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Optimization of the Effect of Temperature, Concentration and pH on Antioxidant Capacity by Gallic Acid by Response Surface Methodology (RSM)

Gilberto Mercado-Mercado [a*] and Elsa Carolina Núñez-Brito [a]

[a] Departamento de Ciencias Químico Biológicas, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, Anillo Envolvente Pronafy Estocolmo, Zip Code 32310, Ciudad Juárez, Chihuahua, México.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

The antioxidant capacity of gallic acid (GA) is affected by temperature and pH.
Aim: To understand this phenomenon, we evaluated the optimization of conditions between temperature (2°C, -20 °C, 40 °C), pH (3,7,10) and concentrations (1 mM, 4 mM and 7 mM) to have a better antioxidant capacity by response surface methodology (RSM).
Methods: The DPPH method was used to measure the percentage inhibition (%Inh). Concentrations, 23 °C and pH 7 were evaluated, respectively. Three temperatures (2°C, -20 °C, 40 °C) were combined with the same concentrations and pH in a Box-Behnken design model.

**Corresponding author: E-mail: gilberto.mercado@uacj.mx, gil_4783@yahoo.com.mx;*

Results: The combination of the three factors significantly influenced the %Inh. The factor levels that resulted in higher %Inh were 2 °C, acidic pH and 4 mM concentration. The RSM showed that the optimum conditions had %Inh values of DPPH of 87.66%, 87.11% and 88.29%, respectively.
Conclusion: the antioxidant capacity of GA. at 2 °C and pH 2 creates highest antioxidant capacity of GA.

Keywords: Gallic acid; antioxidant capacity; DPPH; response surface methodology.

1. INTRODUCTION

Gallic acid (GA) is a phenolic acid found alone or as part of hydrolyzable tannins in various natural sources such as fruits, vegetables, seeds, leaves, tubers, among others. This phenol can form esters with itself, generating digalic and trigalic acids and cyclic ether esters. It is also attributed several biological effects, ranging from anti-inflammatory, antibacterial, anticarcinogenic, antioxidant and antibiotic activity, to cardiovascular protection, preventing oxidation of low-density lipoproteins that carry cholesterol in the blood, preventing diseases such as atherosclerosis [1]. These properties are to its mechanism of donating electrons to neutralize free radicals. They are also highly dependent on the stability of their molecular structure and their concentrations are affected by factors such as low or high temperatures, ultraviolet light exposure, pH, and the presence of co-pigments [2-3]. These factors alter the antioxidant capacity of phenolic compounds (PC) directly and dissociation of -OH groups in their chemical structure. It is logical to suspect that pH will influence the dissociation rates of the -OH groups in these PC [2,4].

Therefore, studies have been carried out to find the appropriate conditions to have the maximum functionality of the PC. One of the ways to determine the appropriate conditions of the PC functions is by means of the response surface methodology (RSM), which is widely used in the optimization of the conditions of the action of a compound of interest. Experimental values are fitted to a polynomial model describing the behavior in relation to several independent variables [5]. Therefore, antioxidant capacity was optimized by the DPPH method through combinations of the variables of GA concentrations, temperature, and pH by means of the RSM.

2. MATERIALS AND METHODS

2.1 Preparation of GA Solutions

A sketch was made to determine the concentration of GA (≥98.0%; Sigma Aldrich®) in thirty acid, neutral and alkaline fruits. The minimum, intermediate and maximum concentrations of GA were recorded for each fruit, averaging them to decide the concentrations for the present study, which were 1 mM, 4 mM and 7 mM. A standard concentration of 20 mM was made in: 1) pure methanol (J.T. Baker®); 2) acidified methanol at pH 2; 3) methanol at pH 7; 4) alkaline methanol at pH 10, all at room temperature (23 ± 2 °C). For the neutral pH, the solution was rectified according to the safety data sheet. Subsequently, the solution at neutral pH was stored at three temperatures: -20 ºC, 3 ºC and 40 ºC, protecting them from light. Also, the following combinations were performed: a) concentration vs pH with constant temperature, b) concentration vs temperature with constant pH, c) pH vs temperature with constant concentration.

2.2 Antioxidant Capacity of GA

The antioxidant capacity of all GA solution treatments was performed by the DPPH (1,1-Diphenyl-2-picrylhydrazyl) method, following the methodology of Mercado-Mercado [6]. The analysis was performed with the solutions at pH at room temperature and at the three temperatures mentioned above. DPPH was diluted to an absorbance of 0.7 ± 0.1 and stored in the absence of light. In triplicate, 25 µL of each GA condition was reacted with 200 µL of DPPH in 96-well microplate wells (Corning®, USA). Percent inhibition (%Inh) was measured at 450 nm at 10 min in a multi-mode spectrophotometric detection with 96-well plates (Thermo Scientific® Mutliskan FC, Winooski, VT, USA) and the Skanlt RE 6.0.2 Program were used. The percentage inhibition was determined from Eq. 1:

$$\frac{\text{Abs DPPH}-\text{Abs GA}}{\text{Abs DPPH}}\ (100) \qquad \text{Eq. 1}$$

Abs DPPH= DPPH reagent absorbance.
Abs GA= Gallic acid absorbance.

2.3 Optimization of the Antioxidant Capacity of GA By RSM

RSM was used to define the optimal parameters to determine the optimal antioxidant capacity. Box-Behnken design was experimented in RSM, with 27 experiments defined by 3 numerical parameters (independent variables), including triplicates at the central point, to access the combined effect of the predetermined independent variables. 3 levels were chosen and coded at -1, 0 and +1, as shown in Table 1.

Table 1. Independent variables and their corresponding levels for antioxidant capacity (%Inh)

Independent variables	Units	Symbols	Code levels		
			-1	0	+1
Temperature	°C	X_1	40	3	-20
pH	----	X_2	2	7	9
Concentration	mM	X_3	1	4	7

Experiments were conducted arbitrarily, to minimize biased results and the effect of indeterminate invariance.

The variables to be optimized were concentration vs pH, concentration vs temperature and pH vs temperature. The dependent variables were modeled with a polynomial model of order 2 (Eq. 2)

$$Y_j = \beta_0 + \sum \beta_i x_i + \sum \beta_i x_i^2 + \sum \beta_j x_i + \sum \beta_j x_j^2 + \sum \beta_{ij} x_i x_j \quad \text{Eq. 2}$$

where:

Y_j is the dependent variable.
β_0 is a constant and βi, βj and βij are the linear coefficients
B_{ij} are the coefficients of interaction of the linear factors
B_i^2, β_j^2 are the quadratic coefficients

The model fit was performed using the lack of fit method and the percentage variability of the optimization parameter was analyzed using the regression coefficient (R^2). The optimal values of the factors (pH, temperature and concentration) were used in the execution of four experimental replicates and compared with the theoretical value of the polynomial model to verify the accuracy of the model. Statica 4.0® software was used in this study to corroborate the results of the optimization routine. A 95% confidence interval was established to test the significant effect of the linear factors and their interaction. The F statistical test was used to evaluate the regression model. Normal probability plots of residuals and plots of residuals versus predicted response were used for model adequacy. The desirability function (di) method was used for optimization. Each response (Yi) is converted into a di that is dimensionless, its values are between 0 and 1, representing the closeness of a response to the ideal value, are combined to obtain a single desirability value (D) [7].

2.4 Validation of the optimized conditions

The optimal conditions for high antioxidant capacity were obtained from the RSM predictive equations. The antioxidant capacity was determined from the suggested combinations of the variables (concentration, temperature and pH) by RSM. The antioxidant capacity was repeated four times and verified by comparing the experimental value with the predicted values obtained from the optimized model.

2.5 Statistical Analysis

The results were analyzed by analysis of variance (ANOVA), applying the LSD method with a significance level of 95 % (α = 0.05) and the statistical package Statistica version 10 was used.

3. RESULTS AND DISCUSSION

3.1 Antioxidant capacity of GA

Table 2 shows the %Inh of DPPH at different concentrations dissolved in methanol.

Table 2. Inhibition percentages (%Inh) of GA at 23 °C

Concentration (mM) methanol	%Inh N/A
1	23.09 ± 2.44[b]
4	24.46 ± 3.28[b]
7	37.59 ± 4.46[a]

N/A: not activity. Lowercase letters represent significant difference (p< 0.05)

The above table shows that the %Inh is related to the stoichiometric reaction between moles of DPPH per molecule of GA [2,8]. Our results were lower than those reported by Nenadis, Lazaridou y Tsimidou[9] who had a %Inh of 50% DPPH; Sadat-Shojaee, Moeenfard & Farhoosh[8] had a %Inh of 69% with methyl gallate, and chlorogenic acid had a %Inh of 43.9% [6]. However, our

results were superior to those obtained for caffeic acid (%Inh 20%) and sinapic acid (%Inh 29%) [9]. The antioxidant capacity of PC depends on the structure, concentration, substituent groups, physicochemical properties of the solvent (polarity, permittivity, hydrogen acceptance or donation, among others) and steric hindrance of the reducing molecule [6,8]. In this sense, the antioxidant capacity of GA dissolved in methanol is higher in comparison when dissolved in apolar solvents (2-propanol > ethanol > methanol > t-butanol > acetonitrile > 2-propanol) [10]. On this basis, methanol favors the stability of phenoxide ions [8]. When GA is dissolved in methanol, PC becomes more acidic due to the formation of the phenoxide ion (formed by the deprotonation of phenol) which is resonance stabilized by the benzene ring, while the methoxide ion formed by the deprotonation of methanol makes it unstable [11].

3.2 Effect of Temperature on the Antioxidant Capacity of GA

Temperature is a crucial variable in the stability and antioxidant capacity of phenolic compounds. Therefore, the effect of temperature on the %Inh of GA was evaluated, as shown in Table 3.

Table 3. Effect of temperature on the percentage inhibition (%Inh) of GA at neutral pH

Temperature (ºC)	Concentration (mM)	%Inh
methanol	**----**	**N/A**
23	1	23.09 ± 2.44^{bA}
3	1	15.97 ± 1.79^{dB}
-20	1	15.10 ± 2.08^{dB}
methanol	**----**	**N/A**
23	4	24.46 ± 3.28^{bA}
3	4	21.58 ± 2.75^{cA}
-20	4	25.59 ± 3.73^{bA}
methanol	**----**	**N/A**
23	7	37.59 ± 4.46^{aA}
3	7	39.71 ± 4.11^{aA}
-20	7	37.39 ± 4.08^{aA}

N/A: not activity. Lower case letters represent significant difference between different concentrations at a single temperature. Capital letters represent significant difference between different temperatures at a single concentration ($p < 0.05$)

The %Inh of GA was favored as temperature decreased and concentration increased (Table 3). This is due to the fact that low temperatures increase the ionization energy of the hydroxyl radical [3,12]. Réblová [13] mentions that as the temperature increases, the antioxidant capacity of GA decreases until it loses 90% at ≥150 ºC. Therefore, it is important to know the tolerance and thermal resistance of phenolic compounds to avoid their oxidation. In this regard, Ross, Hoye & Fernández-Plotka [14] found that the stability of phenolic acids (syringic acid, caffeic acid, benzoic acid) occurs between 70 °C - 90 °C, while flavonoids are preserved between 40 °C - 80 °C. Anthocyanins are another class of PCs that have thermal degradation, particularly monoglycosides (cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside) are more susceptible to heat, while acylation and methoxylation usually improve the stability of anthocyanins at high temperatures [15]. Hayat [16] evaluated the effect of ultrasound-assisted temperature (30 °C, 40°C, 50 °C and 60 °C) and methanol concentration (0, 20, 40 and 60%) for different time intervals (10, 15, 20, 25, and 30 min) of extraction on mango seed kernels. It was observed that at 40 °C for 10 min the highest GA yield (5.30 ± 0.01 mg/g) was achieved, while it decreased with increasing temperature at 60 °C (4.39 ± 0.00 mg/g). From the above, temperatures play an important role in stability, as temperature increases it leads to higher desorption equilibrium and solubility of GA and results in disintegration of GA affecting the antioxidant capacity. In addition, heat decreases the viscosity of the solvent leading to denaturation or precipitation of the GA [2-3,17-18].

3.3 Antioxidant capacity of GA by pH effect

The %Inh of GA was used to evaluate the effect of pH (2, 7 and 9) (Table 4).

Table 4. Inhibition percentages (%Inh) of GA at different pH at 23 °C

pH	Concentration (mM)	%Inh
2	1	81.65 ± 0.93^{aA}
7	1	29.68 ± 3.93^{bB}
9	1	N/A
2	4	85.00 ± 1.10^{aA}
7	4	25.41 ± 1.57^{bB}
9	4	N/A
2	7	85.75 ± 0.37^{aA}
7	7	28.36 ± 3.64^{bB}
9	7	N/A

N/A: not activity. Lower case letters represent significant difference between pH at a single concentration. Uppercase letters represent significant difference between pH at different concentrations ($p < 0.05$)

Table 4 shows better responses at acidic pH than at neutral and alkaline pH, with a null response at alkaline pH. Our results correspond to the data already available in the literature [19-22]. Protocatechuic acid, vanillic acid and *p*-hydroxybenzoic acid showed a higher antioxidant capacity at pH 2.5 than 4.5 [23]. Likewise, quercetin showed a better antioxidant performance at acid pH (2.5) than at alkaline pH (9.5) [24]. On the other hand, anthocyanins become unstable at different pH. Most studies have confirmed that anthocyanins are stable at very acidic pH (<3) [14,25], which may resemble the results obtained in our study with the GA. Skrypnik and Novikova [26] reported that the best optimal pH values that resulted for PC extraction were close to 4. This can be reasoned that depending on the degree of acidity, GA may be present in neutral form or in ionic form which helps to donate hydrogen ion to reduce DPPH. However, this contradicts Hosseinzadeh [27] mentions that simple PC (caffeic acid, hydroxybenzoic acid) are weakly acidic, due to their non-dissociated state at acidic pH, which are more prone to interact with non-polar media. Therefore, our study opens other options to assess antioxidant capacity in other acidic pH parameters and in other systems with acidic character and to observe the behavior of free PC. GA, like anthocyanins, can also be limited to use in very acidic food products, such as fruit juices and certain dairy products (yogurt, kefir, some cheeses).

On the other hand, the null %Inh of GA at alkaline pH (Table 3) is due to the formation of phenolates, leading to a significant reduction in its activity in donating a hydrogen in the DPPH radical [28]. Such results resonate with the theory of Fu [28] who postulate that the hydroxyl groups of PC lose their polarity at alkaline pH due to OH group saturation thus causing a decrease in their reactivity. It should be mentioned that the effect of pH on %Inh is not the same on PC as a whole as it is individually because it depends on the polarity of the interaction between PC, the content and profile of each one, the solvent in which they are dissolved, among others [23,28].

3.4 Effect of Temperature and pH on the Antioxidant Capacity of GA

Fig. 1 shows the result of the %Inh of gallic acid on the influence between concentrations - pH, at temperatures of 40 ºC (Fig 1A), 3 ºC (Fig. 1B) y -20 ºC (Fig. 1C).

The figures show that pH and temperatures have a great influence on the %Inh.

Antioxidant capacity is drastically decreased at alkaline pH. However, the best GA activity occurs at 3 °C and as concentrations increase. Acidic pH generates faster and more efficient GA for its antioxidant capacity; on the other hand, at 40 ºC it causes a deportonation which causes the %Inh to be affected (Table 4). Few studies have reported that the antioxidant capacity of some hydroxybenzoic acids (vanillic, syringic, protocatechuic and *p*-hydroxybenzoic acids) and GA is maintained at acidic pH and freezing temperatures [4,25,29-30]. Sui [30] observed that increasing the temperature and pH to 6 has a major negative impact on the stability of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutoside. Ahmad-Nayik [31] valuated the antioxidant capacity on the DPPH radical of PC extracts of *Plectranthus rugosus*, which worked with temperature (60 ºC, 80 ºC), pH (3, 6) and time (10, 15 min); they observed that the action of PC decreased at 80 ºC at pH 6 con 15 min.

Alkaline pH causes an intrusion in the reaction between DPPH and GA by oxidizing during the reaction. Which, upon subjecting to 40 °C and at alkaline pH loses its ability to inhibit DPPH. Therefore, it is hypothesized that temperature increases the dissociation of GA into the two functional groups (OH and carboxyl) by reducing its ionization energy [4,13,29]. Therefore, further studies are required to demonstrate the effect of the combination of factors influencing the antioxidant capacity.

3.5 Response Surface Methodologies for Antioxidant Capacity of GA

The relationships between the independent variables (concentration, temperature and pH) and the responses (%Inh) were predicted by three-dimensional plots of the response surfaces using the fundamentals of equation (3; concentration - temperature) (4; concentration - pH) (5; temperature - pH).

$$\%Inh = 69.16 + (98.20 * x) - (11.96 * x^2) + (13.72 * y) - (1.57 * y^2) - (7.08 * x * y) + (0.85 * x * y^2) + 0.67(x^2 * y) - 0.08(x^2 y^2) + 0 \quad \text{Eq. 3}$$

$$\%Inh = 79.83 - (0.23 * x) + (0.008 * x^2) - (2.59 * y) - (0.26 * y^2) + (0.05 * x * y) + (0.01 * x * y^2) + 0.002(x^2 * y) - 0.0005(x^2 y^2) + 0 \quad \text{Eq.4}$$

$$\%Inh = 103.64 - (1.41 * x) + (0.056 * x^2) - (8.54 * y) - (0.33 * y^2) + 0.86 * x * y - (0.08 * x * y^2) - 0.03(x^2 * y) + 0.003(x^2 y^2) + 0 \quad \text{Eq.5}$$

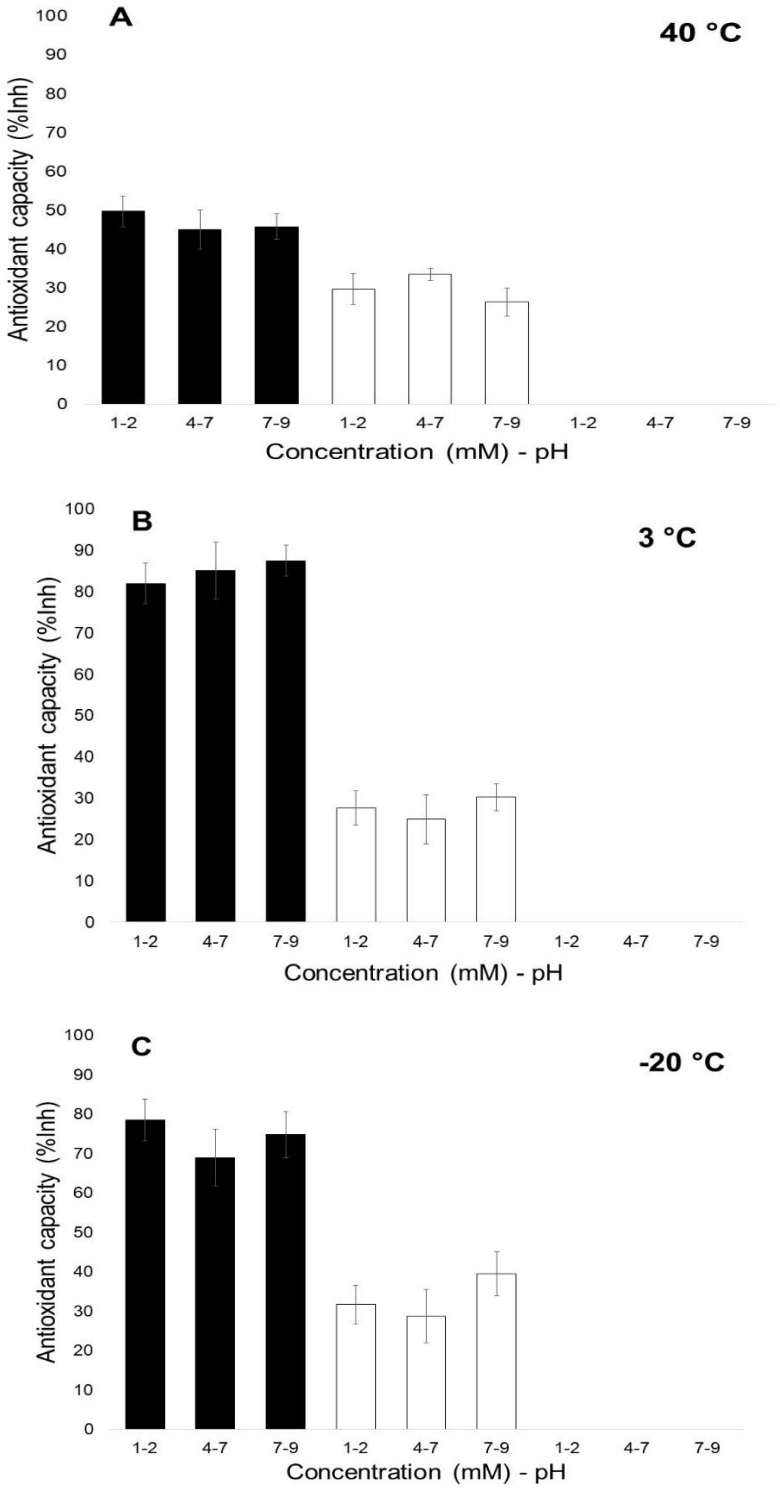
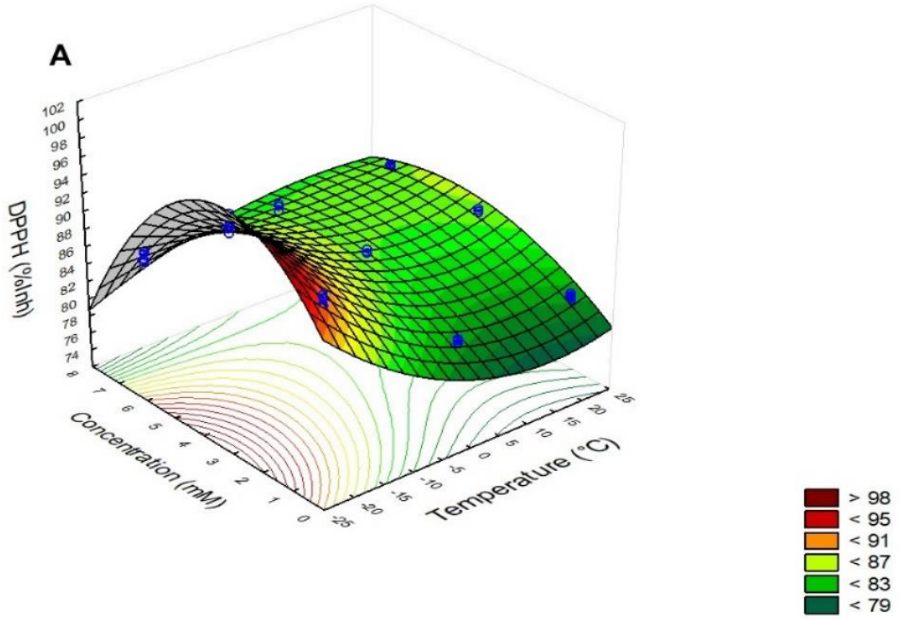
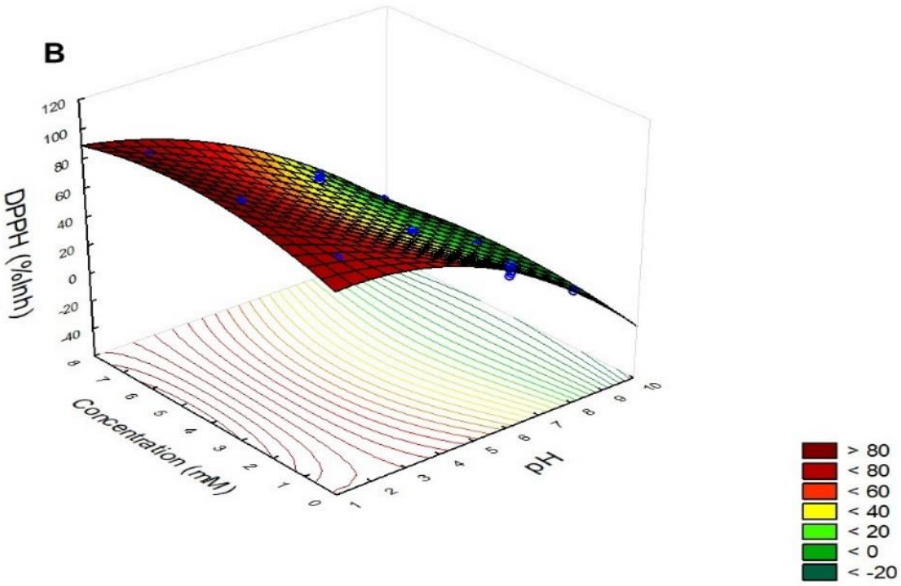


Fig. 1. Antioxidant capacity of gallic acid as a function of pH and temperature

Fig. 2 shows the %Inh influenced from the effect of concentration - temperature (Fig. 2A), concentration - pH (Fig. 2B) and temperature - pH (Fig. 2C). It is worth mentioning that RSM studies are focused on finding the best conditions to better perform its action in biological systems and to increase the antioxidant capacity of different food [21,24,26].

Apparently, the acidic pH - 3 °C ratio gives the highest antioxidant capacities for all concentrations, while it can be noticed that when the pH changes to alkaline, it gradually decreases the %Inh. The result showed that the %Inh increased with 4 mM and 3 °C (Fig. 2A), suggesting that these concentrations are more effective in the antioxidant capacity of GA. Likewise, it can be mentioned that these conditions increases the polarity efficiency of GA. The dielectric constant of methanol may influence its reactivity to maintain the structure of GA and increase the efficiency of its action on DPPH [31]. According to the principle of polarization, a compound dissolves in the solvent having the same polarity, which the dielectric constant of methanol is 32.70 and 32.0 of GA when dissolved in this solvent. Therefore, the polarity of methanol favors the capacity of GA by the deprotonation of the phenol (conjugated base, gallate ion) [32-34].





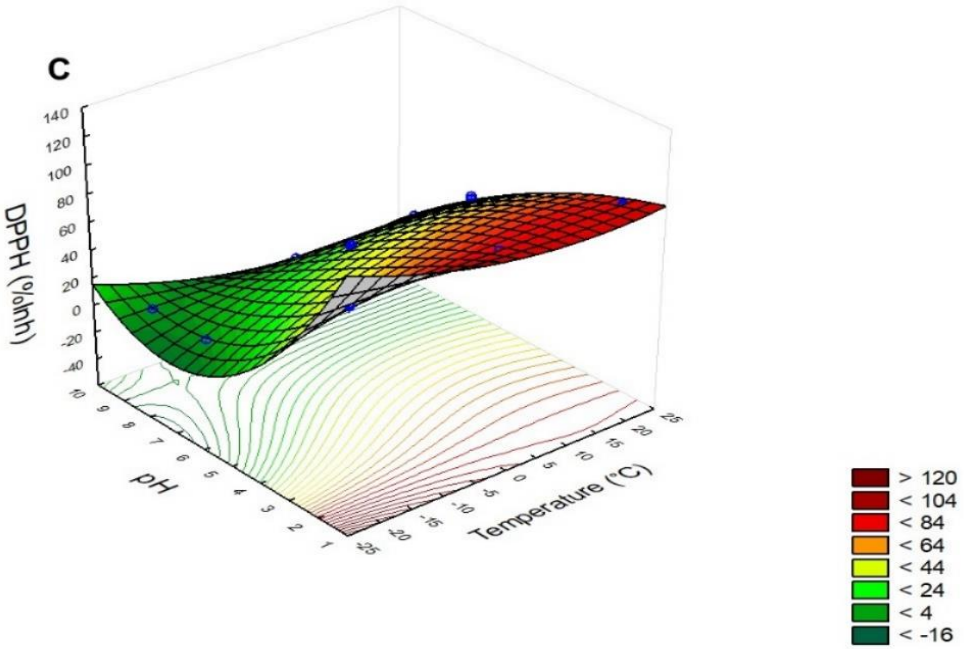


Fig. 2. 3D-response graphs representing representing the antioxidant capacity by DPPH assay of gallic acid obtained by: A) concentration vs temperature; B) concentration vs pH; C) temperature vs pH. The model shown in this figure can be used to predict the behaviour of antioxidant capacity in the variables of GA concentrations, pH and temperature

The RSM graph (Fig. 2A) reveals that the effect of 40 °C and -20 °C significantly affects the %Inh. This same behavior is reflected in the relationship with pH (Fig. 2B) and temperature - pH (Fig. 2). This finding indicates that the antioxidant capacity of GA is very closely related to temperature and pH. The temperatures 40 °C and -20 °C affect the diffusion coefficient of the solvent, causing a decrease in dispersion and diffusion, which accelerates the oxidation of GA [32]. In addition, Fig. 2B and Fig. 2C clearly show that at neutral and alkaline pH, the antioxidant capacity drops drastically, confirming that in these media the GA remains unstable and can generate irreversible changes to its structure and total ionization of the hydroxyl and carboxylic groups [2,4,12,28].

As for the temperature factor, it contributes low %Inh when the pH approaches neutral and alkaline (Fig. 2). When this factor is analyzed with pH (Fig. 2C), a significant loss in %Inh is seen. This is a new data for the literature, since the effect of the factors on the antioxidant capacity of the GA. Daneshfar [10] reported that pH and temperature influence the oxidative effects of GA. Likewise, as GA concentrations increase, instability increases with increasing temperature (Fig. 2A) and pH (Fig. 2B), this is in agreement with several authors, who reported that phenolic properties become pro-oxidant as the concentration of GA increases (Fig. 2B) [10,12-13]. In the literature, there is no mention of experiments correlating the effect of alkaline pH with temperatures of 23 ºC and 3 ºC, in contrast, in that investigated by Fu [28] at 35 ºC (relatively close to 23 ºC) GA becomes unstable at pH 8 and above. This demonstrates the importance of the independent variables (temperature and pH) and their interactions in influencing the antioxidant capacity of a compound of interest.

3.6 Determination and Experimental Validation of the Optimal Conditions

The %Inh was evaluated under optimized conditions, and the results of the analyses are presented in Table 5, which were varied experimentally.

Experimental values ranged from 87% to 89% %Inh of DPPH, regardless of the assay for its determination. The experimental results were consistent with the predicted values (Fig. 3) and were found to be not significantly different at $P > 0.05$ using a paired t-test (Table 5).

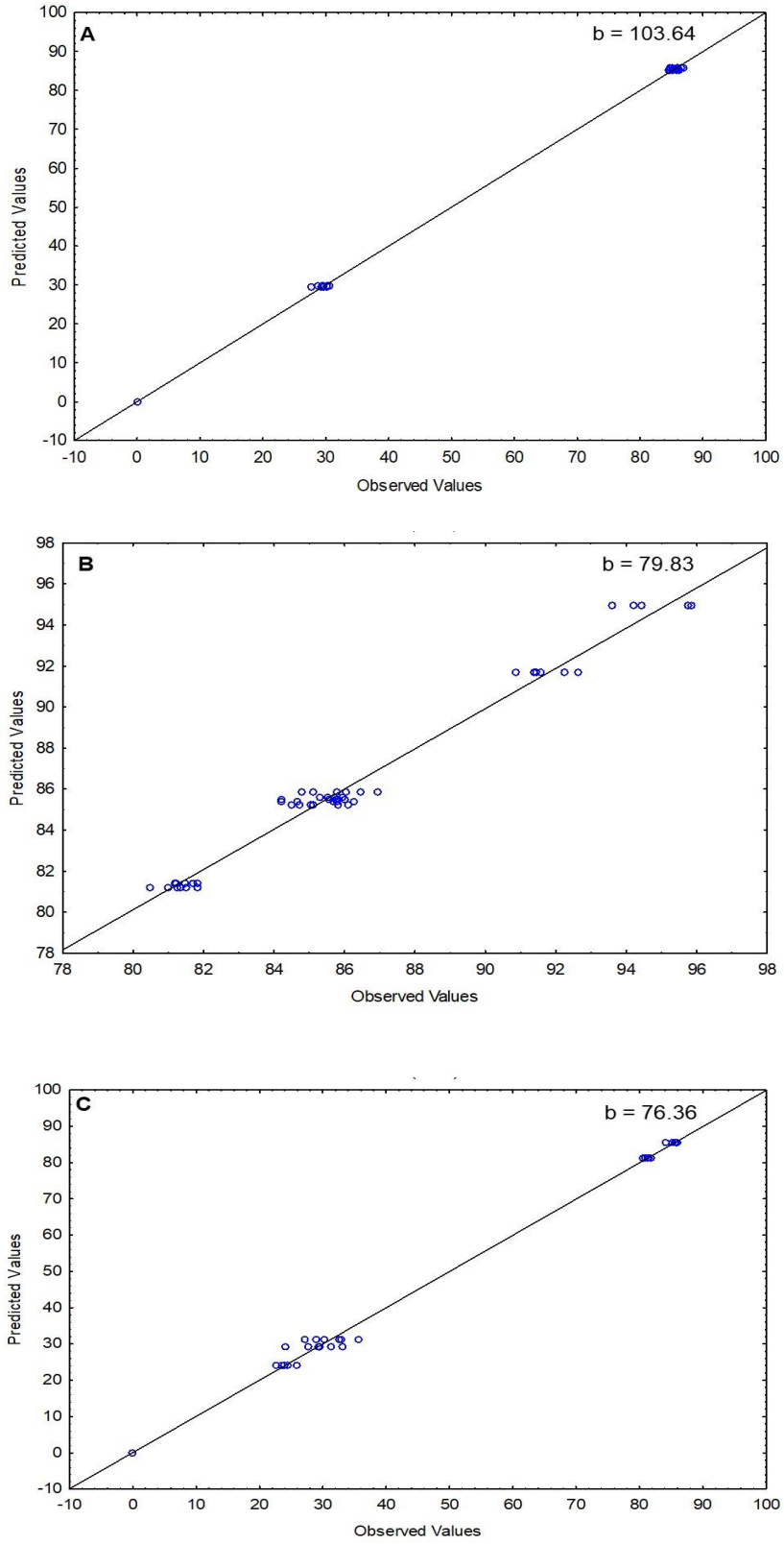


Fig. 3. Predicted versus actual values for the RSM design for DPPH antioxidant capacity. A) concentration vs temperature; B) concentration vs pH; C) temperature vs pH

Table 5. Predicted and experimental values of responses tested at antioxidant capacity (%Inh) of gallic acid

Independent Variables		Optimum Value	
		Predicted	Experimental[a]
Concentration (mM)	Temperature (°C)		
4	2	85.58[a]	87.66 ± 4.02[a]
Concentration (mM)	pH		
4	2	87.11[a]	89.29 ± 4.16[a]
Temperature (°C)	pH		
2	2	88.29[a]	89.68 ± 3.57[a]

[a]Mean of three determinations (n=4) from four replications. Lowercase letters represent significant difference (p< 0.05)

Therefore, the extraction conditions obtained using RSM can be considered accurate and reliable [5]. This confirms that for acidic pH 2 and temperature of 2 ºC is exploitable to evaluate the antioxidant properties of GA. Also, it is found that acidic pH and temperature are relatively significant variables for the antioxidant capacity and would help to postpone auto-oxidation because of hydroxyl groups.

4. CONCLUSION

In summary, this study focused on the effect of concentration, temperature and pH on the antioxidant capacity of GA. Temperature and pH significantly influence the structure of GA, resulting in varying antioxidant capacity. RSM proved to be a useful tool to obtain the best optimal conditions for GA antioxidant capacity. The optimal conditions for the highest antioxidant capacity were 2 °C, pH 2 and 4 mM. Under these conditions, the maximum %Inh values of DPPH were 87.66%, 87.11% and 88.29%, respectively. The experimental values were in agreement with the optimized values. The study could be useful and extended to other PC to evaluate biological processes and properties, in order to improve the efficiency of applications in large-scale biological, cosmeceutical, pharmaceutical and food systems.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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