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Optimization of Total Phenolics Content and Total Antioxidant Activity of Black Tea Using Response Surface Methodology

Tah Clovis Tiku[1], Feumba Dibanda Romelle[2], Yadang Germaine[2], Nyobe Emillienne Carine[2], Achegui Nestor[2], Medoua Nama Gabriel[2], Mbofung Carl Moses[1]

[1]College of Technology, University of Bamenda, Cameroon.
[2]Food and Nutrition Research Centre, Institute of Medical Research and Medicinal Plants Studies, Cameroon.
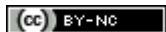
ABSTRACT

Response surface methodology was used to optimize the fermentation conditions for the total phenolics content and antioxidant activity of black tea (Camellia sinensis). Fresh tea leaves were fermented following a rotatable central composite design of eleven experiments replicated thrice. The minimum and maximum fermentation time and temperature were respectively of 1 - 3 h and 30 - 40 °C. The results demonstrated that fermentation time and temperature significantly affect the total phenolics content and antioxidant activity of black tea. The optimum fermentation conditions for the present study were at 36 °C for 35 min which the values for total phenolics content and total antioxidant activity were 38.99 g GAE/100 g dw and 19.74 mg VitC Eq/100 g dw, respectively. Antioxidant activity was strongly correlated with total phenolics content of black tea.

Keywords: Optimization, Response surface methodology, Total phenolics content, Total antioxidant activity, Fermentation, Black tea

Introduction

Tea is one of the most popular and lowest cost beverages in the world and ranks second to water [1]. Tea is a major source of phenolic compounds in human diet. These compounds constitute up to 30% of the tea solids by weight [2]. Recently, tea is considered as a functional food thanks to its high phenolics content and their reported beneficial effects on human health, in particularly as antioxidants [3]. Essentially three main types of Camellia tea are produced annually; black tea (78%), green tea (20%), and 2% which is oolong tea [4].

During black tea processing, fermentation is a critical step. The fermentation stage involves an enzyme-catalysed oxidative reaction to the colored phenolic compounds [5]. The brown colored pigments formed during the fermentation process which give black tea its characteristic color and taste are mostly theaflavins and thearubbigins, the products of phenolics oxidation [6]. The significant reduction of phenolic compounds during black tea production affects the antioxidant properties of the resulting product [7]. This study aims to optimize fermentations conditions of black tea in order to have a black tea with maximum phenolics and antioxidant property.

Materials and Methods

Procurement of tea leaves
The biological material was made of fresh tea leaves (*Camellia sinensis*) that were plucked and collected from the Munjung section of Ndawara Highlands Tea Estate (NHTE), North-West Region of Cameroon.

Black tea processing

Withering of tea leaves
Fresh tea leaves (86 % moisture content) were allowed to wither in order to provide tea leaves with a moisture content of 75 %.

Crushing of tea leaves
Withered tea leaves (15 g) were introduced in a domestic grinder and crushed for 8 s. The crushed tea leaves were scattered as a uniform thin layer of 2 mm on a 10 x 10 cm stainless tray prior to fermentation.

Experimental design for fermentation of tea leaves
In order to assess the effect of fermentation temperature and time on the level of total phenolic compounds and the total antioxidant activity, a full factorial design (3^2) was applied and three replicates in the center point were added to the experiment to fit the surface plot for the responses and to estimate the pure error of the multiple regression models [10], totaling 11 runs as displayed in table 1. So, crushed tea leaves were allowed to oxidize in a pre-heated incubator settled at the various couples of temperature /time as defined by the experimental design (Table 2).

Table 1. Coded and decoded levels of independent variables used in the RSM design

Independent variables	Symbols	Levels				
		-1.414	-1	0	+1	+1.414
Fermentation time (min)	X_1	0.6	1.0	2.0	3.0	3.4
Fermentation temperature (°C)	X_2	28	30	35	40	42

Drying of black tea

Fermented tea leaves were dried at 120 °C for 27 min. Dried tea samples were allowed to cool at room temperature. The cold dried samples were ground and sieve to obtain powder with particle size ≤ 500μm which were used to prepare tea extracts.

Preparation of tea extracts

To prepare tea extracts, 0.5 g of each tea powder was introduced into a round bottom flask and extracted twice with 25 mL of ethanol 80% on a magnetic stirrer for 30 min. After this, the mixtures were filtered with a Wathman filter paper Nº 1 and the filtrates were poured in 50 mL standard flasks and the volume were adjusted to the mark using ethanol 80%. The tea extracts were kept at 4 ºC until analysis.

Evaluation of total phenolics content

Total phenolic content was determined according to [8]. To 125 μL of extract, were added 0.5mL of distilled water and 125 μL of the solution Folin Ciocalteau: water (1:16, v/v). After 6 min, 1250 μL of 7% sodium carbonate were added and the volume was adjusted to 3 mL with distilled water and the absorbance measured after 90 min against a blank tube containing extracting solvent at 760 nm. The results were expressed as gram of gallic acid equivalents per 100 g of dry weight (g GAE/100 g dw). All samples were analyzed in triplicates.

Evaluation of total antioxidant activity

The total antioxidant capacity of tea extracts was determined using the procedure described by [9]. A volume of 20 μL of tea extract was put into a test tube and 280 μL of distilled water added. After this, 3 mL of a reactive solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was added and the tubes were heated or incubated in a water bath at 95 °C for 90 min. The mixture was then allowed to cool at room temperature and the absorbance was measured at 695 nm using a spectrophotometer. The total antioxidant capacity was expressed as mg of Vitamin C equivalents (mg Vit C Eq /100 g dw). All samples were analyzed in triplicates.

Statistical analysis

The models for predicting the total phenolics content ($\hat{y}_1$) and antioxidant capacity ($\hat{y}_2$) of black tea were expressed individually as:

Equation (1): $\hat{y}_1 = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_{11} x^2_{11} + \alpha_{22} x^2_{22} + \alpha_{12} x_1 x_2$
Equation (2): $\hat{y}_2 = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x^2_{11} + \beta_{22} x^2_{22} + \beta_{12} x_1 x_2$

Where $\hat{y}_1$ and $\hat{y}_2$ represent the response variables; α_0 and β_0 the constant coefficients; $\alpha_1, \alpha_2, \beta_1$ and β_2 the coefficients of the linear effects; $\alpha_{11}, \alpha_{22}, \beta_{11}$ and β_{22} the coefficients of the squared effects; α_{12} and β_{12} the coefficients of the interaction effects.

The response surface graphs of predicted values by models were plotted using Sigma Plot v. 12.5. The values of R^2, adjusted-R^2 of models were evaluated to check the model adequacies.

Three experimental replicates were performed under optimized conditions and the experimental and predicted values were compared.

Results and Discussion

Experimental data
Fermentation is a critical step in black tea processing. The total antioxidant activity and total phenolics content of black tea are affected by multiple parameters. In this study, the independent variables were the temperature and time of the fermentation. The resulting total and total antioxidant activity as dependent variables for all runs are reported in table 2. The total phenolics content and total antioxidant activity ranged respectively from from 25.63 ± 1.12 to 40.36 ± 0.10 g GAE/100 g dw and 10.55 ± 0.35 to 20.61 ± 0.66 mg Vit C Eq/100 g dw.
Table 2. Experimental design for total phenolics content and for total antioxidant activity

Runs	Coded and real values for fermentation temperature	Coded and real values for fermentation time	Total phenolics content (g GAE/100 g dw)	Total antioxidant activity (mg VitC Eq/100 g dw)
1	0 (35 $^{\circ}$C)	0 (2 h)	31.42 ± 0.86	12.36 ± 0.72
2	0 (35 $^{\circ}$C)	0 (2 h)	31.65 ± 0.57	12.45 ± 0.33
3	0 (35 $^{\circ}$C)	0 (2 h)	30.85 ± 1.31	12.83 ± 0.90
4	+1.41 (42 $^{\circ}$C)	0 (2 h)	25.63 ± 1.12	10.55 ± 0.35
5	-1 (30 $^{\circ}$C)	1 (3 h)	31.38 ± 0.96	12.67 ± 0.27
6	0 (35 $^{\circ}$C)	+1.41 (3 h 24 min)	31.52 ± 0.13	12.42 ± 0.13
7	+1 (40 $^{\circ}$C)	1 (3 h)	29.06 ± 0.37	10.69 ± 0.17
8	-1.41 (28 $^{\circ}$C)	0 (2 h)	32.30 ± 1.11	14.39 ± 0.56
9	+1 (40 $^{\circ}$C)	- 1 (1 h)	33.48 ± 0.76	17.42 ± 0.27
10	-1 (30 $^{\circ}$C)	- 1 (1 h)	34.18 ± 0.58	15.53 ± 0.81
11	0 (35 $^{\circ}$C)	-1.41 (35 min)	40.36 ± 0.10	20.61 ± 1.85

Data analysis
The experimental data were fitted into the second-order polynomial equations and the regression coefficients were calculated, the significance of the coefficients of the models were determined by analysis of variance (ANOVA) as summarized in table 3.

Table 3. Analysis of variance for the responses of black tea

Responses	Source of variation	Sum of square	Degree of freedom	Mean square	f-value	p-value
Total Phenolics Content						
	X_1 : Time	131.355	1	131.355	141.99	0.0000
	X_2 : Temperature	67.7884	1	67.7884	73.28	0.0001
	X_1X_1	87.2792	1	87.2792	94.35	0.0001
	X_1X_2	5.11446	1	5.11446	5.53	0.0570
	X_2X_2	25.1014	1	25.1014	27.13	0.0020
	Lack of fit	34.7739	19	1.83021	1.98	0.2034
	Pure error	5.5506	6	0.9251		
	Corr total	400.198	32			
$R^2 = 89.9239$ R^2 (adjusted) = 87.1025						
Total Antioxidant Activity						
	X_1 : Time	144.795	1	144.795	474.63	0.0000
	X_2 : Temperature	13.8878	1	13.8878	45.52	0.0005
	X_1X_1	54.4506	1	54.4506	178.49	0.0000
	X_1X_2	14.8025	1	14.8025	48.52	0.0004
	X_2X_2	0.0718739	1	0.0718739	0.24	0.6446
	Lack of fit	12.5576	19	0.660927	2.17	0.1721
	Pure error	12.5576	6	0.30507		
	Corr total	250.107	32			
$R^2 = 94.2472$ R^2 (adjusted) = 92.6365						

Table 3 demonstrates that both fermentation time and temperature have significant linear, interaction and quadratic effects ($p < 0.05$) showing their more significant effect on total phenolics content and total antioxidant activity of black tea [11]. The coefficients of multiple determinations (R^2) for total phenolics content and total antioxidant activity respectively were 89.9239 and 94.2472. So, the calculated model was able to explain 89.92 and 94.25 % of the results in case of total phenolics content and total antioxidant activity, respectively. The adequacy of the model to fit the experimental data was verified also by the lack of fit testing; ANOVA for the lack of fit test for all the responses were insignificant ($p > 0.05$). With a non-significant lack-of-fit ($p \geq 0.05$) and both R^2 and R^2 adj higher than 98%, the analysis indicates that the empirical models fit the experimental data satisfactorily and may be used for prediction purposes [12].

The regression equations obtained for total phenolic content ($\hat{y}_1$) and total antioxidant activity ($\hat{y}_2$)

Equation (1): $\hat{y}_1 = 31.3098 - 2.33948x_1 - 1.68063x_2 + 2.26979x^2_1 - 0.652844x_1x_2 - 1.21725x_2^2$
Equation (2): $\hat{y}_2 = 12.547 - 2.45625x_1 - 0.760697x_2 + 1.7928x^2_1 - 1.11065x_1x_2 - 0.0651351x_2^2$

Response Surface Methodology

Response surface methodology is the best approach to assess the effect of different experimental factors (linear, quadratic and interaction effects) on the analytical responses. To visualize the effects of fermentation temperature and time and the relationships between the factors and the response variables, 2D contour plots of the fitted regression equations were generated.
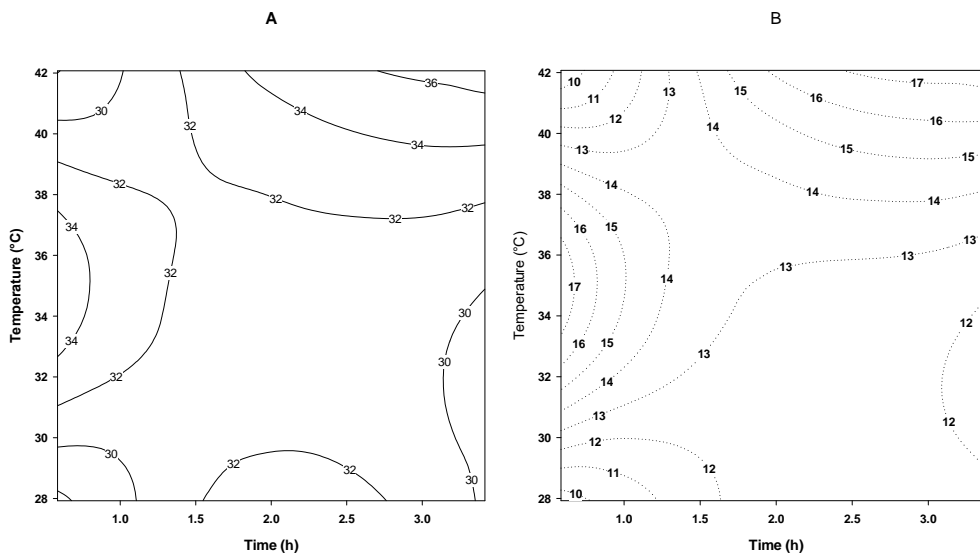

Figure 1. Effect of fermentation time and temperature on total phenolics content (A) and total antioxidant activity (B) of black tea

Figure 1 shows the contour plots of fermentation time and temperature on total phenolics content (a) and total antioxidant activity (b) of black tea. It is noticeable from figure 1a that when fermentation was conducted for 0.5 to 1 h, total phenolics content increased with fermentation temperature between 28 and 38 °C. After 38 °C, total phenolics content started to decrease. When fermentation was conducted for more than 1 h, total phenolics content decreased with fermentation temperature below 38 °C and increased with fermentation temperature beyond 38 °C. A similar trend was observed in case of total antioxidant activity (figure 1b). Concomitant results were found by [13] who reported temperatures between 30 - 38°C to significantly improve polyphenol contents of bush tea fermented for 30 min.

In natural form, phenolic compounds are combined or bound with sugar which reduces their availability to organism. During fermentation, proteolytic enzymes hydrolyze complexes of phenolics into soluble-free phenols and other simpler and biologically more active ones [14, 15]. The bioconversion of the conjugated forms of phenolic compounds into their free forms during fermentation improves their health-linked functionality as antioxidants [16]. However, increasing the fermentation time might allow the microorganisms to use those available compounds as substrates for their growth, thus reducing the sample concentration. Therefore, the fermentation period is critical to ensure optimal breaking down of the compound linkages but not to allow compounds to be substrates for microbial growth [17].

In the other hand, the enzyme polyphenoloxidase is of considerable importance in black tea processing because polyphenoloxidase mainly oxidizes polyphenol to theaflavins. Reference [18] reported that with increase in temperature, the stability of enzyme decreased during fermentation and at 35 °C it can only be observed 90% stability of polyphenoloxidase up to 40 min. This decrease in polyphenoloxidase activity during fermentation could explain the increase in total phenolics content observed after fermentation beyond 38 °C for more than 1 h since polyphenoloxidase oxidizes the phenolic compounds.

Process Optimization and Confirmatory Study

A simultaneous optimization using the desirability function was performed in order to maximize the total phenolic compounds and also to maximize the antioxidant capacity of black tea [19]. The optimum fermentation conditions for the present study were at 36 °C for 35 min which the values for total phenolics content and total antioxidant activity were 38.99 g GAE/100 g dw and 19.74mg VitC Eq/100 g dw, respectively.

The experimental values for the same fermentation conditions include total phenolics content and total antioxidant activity of 40.36 ± 1.99 g GAE/100 g dw and 20.62 ± 1.85 mg VitC Eq/100 g dw, respectively.

The experimental and predicted values were within the range and did not vary significantly at 5% level. So, the regression equations obtained can be used to predict the total phenolics content and total antioxidant activity of black tea.

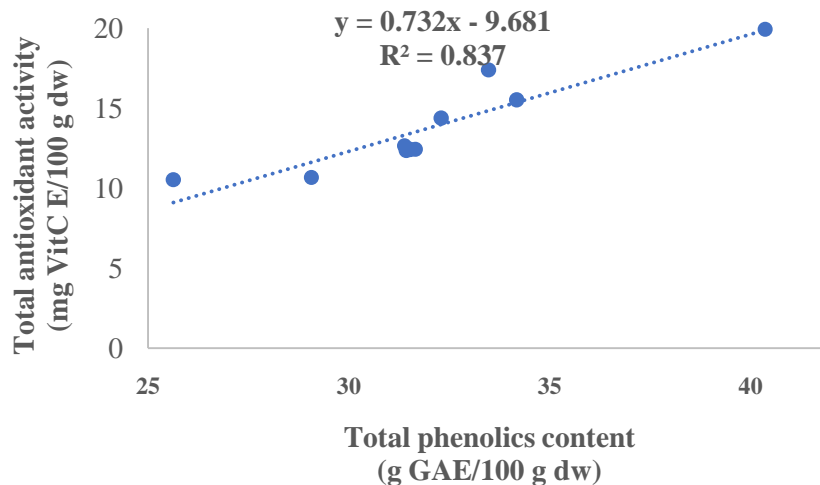**Correlation between Total Phenolics Content and Total Antioxidant Activity**

Figure 2. Regression plot between total phenolics content and total antioxidant activity of black tea

Figure 2 showed that total antioxidant activity was strongly correlated ($R^2 = 0.84$) to total phenolics content in black tea meaning that black tea possesses antioxidant properties through its chemical components (thearubigins, phenolic acids, catechins, and theaflavins). This strong correlation was earlier reported by [20, 21] and implying thus that phenolics possess antioxidant property that provides protection against oxidative stress.

Conclusion

Fermentation time and temperature significantly influence the total phenolics and antioxidant activity of black tea. The above study provides an insight on the need of fermenting tea leaves at 36 °C for 35 min in order to obtain a functional black tea. It is worthy to mention that the modeling of the total phenolics content and the antioxidant activity of black tea is important because food companies can use this statistical approach as the basis for developing products with a functional claim.

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