are expressed as the equivalent weight of the chosen phenolic compound (e.g., pyrocatechol) per gram (or per 100 g) of extract (Rumpf *et al.*, 2023). This method is simple, as it involves few variables. However, individual laboratories often introduce "slight modifications" to their parameters without evaluating their impact on the response, in addition to using different phenolic compounds as standards.



Scheme 1. Reduction reaction of Folin-Ciocalteu's Reagent.

Despite being a very simple method, it is necessary to optimize the variables involved in the process to obtain a more accurate response (Martins et al., 2021). In particular, it has been observed that the linear range of calibration curves reported with gallic acid has a narrow absorbance window. Therefore, this study aimed to optimize the experimental conditions for total phenol quantification. Subsequently, analytical validation was performed, and the method was applied to a series of plant extracts.

Material and Methods

Determination of total phenols in tube

The standardized method long used within our research group (Salazar *et al.*, 2008) was employed, although it was originally published by Kaur & Kapoor (2002). The tube-based method was carried out using the standards pyrocatechol, gallic acid, quercetin, and Trolox, in all cases, triplicate solutions of 1000 ppm in ethanol were prepared at eight concentration levels (17.86–0.14 ppm). Table 1 presents the general assay procedure. Linear regression curves were obtained using the least squares method based on the absorbance values recorded at each concentration level.

Table 1. Folin-Ciocalteu method in tube.

Final standard concentration (17.86 – 0.14 ppm)	100 µL
Distilled water	3 mL
Folin-Ciocalteu's reagent	0.5 mL
Incubate	3 min
Sodium carbonate (20 %)	2 mL
Incubate	60 min
** Measure absorbance	750 nm
** Genesys 10s UV-Vis Spectrophotometer. Thermo Scientific.	

Application of the tube method

Five plant extracts available in the laboratory were selected, obtained in previous studies from plants collected in Northeastern Mexico (Rodríguez-Martínez, 2018): *Tecoma stans*, *Malus pumila*, *Chrysactinia mexicana*, *Eucalyptus globulus*, and *Quercus virginiana*. In all cases, the aerial parts of the plants were dried, ground, and stored in plastic bags at room temperature until extraction. Three hydroalcoholic extractions were performed using 100 g of plant powder with 100 mL of solution for 20 minutes. The resulting extract was evaporated to dryness using a rotary evaporator at 37 °C. The final extract was stored in amber vials under dark conditions, sealed under a nitrogen atmosphere, and refrigerated (2–4 °C) until use.

A solution of each extract was prepared at a concentration of 1000 ppm in 1 mL of ethanol, followed by two serial 1:2 dilutions. Each solution underwent the procedure for the tube-based total phenol determination. Absorbance values were interpolated using the gallic acid calibration curve, and the Total Phenol Content was calculated and expressed as milligrams of Gallic Acid Equivalents (GAE) per gram of extract. Each extract was analyzed in triplicate.

Solvent selection

The tube-based total phenol determination method was applied to 1 mL solutions of gallic acid (1000 ppm) prepared in ethanol and dimethyl sulfoxide (DMSO, 0.05 % in water). A 4.46 ppm gallic acid concentration was used in each solvent to obtain a spectral scan in the 300–800 nm range using a Genesys 10s UV-Vis spectrophotometer (Thermo Scientific). Absorbance values of each curve were recorded at their respective maximum absorption wavelength.

Total phenol determination at microscale

The reagent volumes used in the macro-dilution method were reduced to a final reaction mixture volume of 200 μ L, maintaining the final concentrations of each reagent. However, the final concentration levels of the standard were adjusted between 4 and 20 ppm. Table 2 presents the general procedure for the microplate method, including the volumes used in each reaction well. Under these conditions, a calibration curve was constructed at five concentration levels (4 and 20 ppm) in triplicate, with measurements taken on different days.

Table 2. Reduction of variable values for the microplate method.

Factor or variable	Well
Final gallic acid concentration (20 – 4 ppm) (μ L)	20
Distilled water (μ L)	90
Folin-Ciocalteu's reagent (μ L)	20
Incubate (min)	3
20 % sodium carbonate (μ L)	70
Incubate (min)	60
* Measure absorbance (nm)	750
* Multiskan fc Microplate Reader. Thermo Scientific	

Experimental design: sequential simplex method

The method variables selected for optimization were the amount of Folin-Ciocalteu's reagent, the amount of 20 % sodium carbonate, and the reaction time. Initially, a screening process was conducted to identify variables with a significant effect on the response. For this purpose,

a sequential simplex method design was applied, consisting of a matrix of four experiments with 3 variables. The variable values for these four experiments are presented in Table 3. Each experiment was performed in duplicate for both 20 ppm and 4 ppm concentrations of gallic acid. In all cases, the final well volume was adjusted to 200 μL with distilled water, after the reaction time, absorbance was measured at 750 nm using a Multiskan FC microplate reader (Thermo Scientific).

Following the Simplex rules (Granados-Guzmán *et al.*, 2017), the measured responses were compared, and the experiment with the lowest response was discarded. Subsequently, new variable values were calculated for the next experiment using the following formula:

$$V_N = (2 \cdot X_{\text{optimal}}) - (V_{\text{low}})$$

V_N = New value for each factor.

X_{optimal} = Average value of the factors that produced an optimal response.

V_{low} = Value that produced the lowest response.

Table 3. Design of experiments 1 to 4.

Experiment	Incubation time (min)	Folin-Ciocalteu volume (μL)	Sodium carbonate volume (μL)
1	80	20	70
2	60	20	70
3	40	18	70
4	40	6	55

Validation of the optimized method

The analytical validation of the optimized method was performed by constructing external standard calibration curves of gallic acid at 5 concentration levels (4 - 20 ppm), in triplicate, on different days. Linearity, intra- and inter-day precision at each concentration level, and accuracy were established by recalculating the concentration of standards at 3 concentration levels. Detection and quantification limits were determined using the absorbance values of 30 blanks, applying the formulas $3 SD_b/m$ and $10 SD_b/m$, respectively, where SD_b represents the standard deviation of blank readings and m is the slope of the obtained calibration curve.

The robustness of each condition was evaluated by slightly modifying the Folin-Ciocalteu's reagent volume (33 and 34 μL), Na_2CO_3 concentration (19 % and 21 %), and reaction time (46 and 47 minutes).

Matrix effects were assessed using a *Tecoma stans* extract solution (1000 ppm) spiked with gallic acid (200 ppm), dilutions were prepared to achieve a final gallic acid concentration range from 4 to 20 ppm. The curve was generated in triplicate over three different days.

Application

The optimized and validated microplate method was applied to 30 plant extracts available in the laboratory, originally obtained in previous studies from plants collected in Northeastern Mexico (as described earlier). Standard addition curves of gallic acid (0 - 16 ppm) were constructed using 4 μ L of a 1000 ppm extract solution to assess matrix effects. The procedure followed is detailed in Table 4.

From the standard addition curve, the gallic acid concentration (ppm) in 4 μ L of the 1000 ppm extract was determined. The Total Phenol Content was then calculated and expressed as milligrams of Gallic Acid Equivalents (GAE) per gram of extract.

Table 4. Microplate method by standard addition curve.

Condition or variable	Value
<i>Tecoma stans</i> extract (1000 ppm)	4 μ L
Gallic Acid (Final conc. 0 - 16 ppm) in distilled water	76 μ L
Folin-Ciocalteu's reagent	33.5 μ L
Incubate	3 min
20 % sodium carbonate	86.5 μ L
Incubate	46.5 min
* Measure absorbance	750 nm

Statistical analysis

All experiments were performed in triplicate on three different days, except for the sequential simplex method. Calibration curves were compared using one-way Analysis of Variance (ANOVA, $p < 0.05$). The initial and final method results were compared using Student's t-test ($\alpha > 0.05$).

Results and Discussion

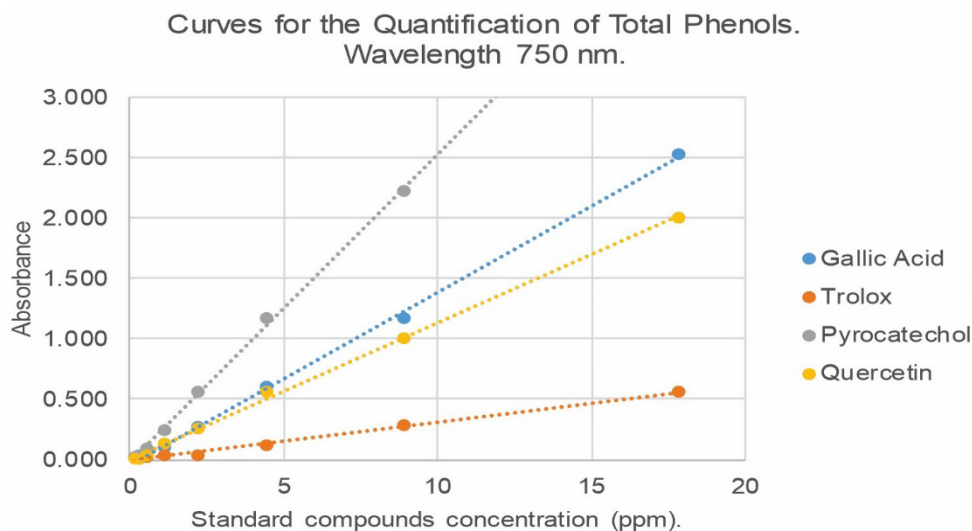
The Total Phenolic Content (TPC) present in an extract can explain the antioxidant activity of a plant-derived product; therefore, it is important to report the estimated value as part of its characterization (Ponnampalam *et al.*, 2022). However, the TPC determination method may

present some limitations, which must be evaluated and resolved in each laboratory before reporting the determination (Martins *et al.*, 2021).

Total phenolic content determination in test tubes

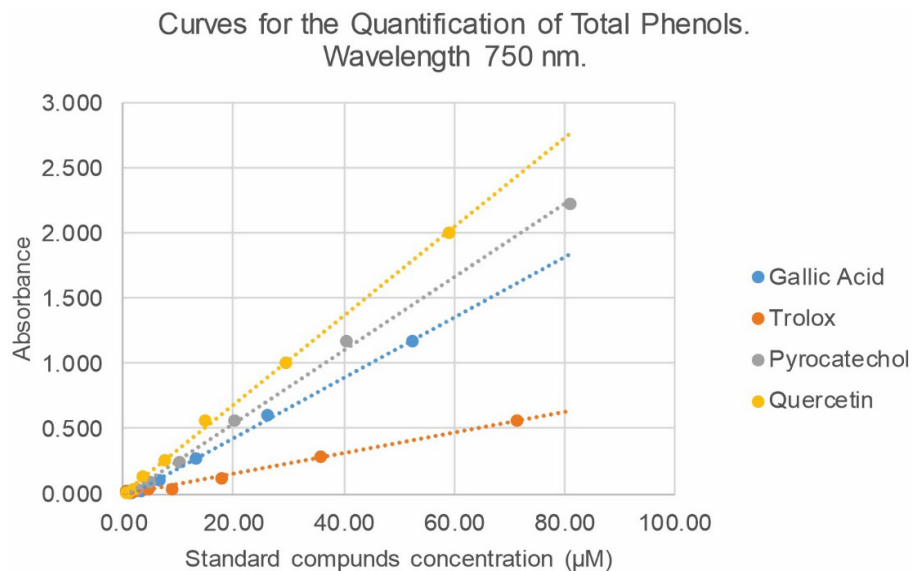
A common issue in various studies is the homogeneity of the standard used. The use of pyrocatechol, gallic acid, quercetin, and Trolox, among other phenolic compounds, has been reported (Pérez *et al.*, 2023). As previously mentioned, this method is based on the capacity of phenolic compounds to reduce a phosphotungstic-phosphomolybdic acid mixture; therefore, each standard will produce a different degree of reduction, and as a consequence, the values reported will be different and not comparable. The reduction capacity of each compound is closely related to its chemical structure (Pérez *et al.*, 2023). Pyrocatechol is the clearest example of a phenolic compound; in its structure, it has one more phenolic group than phenol itself, gallic acid has three phenolic groups and one carboxyl group as ring substituents, quercetin has a more complex structure, as it features a flavonoid nucleus with five phenolic groups, while Trolox has a polysubstituted phenolic ring (Pérez *et al.*, 2023).

In Figure 1, the calibration curves obtained are displayed in concentration units (ppm). The molar concentrations of each standard were calculated, and in Figure 2, the curves are presented in micromolar units (μM). Each obtained curve exhibited different slope values and linear ranges; these values are related to the structure of each compound and, consequently, its response (absorbance). The curve for pyrocatechol showed the highest slope value ($m = 0.2547$) within a linear range ($R^2 = 0.9987$) from 0.14 to 8.93 ppm, equivalent to 1.27 to 81 μM . At higher concentrations, linearity was lost. The curves for gallic acid and quercetin displayed slope values of $m = 0.1428$ and $m = 0.113$, respectively, with the widest linear range from 0.14 to 17.86 ppm, corresponding to 0.82 to 104 μM for gallic acid ($R^2 = 0.9966$) and 0.46 to 59.09 μM for quercetin ($R^2 = 0.997$). It could be argued that concentrations higher than 17.86 ppm (59.09 μM) should be considered for quercetin, as it has the highest molecular weight among the analyzed standards, but the absorbance values at the highest concentration were around 2. The curve for Trolox exhibited the lowest slope value ($m = 0.0335$) with the shortest linear range ($R^2 = 0.9902$), which included the highest concentration levels from 2.23 to 17.86 ppm, equivalent to 8.91 to 71.36 μM . This result was anticipated since Trolox has only one phenolic group and a high molecular weight, necessitating a larger quantity of the compound to yield absorbance values comparable to those of the other compounds.



**Figure 1. Total phenols curves by the tube method with Folin-Ciocalteu's reagent.
Concentration in ppm of phenolic compounds**

Source: Own elaboration based on results in Excel Program.



**Figure 2. Total phenols curve by tube-based method with Folin-Ciocalteu's reagent.
Molar concentration of phenolic compounds.**

Source: Own elaboration based on results in Excel Program.

Based on the previous results, gallic acid was selected as the standard that exhibits the best linearity and analytical response as a function of concentration. Therefore, the following experiments were conducted using this compound.

The Total Phenolic Content (TPC) determination was performed on five plant extracts using the tube-based procedure, employing the calibration curve constructed with gallic acid. Table 5 presents the results obtained for each extract, which were compared with those reported in previous studies, except for *Chrysactinia mexicana*, as no reports were found within the scope of our search.

Table 5. Total phenol content in plant extracts in triplicate with the tube-based method.

Plant extract	mg GAE/g	Reference	mg GAE/g
<i>Tecoma stans</i>	117.66 ± 2.04	Sbihi <i>et al.</i> , 2015	1.68 in seeds
<i>Malus pumila</i>	129.49 ± 3.10	Ko <i>et al.</i> , 2022	0.40
<i>Chrysactinia mexicana</i>	186.55 ± 3.48		
<i>Eucalyptus globulus</i>	212.43 ± 4.65	Rodríguez- Solana <i>et al.</i> , 2016	36.12
<i>Quercus virginiana</i>	262.14 ± 3.33	Valero-Galván <i>et al.</i> , 2021	29.4

GAE: Gallic acid equivalent.

For cases where previously reported data was found, the results were significantly lower than those determined in this study. This discrepancy may be due to the different procedures used by each research group. For example, Ko & Ku (2022) and Valero-Galván *et al.* (2021) used a lower volume of sodium carbonate compared to our procedure. This results in a lower final concentration of this reagent in the medium, making the pH insufficiently alkaline to fully complete the reaction. Additionally, these authors reported shorter reaction times and lower absorption wavelengths, which directly affect the obtained responses.

Solvent selection

Despite obtaining good, reliable, and reproducible results, it is currently necessary to design environmentally friendly chemical methods. Therefore, it is advisable to consider the amount of reagents used, as well as their hazardousness and toxicity. The solvent used in the macrodilution method and commonly reported for the Total Phenols Quantification in natural extracts is ethanol, since polyphenols with more complex structures (condensed tannins, lignins, and hydroxycinnamic acids) are insoluble in water (Valencia-Avilés, 2017). The use of distilled water has also been reported in the Total Phenols Quantification, although there is controversy for the same reason (Pérez *et al.*, 2023). To address the low or null solubility of polyphenols in water, we considered using a small amount of dimethyl sulfoxide (DMSO) for dissolution, followed by adding water to reach the desired volume. To compare the performance of both solvents, calibration curves were prepared using gallic acid as the standard, with ethanol or DMSO (0.05 % in water) as solvents,

at the concentration levels of the linear range (17.86 - 0.14 ppm), following the protocol of the tube-based Total Phenols Determination method. It is important to highlight that before measuring absorbances, the spectra of gallic acid in each solvent were obtained, and as shown in Figure 3, no differences were found in the obtained spectra.

Subsequently, the absorbances of each standard were measured, calibration curves were constructed, and the corresponding graphs and equations were obtained. Both calibration curves were linear and exhibited similar slope values (Figure 4). The set of absorbances obtained at different concentration levels was subjected to a one-way analysis of variance (ANOVA), and no significant difference ($p < 0.05$) was found between them. Therefore, water can be used as a solvent for this assay with the same analytical performance as ethanol, with the advantage that water is a more economical and less toxic reagent.

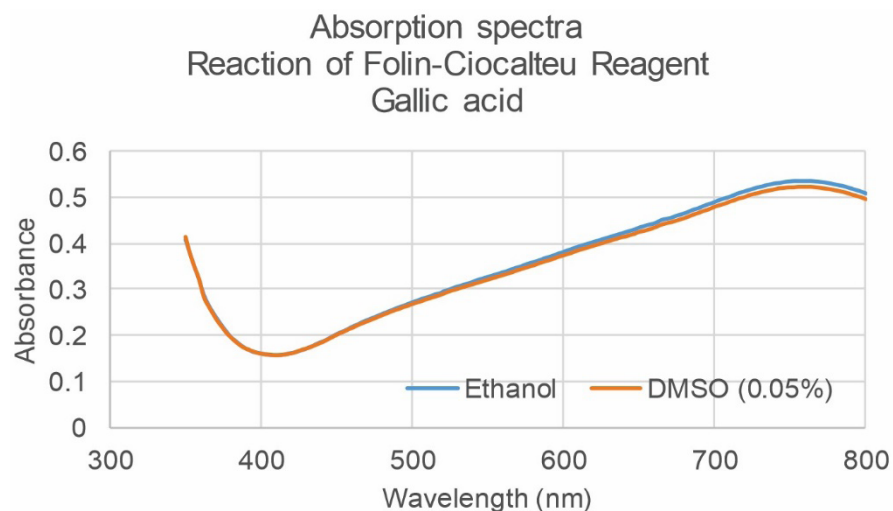


Figure 3. Absorption spectra obtained from the reaction of Folin-Ciocalteu's reagent with gallic acid in ethanol and DMSO (0.05 % in water).

Source: Prepared by the authors based on the results of UV-vis spectra and graphed in Excel.

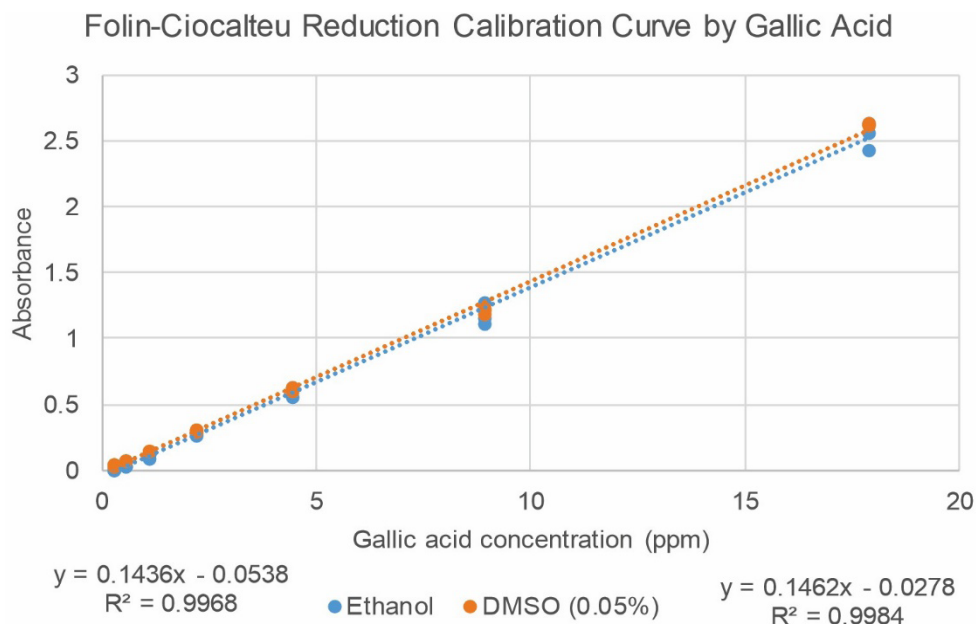


Figure 4. Calibration curve obtained from the reaction of gallic acid with the Folin-Ciocalteu's reagent in ethanol and DMSO (0.05 % in water) using the tube method.

Source: Own elaboration based on results in Excel Program.

Total phenolic content determination in microplate

Oresanya et al. (2024) and Ko & Ku (2022) reported the use of a microplate method for the quantification of total phenolic content in plant extracts. However, both studies referenced a previous article that employed a test tube method, without evaluating the effect of this modification on the analytical response. Conducting the method at a microscale offers a more economical, simple, less toxic, and faster approach, as absorbance measurements can be performed simultaneously in a microplate reader. These features make it a high-efficiency method (Granados-Guzmán *et al.*, 2017).

As previously mentioned, the test tube method used a final volume of 5.6 mL. In this experimental phase, we aimed to reduce the final volume to 200 μ L. To achieve this, the volumes of each solution were proportionally scaled. However, the standard volume resulted in a very small amount (3.57 μ L), so it was decided to increase the standard solution volume to 20 μ L while reducing the water volume to 90 μ L, ensuring that the final standard concentrations remained constant. Additionally, the Folin-Ciocalteu's reagent volume was adjusted to 20 μ L, and the sodium carbonate volume to 70 μ L. Under these conditions, a linear calibration curve was obtained within

the evaluated range. However, absorbance values and the slope differed from those obtained in the test tube method (Figure 5).

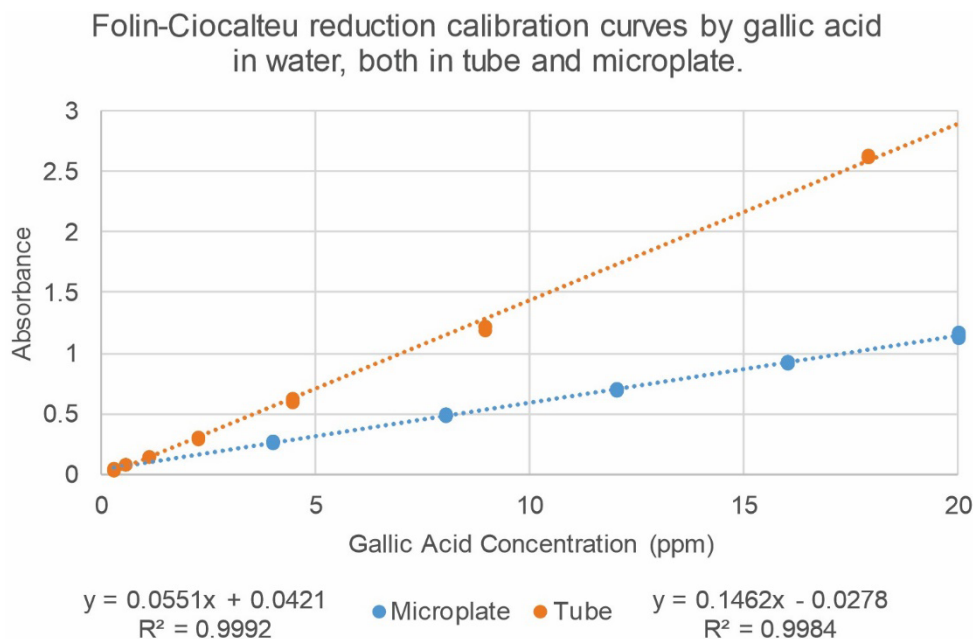


Figure 5. Calibration curve obtained from the reaction of gallic acid with the Folin-Ciocalteu's reagent in DMSO (0.05 % in water) using the tuber and microplate methods.

Source: Own elaboration based on results in Excel Program.

This effect is possibly the result of the modification of using a very small volume of Folin-Ciocalteu reagent, so the amount of phosphomolybdic and phosphotungstic acids is low compared to the test tube method, and therefore their absorbances are lower.

Experimental design

A Basic Sequential Simplex was performed to find the best conditions to expand the absorbance window between the extremes of the linear interval (4 ppm and 20 ppm) to increase sensitivity. The variables optimized were: The volume of the Folin-Ciocalteu's reagent, 20 % sodium carbonate, and reaction time. pH is a variable that affects the result; however, it was necessary to keep it constant to ensure the reaction in all assays.

The experiments were carried out in duplicate, and the average absorbance at both concentration levels (4 ppm and 20 ppm) was obtained. The difference between the absorbances was calculated, which indicates the absorbance window that can be used for interpolation. Strictly speaking, the larger the window, the more reliability we will have in interpolating to determine the concentration of Total Phenols. After obtaining the results of experiments 1 to 4 (first set of experiments), the one that presented the lowest difference was discarded, as it was considered the experiment with conditions not suitable for the objective of the experiments. The conditions for a new experiment were calculated and performed. Again, the results were compared, and the worst experiment was discarded. This procedure of comparing results, eliminating the experiment with the lowest responses, applying the formula, and calculating new values for new experiments was carried out until reaching experiment 9. All results appear in Table 6, including the order in which each experiment was discarded.

Table 6. Results of the experimental design.

Exp	Incubation time (min)	Folin-Ciocalteu volume (μL)	Sodium carbonate volume (μL)	4 ppm (Abs)	20 ppm (Abs)	Difference in absorbances	Order of elimination
1	80	20	70	0.231	0.973	0.742	2
2	60	20	70	0.226	0.982	0.756	4
3	40	18	70	0.205	0.954	0.749	3
4	40	6	55	0.204	0.827	0.623	1
5	50	32.66	85	0.197	1.014	0.817	5
6	20	27.3	80	0.29	1.202	0.912	
7	46.66	33.3	86.6	0.331	1.367	1.036	
8	17.7	43.8	97.7	0.317	1.231	0.914	
9	6.24	38.14	91.28	0.207	0.745	0.538	6

When comparing the results obtained with the last set of experiments (6, 7, 8, and 9), it was determined that the lowest responses were from experiment 9. According to the Basic Sequential Simplex rules, if the new experiment (9) results in the worst conditions, it must be discarded. Subsequently, the next worst experiment should be identified to calculate new conditions. However, upon reviewing the differences in the other experiments (6 and 8), they were almost identical. Additionally, experiment 7 was the best of all, providing the highest absorbance difference: 1.036. Based on these results, the experimental design was stopped, and the optimal reaction conditions were established as shown in Table 7.

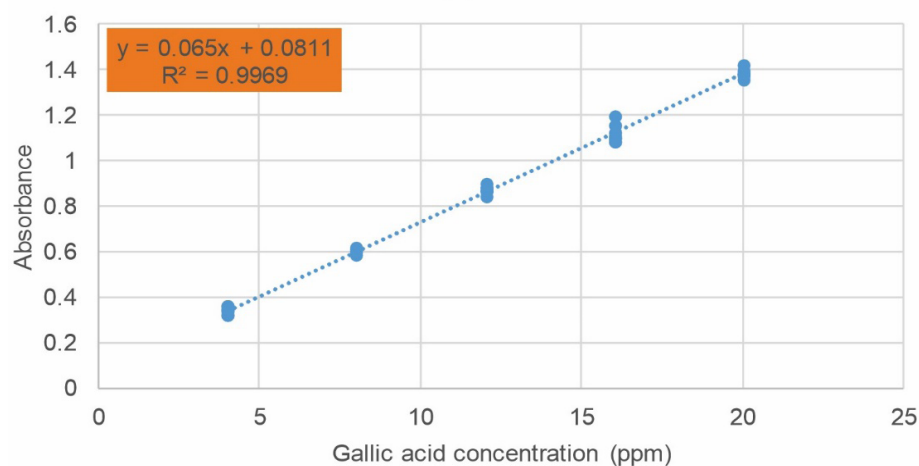
Table 7. Optimal conditions for the microplate method.

Condition or variable	Value
Gallic Acid (4 - 20 ppm) in distilled water	80.0 µL
Folin-Ciocalteu's reagent	33.5 µL
Incubate	3 min
Sodium carbonate (20 %)	86.5 µL
Incubate	46.5 min
Measure absorbance	750 nm

Validation of the optimized method

The analytical validation of the optimized method was performed by constructing calibration curves using an external standard with gallic acid at 5 concentration levels. The regression line was calculated using the least squares method (Figure 6), and linearity was established based on the determination coefficient and the relative standard deviation percentage of the response factors across the entire linear range ($R^2 = 0.9969$ and % RSD = 8.31). The absorbance window was 1.104, ranging from 0.346 for 4 ppm to 1.420 for 20 ppm.

Calibration curves for quantification of total phenols in microplate, using the optimized Folin-Ciocalteu reduction method by gallic acid.

**Figure 6. Gallic acid calibration curve with Folin-Ciocalteu's reagent using the optimized microplate method.**

Source: Own elaboration based on results in Excel Program.

Intra- and interday precision was established at each concentration level, with % RSD values < 4.27 and < 4.46, respectively. Accuracy was determined based on the percentage error obtained by recalculating the standard concentrations at 3 levels: 4 ppm with a % Error < 9.5 %, 12 ppm with a % Error < 3.3 %, and 20 ppm with a % Error < 1.04 %. The limits of detection and quantification were established as LOD = 0.84 ppm and LOQ = 1.09 ppm, respectively.

The robustness of each condition was assessed by modifying the Folin-Ciocalteu's reagent volume (33 and 34 μL), Na_2CO_3 concentration (19 and 21 %), and reaction time (46 and 47 min). Absorbances obtained throughout the linear range were compared using one-way ANOVA, and no significant differences were found (ANOVA $p < 0.05$), confirming that the method was robust under the evaluated conditions.

Plant extract dilutions are commonly reacted with Folin-Ciocalteu's reagent, and absorbances are interpolated directly on the calibration curve (Rumpf *et al.*, 2023). However, the effect of the extract matrix is not typically evaluated, despite plant extracts displaying varying shades of green-brown, which can absorb at 750 nm. The matrix effect was assessed using a *Tecoma stans* extract solution (1000 ppm) + gallic acid (200 ppm), which was diluted to obtain final gallic acid concentrations between 4 and 20 ppm. The calibration curve was performed in triplicate on three different days. A variance analysis (ANOVA) comparing absorbances at each concentration level revealed a significant difference (ANOVA $p < 0.05$), indicating a matrix effect (Figure 7). The absorbance values at the highest gallic acid concentration (20 ppm) within the extract reached 2.3 AU, which may have caused the loss of linearity ($R^2 = 0.9856$). A technique was needed to differentiate the sample's absorbance from that generated by the Folin-Ciocalteu's reaction to compensate for the matrix effect.

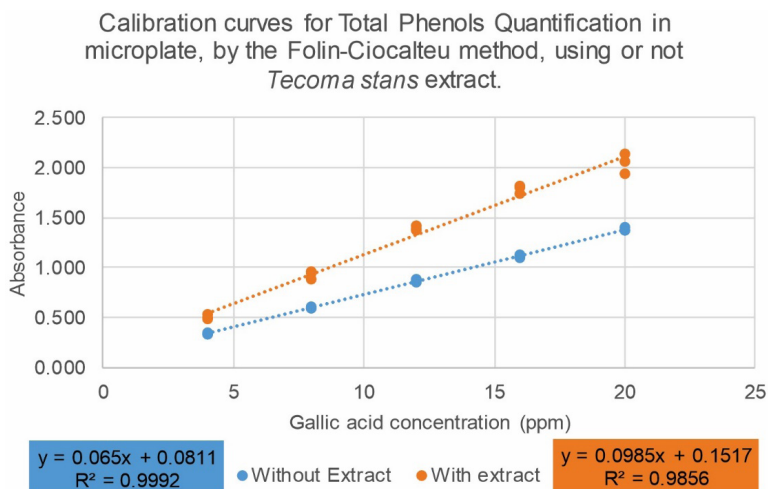


Figure 7. Gallic acid calibration curves with Folin-Ciocalteu's reagent using the optimized microplate method, with and without *T. stans* extract.

Source: Own elaboration based on results in Excel Program.

Application

As a strategy to compensate for the matrix effect, standard addition curves of gallic acid were constructed for each extracted sample. Gallic acid was added at concentrations ranging from 0 to 16 ppm to a 4 μ L solution of *Tecoma stans* extract (1000 ppm), following the protocol outlined in Table 4. The resulting curve is shown in Figure 8, where 3.47 ppm of gallic acid equivalents were quantified in the extract after considering dilution effects. The Total Phenolic Content was determined as 172.7 ± 16.1 mg gallic acid equivalents (GAE)/g extract.

Unlike the tube method, which was applied to only 5 extracts, the microplate method with standard addition was applied to 30 plant extracts, a significantly larger number. The results are presented in Table 8, including the mean and standard deviation (SD). The extracts with the highest Total Phenolic Content were from oak species (*Quercus spp.*), while the lowest phenolic content was found in the Jerusalem Thorn (*Parkinsonia aculeata*). Interestingly, two extracts of *Chrysactinia mexicana* collected by different researchers at different times were evaluated, and the results were very similar, though the extract collected in spring had a slightly higher Total Phenolic Content.

Only five extracts were evaluated using both the tube method and the microplate method with standard addition. The results from both methods are compared in Table 9. A *Student's t*-test demonstrated a significant difference ($\alpha > 0.05$) in three of the five extracts: *Tecoma stans*, *Chrysactinia mexicana*, and *Eucalyptus globulus*. In cases where no significant difference was found, the concentration determined by the tube method was lower than that obtained through standard addition, possibly due to a matrix effect in the macrodilution method, which may have influenced quantification. In contrast, the standard addition method helped to mitigate or at least compensate for this effect.

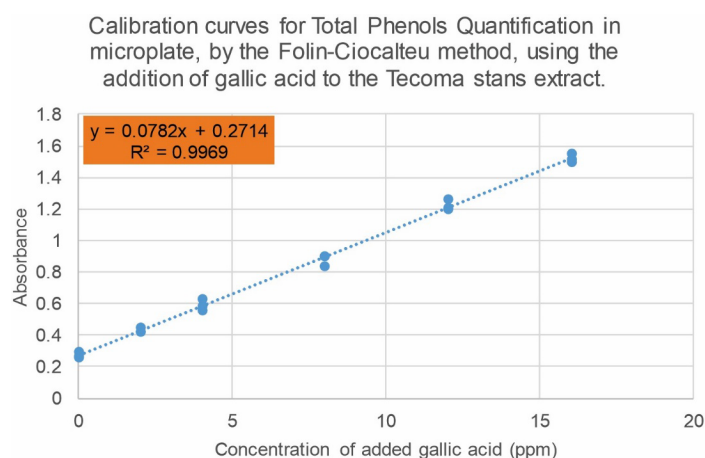


Figure 8. Addition curve of gallic acid standard with Folin-Ciocalteu's reagent in microplate using the optimized method.

Source: Own elaboration based on results in Excel Program.

Table 8. Total phenol content in plant extracts (mgGAE/g).

Plant	Average	SD	Plant	Average	SD
1 <i>Quercus canbyi</i>	438.9	39.7	16 <i>Cephalanthus occidentalis</i>	156.3	24.7
2 <i>Quercus virginiana</i>	371.2	75.1	17 <i>Lantana camara</i>	154.9	16.6
3 <i>Chrysactinia mexicana</i>	365.3	23.9	18 <i>Xanthosoma robustum</i>	149.1	3.3
4 <i>Lippia graveolens</i>	348.9	37.7	19 <i>Parthenium hysterophorus</i>	111.5	11.9
5 <i>Chrysactinia mexicana</i>	333.9	18.4	20 <i>Ruta chalapensis</i>	99.3	20.3
6 <i>Leucaena greggii</i>	246.2	69.4	21 <i>Ricinus communis</i>	90.4	19.7
7 <i>Porophyllum scoparium</i>	235.0	11.1	22 <i>Solanum rostratum</i>	90.4	2.2
8 <i>Taraxacum officinale</i>	204.1	19.7	23 <i>Porlieria angustifolia</i>	86.7	5.5
9 <i>Hunnebmanna fumariifolia</i>	192.5	22.7	24 <i>Physalis ixocarpa</i>	84.5	10.9
10 <i>Cordia boissieri</i>	191.3	9.8	25 <i>Teucrium bicolor</i>	79.1	3.9
11 <i>Tecoma stans</i>	172.7	16.1	26 <i>Ebenopsis ebano</i>	78.8	1.7
12 <i>Salvia coccinea</i>	168.8	2.1	27 <i>Juglans nigra</i>	75.3	8.8
13 <i>Malus pumila</i>	163.0	3.5	28 <i>Pinus teocote</i>	59.8	2.5
14 <i>Eucalyptus globulus</i>	161.6	2.1	29 <i>Pithecellobium dulce</i>	56.0	18.9
15 <i>Citrus aurantium</i>	158.9	39.5	30 <i>Parkinsonia aculeata</i>	55.5	13.7

Table 9. Comparison of tube and microplate methods.

	Macrodilution mg GAE/g	Standard Addition mg GAE/g
<i>Tecoma stans</i> *	117.66 ± 2.04	172.7 ± 16.1
<i>Malus pumila</i> *	129.49 ± 3.10	163.0 ± 3.5
<i>Chrysactinia mexicana</i> *	186.55 ± 3.48	333.9 ± 18.4
<i>Eucalyptus globulus</i> *	212.43 ± 4.65	161.6 ± 2.10
<i>Quercus virginiana</i>	262.14 ± 3.33	371.2 ± 75.1

* Significant Difference $\alpha > 0.05$

Conclusions

The Total Phenolic Content (TPC) in plant extracts can be determined through the oxidation reaction of the acid mixture in the Folin-Ciocalteu's reagent, which generates a broad absorption band at 750 nm. Among the four polyphenolic compounds evaluated, gallic acid provided the best analytical response. In this study, the reaction conditions were miniaturized and optimized, achieving an absorbance window greater than 1 within a linear concentration range of gallic acid.

The proposed method demonstrated good performance when using water as a solvent instead of ethanol, making it a more environmentally friendly approach. The quality parameters of the optimized method were established, confirming that it is linear, precise, accurate, sensitive, and robust. A matrix effect was observed, likely caused by the composition and/or color of the extract. Therefore, it is recommended to perform quantification using a standard addition curve to reduce matrix interference. The method was successfully applied to 30 plant extracts, which exhibited a Total Phenolic Content range of 55.5 to 438.9 mg GAE/g extract.

Author contributions

Work conceptualization, R.S.A.; methodology development, C.P.G.; software management, C.P.G., B.A.A.G.; experimental validation, C.P.G., O.J.P.C.; analysis of results, R.S.A., B.A.A.G., O.J.P.C.; data management, R.S.A.; manuscript writing and preparation, R.S.A.; writing, review, and editing, R.S.A., B.A.A.G., O.J.P.C.; project administrator, R.S.A.; funding acquisition, R.S.A.

All authors of this manuscript have read and agreed to the published version.

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Ethics declarations

This research is part of Research Project QA21-00002, approved by the Ethics Committee of the Research Subdirectorate of the Faculty of Medicine, UANL.

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Conflict of Interest

The authors declare no conflict of interest.

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