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ORIGINAL PAPER

Optimizing homogenizer-assisted extraction of chlorophylls from plantain epicarp (*Musa paradisiaca* L.)

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Abstract
The objective of this study was to establish parameters of optimization of the factors such as solid/solvent ratio, stirring speed and extraction time in homogenizer-assisted extraction (HAE) of chlorophyll a, b and total from plantain epicarp (*Musa paradisiaca* L.) using the Box–Benhken design. The individual effect and the interactions of the process variables (solid/solvent ratio, stirring speed (revolutions per minute, rpm), and extraction time in HAE) on the chlorophyll a, b and total contents in the extract were studied. The optimization was carried out with a Derringer desirability function, and it was found that the optimal extraction conditions included a solid/solvent ratio of 0.025 g/mL, an stirring speed of 13,508 and 90 s of treatment with Ultra-turrax. The corresponding predicted values were 33.67 mg/100 g of chlorophyll a, 32.915 mg/100 g of chlorophyll b and 66.585 mg/100 g of total chlorophyll. Finally, the optimal conditions were experimentally verified, and it was established that extraction with an Ultra-Turrax treatment is more efficient than the conventional method (maceration).

Keywords Box–Benhken design · Derringer desirability function · Extract · Chlorophyll

Introduction

Plantain (*Musa paradisiaca* L.) plays an important role in diets in tropical countries. In Colombia, the production of this product has grown significantly, becoming the third largest producer worldwide with approximately 3.948.216 tons annually [1]. However, the ingestion and transformation of plantains generate waste that has little use at the agroindustrial level, including the epicarp [2]. This by-product represents approximately 30% of the fruit weight, has a high content of dietary fiber, proteins and essential amino acids [3]. These residues bring economic and environmental problems as they require high values of biochemical demand (BOD) and oxygen chemistry (COD) for their degradation, as they are biological materials with high contents of hemicellulose, cellulose and lignin [4, 5]. However, the presence of compounds of interest such as pigments, and phenolic compounds in these residues, makes them attractive resources for agroindustrial valuation as an important source of additives in the food, cosmetic and pharmaceutical industry [6, 7]. In Cavendish plantains (*Musa acuminata* "Williams"), a close relative of the species *Musa paradisiaca* L., up to 36.69 µg of total chlorophyll/g fresh weight has been found in the peel of late-maturity-stage fruits [8]. Chlorophyll is a liposoluble pigment that originates in chloroplasts [9] and its function is to capture the energy of sunlight to synthesize carbohydrates from CO_2 during photosynthesis [10]. It consists of a porphyrin ring with a magnesium atom (Mg^{2+}) in the center and an esterified and hydrophobic phytol tail [11]. Generally, five classes of chlorophylls are found in photosynthetic organisms and plants, but chlorophyll a and b are predominant in food at a 3:1 ratio. Notably, chlorophyll a has a methyl group and b a formyl group at position 7 [12]. This pigment has been described as a beneficial compound for humans, helping in the growth and repair of tissues, stimulating red blood cells for an improved oxygen supply, neutralizing free radicals with other vitamins such as A, C and E, and reducing the ability of carcinogens to bind to DNA in different major organs of the body [13]. Recently, it has been used to replace synthetic dyes derived from coal tar, which are not fully assimilated and absorbed in the body [14]. The sources from which chlorophyll has been extracted have mainly included green leaves (spinach, alfalfa, mulberry, and elephant grass), and this pigment has been applied in dairy products, soups,

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beverages and candy [15]. The extraction process of a natural colorant in a plant matrix, requires an extraction stage that allows extraction of the crude pigment from the matrix of interest [16]. The extraction of pigments in plants is traditionally done using Soxhlet extractor, magnetic stirrer, maceration, and heat reflux, and these conventional methods demand high energy, time and solvent consumption, and register low efficiency and high costs [17]. These limitations in the traditional extraction of pigments have allowed the application of emerging methods that allow to increase extraction efficiency, among the method methods used is ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), pulsed-electric field (PEF) extraction, enzyme-assisted extraction (EAE), and homogenizer-assisted extraction (HAE) [16, 18–21] report that HAE has greater effectiveness in the extraction of phenolic compounds and carotenoids in terms of time and efficiency, if it is evaluated against UAE and MAE. Recent studies report the benefits of extracting bioactive compounds in different plant tissues [18–23]. Despite being an extraction alternative in the extraction of bioactive compounds in plant tissues, it is necessary to evaluate the effect of extraction time, stirring speed, polarity, solvent and the solid-solvent ratio in the extraction of other bioactive compounds of interest. Thus in this study, the objective was to establish parameters of optimization of the factors such as solid/solvent ratio, stirring speed and extraction time in HAE of chlorophyll a, b and total from plantain epicarp (*Musa paradisiaca* L.) using the Box–Benhken design.

Materials and methods

Material and reagents

The plantain (*Musa paradisiaca* L.) was purchased at a local supermarket and stored at room temperature before conditioning. The solvent was ethanol of analytical grade. It was acquired from Merck® chemicals, Colombia.

Conditioning the raw material

Four kilograms of green banana epicarp were extracted, and the samples were manually cut into pieces of approximately 2 cm^2, using a stainless steel knife, to favor freeze-drying (Freezone 6 plus, LABCONCO, Kansas City, MO, USA). This operation was performed in 3 batches, each for 24 h, at pressures close to 0.120 mbar and chamber temperatures from − 25 to − 30 °C. With this process, 0.54 kg of dry sample was obtained, which was ground (Labortechnik M20, IKA, Campinas, SP, Brasil) and sieved in the same batch. For extraction, a powder with a particle size between 250 and 600 µm was used.

Characterization of the raw material

For the physico-chemical analysis of the fresh plantain epicarp, moisture was determined with the moisture balance HB43-S Halogen (METTLER TOLEDO, Columbus, OH, USA), and dry matter was calculated. Likewise, an extract of the fresh epicarp was used to measure the water activity using the AQUALAB Series 3 TE (Meter Group, Pullman, WA, USA), the titratable acidity was determined as the percentage of malic acid, and the soluble solids and pH were determined according to AOAC [24]. It should be noted that these parameters were evaluated in the lyophilized sample, except for the soluble solids.

Ultra-turrax extraction of chlorophyll

The study factors in the extraction of the chlorophylls in the lyophilized plantain epicarp corresponded to the solid/solvent ratio (g/ml), adding 0.5, 1 and 1.5 g in a falcon tube, along with 20 mL of 80% ethanol (v/v) as the solvent. The other two factors were extraction rate: 12,000, 13,500 and 15,000 rpm and duration: 30, 60 and 90 s (18 digital ULTRA TURRAX, IKA, Campinas, SP, Brasil). After the treatments, the extracts were centrifuged at 4500 rpm for 20 min (Centrifugen Universal 320, Hettich, Vlotho, Herford, Alemania) and kept at 4 °C before the quantification of the pigments. The experiments were carried out in a random order to decrease the effects of the variability in the responses as a result of external factors.

Chlorophyll a, b and total contents

The determination of chlorophylls was carried out with the measurement of the absorbance by spectrophotometry using the technique described by Richardson et al. [25] with ethanol as a blank. Subsequently, the equations proposed by Ignat et al. [26] to calculate the concentration of chlorophyll a, chlorophyll b and total chlorophyll were used to the Eqs. 1–3.

$$C_a(\mathrm{mg}/100\,\mathrm{g}) = \left(13,36A_{664} - 5,19A_{649}\right) \times \left(\frac{V}{1000 \times W}\right) \times 100 \quad (1)$$

$$C_b(\mathrm{mg}/100\,\mathrm{g}) = \left(27,43A_{649} - 8,12A_{664}\right) \times \left(\frac{V}{1000 \times W}\right) \times 100 \quad (2)$$

$$C_{total}(\mathrm{mg}/100\,\mathrm{g}) = \left(5,24A_{664} + 22,24A_{649}\right) \times \left(\frac{V}{1000 \times W}\right) \times 100 \quad (3)$$

where C_a is the chlorophyll a content, C_b is the chlorophyll b content, C_{total} is the total chlorophyll content, A_{664} is the absorbance at 664 nm, A_{649} is the absorbance at 649 nm, V is the volume of the used solvent (mL of 80% ethanol (v/v), and W is the weight of the sample (g).

Experiment design

In order to optimize the extraction of chlorophylls, the methodology proposed by Tong et al. [27] was followed. Therefore, a Box–Benhken design was used, which is an independent and rotating quadratic design with no star points and combinations of factors that are found in the midpoints of the edges of the variable space and in the center [28]. The experiment ranges selected for the independent variables were: solid/solvent ratio (0.025–0.075 g/mL), stirring speed (12,000–15,000 rpm) and extraction time (30–90 s) of the (HAE). After the selection of independent variables and their ranges, the experiments were established based on the Box–Benhken design with three factors on three levels, and each independent variable was coded according to Maran et al. [29] with Eq. 4, between + 1, 0 and − 1, corresponding to a low level, medium level and high level, respectively (Table 1). It should be noted that the central points were chosen according to previous studies [30, 31].

$$x_i = \frac{X_i - X_{cp}}{\Delta X_i}; \quad i = 1, 2, 3, \ldots, k \tag{4}$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_{cp} is the real value of an independent variable in the central point and ΔX_i is the rate of change of the real value of the I variable corresponding to a variation of one unit for the dimensionless value of the variable I.

In this research, a Box–Benhken 2^3 design was developed, which consisted of 15 experiments in duplicate, including three central points. The total number of experiments was calculated with Eq. 5. Where K is the number of factors and C_p is the number of replicas in the central point [28].

$$N = K^2 + K + C_p \tag{5}$$

On the other hand, the development of the process was evaluated by analyzing the relationship between the response (Y) and the input parameters of the process $(X_1, X_2, \ldots X_k)$ using Eq. 6 [29].

$$Y = f(X_1, X_2, \ldots X_k) + e \tag{6}$$

where Y is the actual response function, whose format is unknown, and e is the error that describes the differentiation. The dependent parameter (Y) was related to the independent variables $(X_1, X_2, \ldots X_k)$ by means of a second-order polynomial model. The generalized response surface model is presented in Eq. 7 [32].

$$Y = \beta_o + \sum_{a=1}^{c} \beta_a X_a + \sum_{a=1}^{c} \beta_{aa} X_a^2 + \sum_{a} \sum_{b \leq 2}^{c} \beta_{ab} X_a X_a + e_a \tag{7}$$

where Y is the response, the X_a and X_b variables (the range of a and b was from 1 toc), β_o the intercept coefficient of the model; β_a, β_{aa} and β_{ab} are the coefficients of interaction of linear, quadratic and second order terms, respectively. While, c is the amount of independent parameters ($c = 3$ in this study), and e_a is the error.

Verification of optimal conditions

The Derringer desirability function was used to generate the optimal weight conditions of the lyophilized epicarp, rate and duration of the Ultra-turrax treatment for the extraction of chlorophylls. In order to determine the validity of the model, the predicted chlorophyll a, b and total contents were compared as maximums to those quantified from extracts obtained under optimal conditions and from a conventional method. The conventional method used maceration for 24 h at 4 °C [33, 34].

Statistical analysis

Minitab 18.0 Free Trial software (Minitab Inc., State College, PA, USA) was used for a t-test of two samples for the physicochemical parameters, a one-way ANOVA, Tukey test for verification of optimal conditions, and $p < 0.05$ was applied to determine the level of significance between the mean values. With Design Expert 11.0 software (Stat Ease Inc., Minneapolis, USA), the significant terms of the model were found through the analysis of variance (ANOVA) for the response variables, the lack of fit, the determination coefficients (R^2) and the coefficients of variation (CV%). The experiment data were used to plot response surfaces and contour plots.

Table 1 Coded factors, ranges and levels for the Box–Benhken design

Independent variables	Factors	Coded levels		
	X	− 1	0	+ 1
Solid/solvent ratio (g/mL)	X_1	0.025	0.50	0.075
Rate (rpm)	X_2	12,000	13,500	15,000
Duration (s)	X_3	30	0	90

Results and discussion

Characterization of the raw material

As seen in Table 2, the lyophilization process significantly affected ($p < 0.001$) all of the physicochemical variables evaluated in the epicarp. The drying led to the removal of approximately 95% of the free water of the plantain epicarp, which reduced the water activity and increased the dry matter (Table 2). Likewise, there was an increase in pH and a decrease in acidity after drying, corroborating the loss or degradation of water-soluble organic acids [35]. The elimination of water in the samples probably allowed the interaction of oxygen with organic acids, triggering oxidative degradation processes or the generation of new products, which allowed the reduction of the concentration of organic acids in the study samples [36].

Analysis of the Box–Benhken design

Table 3 shows the mean values of the response variables evaluated in the Box–Benhken design. The range of the chlorophyll a, b and total in the study samples corresponded to 22.45–28.86 mg/100 g, 12.50–34.47 mg/100 g and 35.65–68.53 mg/100 g of sample, respectively. In other studies, it was found that the total chlorophyll content in the evaluated samples exceeded those recorded by Isaak et al. [37] and Kudachikar et al. [38] in the epicarp of two banana varieties ('Monthan' and 'Robusta'), respectively, before storage under modified atmosphere. The statistical tests of the model were carried out with the experiment values in Table 3. The summary of the tests and the regression coefficients are shown in Table 4. With the R^2 values, it was established that the chlorophyll a, b and total extraction process fit a quadratic model; however, the coefficients of variation (CV%) were relatively high, especially for chlorophyll b.

Second order polynomial equation fitness

The model, the solid–liquid ratio, the interaction of the solid–liquid ratio factors, and the duration of extraction, and the quadratic effect of rate, significantly affected the three response variables. The square of the solid–liquid ratio, and the extraction duration only significantly affected chlorophyll a, and the square of the extraction duration only

Table 2 The physicochemical parameters in fresh and lyophilized plantain epicarp

Sample	Physicochemical parameter				
	Moisture (%)	Dry matter (%)	pH	Titratable acidity (%)	Aw
Fresh epicarp	85.24 ± 1.32	14.76 ± 1.32	5.43 ± 0.01	2.25 ± 0.10	0.97 ± 0.01
Lyophilized epicarp	4.23 ± 0.18	95.77 ± 0.18	5.68 ± 0.01	0.36 ± 0.04	0.29 ± 0.01
t-test	***	***	***	***	***

t-test: ***$p < 0.001$

Table 3 Combinations of experiment conditions for the extraction of chlorophyll a, b and total and observed results

Run	X_1	X_2	X_3	C_a**	C_b***	***
1	0	1	−1	23.74 ± 1.99^{b}	12.50 ± 2.16^{c}	36.24 ± 4.16^{c}
2	1	−1	0	23.40 ± 0.26^{b}	13.24 ± 1.01^{c}	35.65 ± 1.28^{c}
3	−1	0	1	34.05 ± 4.02^{a}	34.47 ± 4.10^{a}	68.53 ± 8.12^{a}
4	−1	1	0	28.53 ± 2.55^{ab}	19.57 ± 1.49^{bc}	48.10 ± 1.06^{bc}
5	0	0	0	23.18 ± 2.34^{b}	13.70 ± 0.02^{c}	36.88 ± 2.32^{c}
6	0	−1	1	25.49 ± 0.57^{ab}	14.04 ± 1.29^{c}	39.54 ± 1.87^{bc}
7	1	0	−1	27.76 ± 0.75^{ab}	16.56 ± 0.04^{bc}	44.32 ± 0.79^{bc}
8	0	0	0	23.07 ± 1.56^{b}	13.63 ± 0.57^{c}	36.70 ± 1.00^{c}
9	0	−1	−1	28.86 ± 0.06^{ab}	14.34 ± 0.52^{c}	43.19 ± 0.46^{bc}
10	0	0	0	22.67 ± 1.01^{b}	15.88 ± 0.27^{bc}	38.55 ± 0.74^{bc}
11	0	1	1	25.35 ± 0.06^{ab}	17.24 ± 1.96^{bc}	42.59 ± 2.02^{bc}
12	1	1	0	22.45 ± 0.60^{b}	13.21 ± 1.81^{c}	35.66 ± 2.41^{c}
13	−1	−1	0	28.57 ± 0.93^{ab}	25.84 ± 3.69^{ab}	54.41 ± 4.61^{ab}
14	−1	0	−1	26.26 ± 1.86^{ab}	16.13 ± 2.97^{bc}	42.39 ± 1.12^{bc}
15	1	0	1	22.75 ± 0.56^{b}	13.77 ± 1.17^{c}	36.51 ± 1.72^{c}

Values with different letters in the same column are significantly different ($p < 0.05$) from each other

ANOVA: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

Table 4 Regression coefficients and summary of statistical tests

Factor	C_a	C_b	C_{total}
Model	0.004**	0.024*	0.004**
Intercept	31.09	22.68	53.77
A-solid/solvent ratio	−2.63**	−4.91**	−7.51***
B-rate	−0.78	−0.62	−1.40
C-duration	0.13	2.50	2.63
AB	−0.23	1.56	1.33
AC	−3.20**	−5.28*	−8.48**
BC	1.24	1.26	2.50
A^2	−1.75*	0.49	−1.26
B^2	−3.60**	−5.21*	−8.81**
C^2	−1.63*	−2.94	−4.58*
Lack of fit	0.27	0.58	0.33
R^2	0.96	0.92	0.97
CV (%)	4.11	15.41	6.30

ANOVA: *p < 0.05, **p < 0.01 and ***p < 0.001

significantly affected total chlorophyll (Table 4). The lack of adjustment was not significant (p > 0.05) in the three response variables, and the coefficients of determination R^2 between 0.92 and 0.97 (Table 4) show that the model fits the data of the experiment, and can predict the variations within the system, and the values of the experiment showed a good fit with the following regression of the mode, the final equations obtained (8–10) in terms of coded and significant factors in the extraction process are presented below.

$$C_a = 31,090 - 2632A - 3201AC - 1753A^2 - 3599B^2 - 1633C^2 \quad (8)$$

$$C_b = 22,684 - 4905A - 5283AC - 5209B^2 \quad (9)$$

$$C_{total} = 53,774 - 7537A - 8484AC - 8808B^2 - 4576C^2 \quad (10)$$

Effect of the process variables

The response surface and contour plots were used to represent the effect of the chlorophyll a, b and total extraction process variables. It is important to note that the treatment with Ultra-turrax exerted an influence on the process since it broke the cell wall of the epicarp and allowed the chlorophylls to be released easily [27].

For the extraction, it was noted that the most significant factor was the solid/solvent ratio, followed by the interaction between variables A and C (Table 4). Also, the highest amount of chlorophyll a, b and total, was achieved with a solid/solvent ratio of 0.025 g/mL, a rate close to the central point (13,500 rpm) and a duration of 90 s (Figs. 1, 2, 3). As observed by Swamy et al. [32], the solid/solvent ratio is a

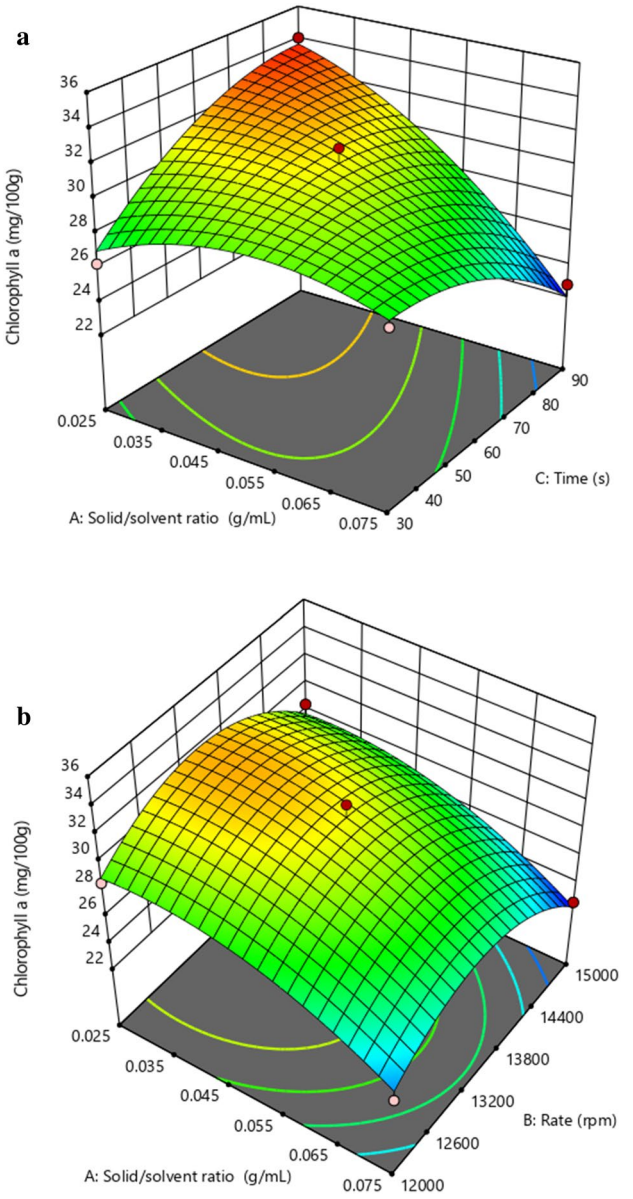

Fig. 1 Effect of the process variables on the chlorophyll a content. **a** Effect of the solid/solvent ratio and duration. **b** Effect of solid/solvent ratio and rate

significant factor in the extraction of betalains from *Beta Vulgaris*, and a longer process time increases the content of pigments studied in this research.

According to Sumanta et al. [39], the polar and apolar nature of the chlorophyll molecule can explain the behavior of chlorophyll during extraction processes. Chlorophyll has an Mg^{2+} at its center, which gives it ionic and hydrophilic characteristics, and a ring with a carbonyl group in its tail makes it polar. Given the polarity of the studied compounds, the extraction was carried out thanks to the electrostatic interactions between the dipoles, which helped the different chlorophyll classes to be dissolved with a polar solvent