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Optimization of Cocoa Butter (*Theobroma cacao* L.) Extraction with Ethanol Using Response Surface Methodology

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Abstract Cocoa butter is the natural and edible fat extracted from the cocoa bean, it has a characteristic aroma and flavor of chocolate and is mainly composed of lipids, including saturated and monounsaturated fatty acids. The classic extraction technique for these lipids is called Soxhlet, where highly non-polar solvents, such as hexane or petroleum ether, are used; however, the use of these solvents is not only harmful to the environment, but also represents high costs for their acquisition. The objective of this research was to evaluate the content of cocoa butter extraction using ethanol as a solvent. The Box-Behnken Design was used to investigate the effects of the intervening variables of the Soxhlet technique such as extraction time, cocoa powder nib weight and solvent volume, and the Response Surface Methodology (RSM) was applied to determine the optimum cocoa butter content. The polarity of ethanol generated two phases in the extraction, a liquid and an oily phase, which were separated by centrifugation. It was shown that the optimum extraction content (47.317 g/100g) was obtained in a time of 3.35 h, nib weight of 15 g and solvent volume of 157.17 mL. The preliminary results of this study can serve as a basis for extractions in continuous systems at industrial level.

Keywords: *nibs, spectroscopy, hexane, gas chromatography, fatty acids*

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1. Introduction

Cacao is a small plant that originates in the Amazon basin, and its cultivation has been fundamental in the agriculture of several Latin American countries since colonial times [1]. This tree species, typical of tropical and humid areas of South America, has played a prominent role in history [2]. In Peru, this crop has increased making it one of the world's leading exporters of cocoa beans [3].

Cocoa beans are mainly composed of butter (50-55% w/w), which is considered the most expensive and important ingredient in chocolate making; its physical and chemical characteristics confer unique and desirable properties on the final product [4]. The production of high quality cocoa butter from cocoa liquor or cocoa beans involves the use of cold pressing systems and organic solvent extraction; However, there are concerns with both types of extraction since pressing (hydraulic and expeller) often introduces contaminants into the butter and its extraction yield is lower than solvent extraction; on the other hand, extraction with organic solvents such as hexane is of growing concern because of the risks to human health and detrimental damage to the environment [5].

Therefore, there is a migration towards cleaner extraction methods, such as supercritical fluid extraction using mainly carbon dioxide (CO2) as extraction solvent, which represents a potential alternative to the usual methods of cocoa butter production, offering the advantages of fast, non-toxic, environmentally friendly, pollution-free and easily manipulated conditions [6]. However, the equipment used for supercritical extraction requires an extremely high capital investment, even at low production capacities, becoming a major drawback for the exploratory research and risk reduction stages [7].

Cocoa butter extraction using ethanol as a solvent also represents an effective and environmentally friendly alternative to traditional extraction methods, whether mechanical or solvent extraction, ethanol, being a polar solvent, has the ability to selectively dissolve fats, which makes it suitable for extracting cocoa butter from cocoa beans. Under optimal conditions, the yield of this extraction can be quite high, usually between 50% and 70% of the total cocoa butter content [8].

The soxhlet extraction technique is used to extract lipids due to its safety, simplicity and its potential to be implemented on a large industrial scale, the Soxhlet extraction equipment is composed of a condenser, an extraction chamber, a cellulose cartridge and a round bottom flask, during extraction, the temperature varies between 50 and 90 °C, and the reaction time is between 5 and 25 hours, this method uses a combination of solvents both mixed and separated [9]. A new technique that has recently been applied in seed oil extraction is ultrasound-assisted extraction, this technique seeks to replace the traditional Soxhlet extraction method, which involves a significant use of solvents and a long processing time [10].

Response surface methodology (RSM) has several advantages compared to traditional optimization methods, these advantages include the ability to optimize multiple responses, reduced experimentation required, better understanding of process interactions, and improved accuracy in predictions as it combines mathematical and statistical tools to develop models that describe the relationship between input variables (independent factors) and output variables (independent variables) [11,12]. The present work aims to optimize the parameters of the Soxhlet extraction technique (temperature (°C), solvent volume (mL) and time (h)) on the cocoa butter content extracted in nibs and using ethanol as solvent.

2. Materials and Methods

2.1. Raw Material

The cocoa beans were obtained from the District of Bellavista, province of Jaén in the Department of Cajamarca - Peru. The nibs of the cocoa beans were manually shelled, ground in a mortar and sieved in a sieve mesh 40 (opening 0.420 mm) to obtain cocoa nibs powder with uniform particle size.

2.2. Characterization of Cocoa Nibs

The moisture content in the cocoa nibs powder was determined using an oven (POL-EKO-APARATURA®, SW115STD, USA) according to AOAC 931.04 (JAOAC, 1931) [13]. Ash was determined according to AOAC 923.03 (AOAC, 1997) [14] by ashing 1.5 g of nibs at 600 °C for 5 h in a muffle (Thermolyne, 347034984, USA). To determine the protein content, the Dumas method was used according to AOAC methodology (2005) [15]. Fat content was determined by soxhlet fat extractor (FOSS, Soxtec TM-2043, Denmark) using hexane as solvent (CDH Fine Chemical, India). Finally, carbohydrate content was determined according to equation (1):

$$\% \text{ carbohydrats} = 100 - \% \text{ moisture} - \% \text{ ash} - \% \text{ protein} - \% \text{ fat} \quad (1)$$

2.3. Cocoa Butter Extraction

Cocoa butter extraction by leaching was carried out using the soxhlet equipment (TOPT.SOFHLET FOSS, Soxtec TM 2043, China) using ethanol (99% purity) and the parameters and levels in Table 1. Cocoa butter was collected in the glass balloon area of the soxhlet, then emptied into 50 mL amber vials with nitrogen injection and finally stored under refrigeration until further analysis.

2.4. Response Surface Methodology (RSM)

The RSM used a Box - Behnken design (BBD) with three central points. Minitab version 18 statistical software was used to obtain the ANOVA of the regression equation and the analysis of coefficients (Pareto). The model used was of second order and adjusted each response in terms of X_1, X_2, and X_3 (equation 2) at the levels indicated in Table 1.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_1 + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j + \varepsilon \quad (2)$$

Where: Y is the content of cocoa butter; X_1, X_2, ..., X_k are the extraction factors; β_0 is the intercept, β_i (i=1,2, …, k) are the linear coefficients, β_{ii} (i=1,2, …, k) are the quadratic coefficients, β_{ij} (i=1,2, …, k; j=1,2, …, k) are the interaction coefficients of the regression model; ε is the experimental error of the model. RSM optimization was accomplished using a desirable function provided by Minitab version 18.

Table 1. Independent factor levels

Factor	Level			Units
	-1	0	+1	
Time (X_1)	2	3	4	h
Weight (X_2)	5	10	15	g
Volume (X_3)	120	160	200	mL

The values predicted ($y_{i,pred}$) by the quadratic RSM model were correlated with the experimental values ($y_{i,exp}$) to calculate the coefficient of determination and the root mean square error (RMSE).

$$R^2 = 1 - \sum_{i=1}^{n} \frac{\left(y_{i,pred} - y_{i,exp}\right)^2}{\left(\hat{y}_{i,exp} - y_{i,exp}\right)^2} \quad (3)$$

$$RSME = \sum_{i=1}^{n} \frac{1}{n}\left(y_{i,pred} - y_{i,exp}\right)^2 \quad (4)$$

Where: n is the number of experimental data and $\hat{y}_{i,exp}$ is the average experimental value. The models were acceptable as R^2 was closer to the value of 1 (equation 3) and the RSME values were as small as possible (equation 4).

After designing the optimal treatment, the results of the generated RSM model were validated for the extracted cocoa butter content. External samples of cocoa nibs were subjected to the optimal treatment of the RSM model in triplicate. Subsequently, the calculated (V_{cal}) and experimental (V_{exp}) values obtained using the optimal parameters of the RSM model were compared in terms of percentage validation (% Val), using equation 5.

$$\%\ \text{Val} = \left(1 - \left|\frac{V_{cal} - V_{exp}}{V_{cal}}\right|\right) x100 \qquad (5)$$

2.5. MIR Spectrum of Cocoa Butter

Infrared spectra of cocoa butter extracted with hexane and ethanol were obtained using an FTIR System (PerkinElmer, Waltham, MA, USA) with an attenuated total reflectance (ATR) sampling area of 2 mm diameter (200 µm active area). The amount of sample deposited in the ATR was 0.5 mL of cocoa butter in liquid state, previously heated in water bath at 70°C temperature. The spectra were scanned at room temperature in a wavenumber range of 4000 - 650 cm^{-1}, with a resolution of 4 cm^{-1} and 32 scans. The samples were measured prior to the background signal obtained without sample (Background). Since cocoa butter is highly lipophilic, the ATR surface was cleaned with 95% acetone and dried with tissue paper.

2.6. Gas Chromatography Analysis

The AOAC 991.39 [16] method for fatty acid methyl esters (FAMES) was used to analyze the cocoa butter samples. The FAMES were analyzed in a gas chromatograph (SHIMADZU, GC2010, Japan), equipped with a flame ionization detector (FID) and an AOC-20I auto injector using helium as carrier gas. The cocoa butter samples, in solid state, were heated in a water bath to take 0.025 g and deposit them in glass tubes (maximum volume of 20 mL with lid), the cocoa butter sample was dissolved in 1.5 mL of NaOH (0.5N in methanol), covered with nitrogen and placed in a water bath for 5 minutes at 100°C temperature. Then 2 mL of Boron Trifloride (BF3) at 12% in methanol was added, again covered with nitrogen and placed in a water bath at 100°C for 30 minutes, then cooled and 5 mL of saturated NaCl solution and 1 mL of isooctane were added, under agitation the surface phase (FAMES, colorless) was extracted to the vial in a maximum volume of 1.5 mL. Finally, the vials were again covered with nitrogen and stored under refrigeration for subsequent injection into the auto sampler of the chromatographic equipment. A volume of 1 uL of FAMES sample was injected into the gas chromatograph, the oven temperature was programmed at 100°C with a rate of 1.20 mL/min, the entrained helium had a constant flow rate of 124 mL/min at a temperature of 225°C and pressure of 261.5 kPa. Finally, the quantification of the FAMES was proportional to the total content of fatty acids, whose percentage was proportional to the area of the curve formed in the chromatogram.

3. Results

3.1. Cocoa Nibs Characterization

The characterization of the cocoa nibs is presented in Table 2. It can be identified that the major component was the fat content (approximately 50% of the total), while the carbohydrate content was the second major component. The cocoa nibs had a moisture content of less than 7%, which is normally found in dried and fermented beans. Another interesting component was the presence of protein and finally ash, approximately 20% of the total.

Table 2. Nib composition chemical of cocoa nibs

Characteristics	Composition (%)
Moisture	6.556 ± 0.005
Fat	46.283 ± 0.751
Protein	16.440 ± 0.575
Ash	4.167 ± 0.006
Carbohydrate	26.557 ± 0.005

Table 3. Experimental (Y_{exp}) and predict (Y_{pred}) cocoa butter content (g/100 g) by RSM

X_1: Time (h)	X_2: Weight (g)	X_3: Volumen (mL)	Y_{exp}	Y_{pred}	Residual
2	5	160	23.740	25.496	1.756
4	5	160	39.040	37.854	1.186
2	15	160	34.073	34.956	0.883
4	15	160	46.160	44.094	2.066
2	10	120	30.420	27.198	3.222
4	10	120	41.940	41.434	0.506
2	10	200	28.860	29.060	0.200
4	10	200	33.400	36.320	2.920
3	5	120	36.160	37.291	1.131
3	15	120	41.187	43.181	1.994
3	5	200	36.000	33.705	2.295
3	15	200	44.947	43.515	1.432
3	10	160	44.600	43.360	1.240
3	10	160	43.200	43.360	0.160
3	10	160	42.730	43.360	0.630

3.2. Modeling and Optimization of Cocoa Butter Extraction

The different butter contents in cocoa nibs obtained using the soxhlet extraction method are presented in Table 3. The DBB allowed obtaining cocoa butter contents in the range of 23.74 g/100g (2 h, 5 g and 160 mL) - 46.16 g/100g (4 h, 15 g and 160 mL). The predicted values ($\mathbf{Y_{pred}}$) were obtained with the quadratic model of equation 5, the ANOVA analysis of the quadratic model was significant ($p<0.05$) and the significant factors were X_1, X_1^2 and X_3.

The coefficient of determination for the fitted model was R^2=0.9310 with a standard deviation (SD) of 2.923 for cocoa butter extraction content, indicating that the model obtained explains 93.10% of the variation in the observed data for cocoa butter extraction yield.

$$Y = -108.8 + 55.0X_1 + 1.22X_2 + 0.665X_3 - 6.84X_1^2 - 0.0368X_2^2 - 0.001886X_3^2 - 0.161X_1X_2 - 0.0436X_1X_3 + 0.00490X_2X_3 \quad (5)$$

The optimized cocoa butter extraction content can be visualized in the contour lines (Figure 1A) and surface plots (Figure 1B). An optimum cocoa butter content of 47.317 g/100g was found for the factors of: time (X_1 = 3.35 h), weight (X_2 =15 g) and solvent volume (X_3 =157.17 mL), the contour lines confirmed the curvatures of the surface plots for the extraction optimization.

The validation of the optimized result was performed by repeating the extraction procedure with the optimal factors (X_1, X_2 and X_3), the result was performed in triplicate obtaining a cocoa butter content of 47.010 ± 0.120 g/100g, originating a % Val= 99.351%.

Figure 2, showed the correlation between the experimental values and the values predicted by the predictive model of the RSM optimization. The correlation showed a significant ($p<0.05$) coefficient of determination (R^2 = 0.931). The root mean square error (RSME) was also determined to be 1.699, slightly high for predicting the cocoa butter content under the parameters used in the soxhlet method using ethanol as solvent.

3.3. Infrared Spectra Analysis of Cocoa Butter

The mid-infrared spectrum (MIR) of cocoa butter extracted with hexane and ethanol (Figure 3), evidences a great similarity between the main characteristic spectral bands corresponding to fatty acids (saturated, monounsaturated and polyunsaturated), for example the bands detected between 2918 and 2850 cm^{-1} associated to the asymmetric and symmetric CH_2 stretching vibration respectively, are associated to the presence of lipids.

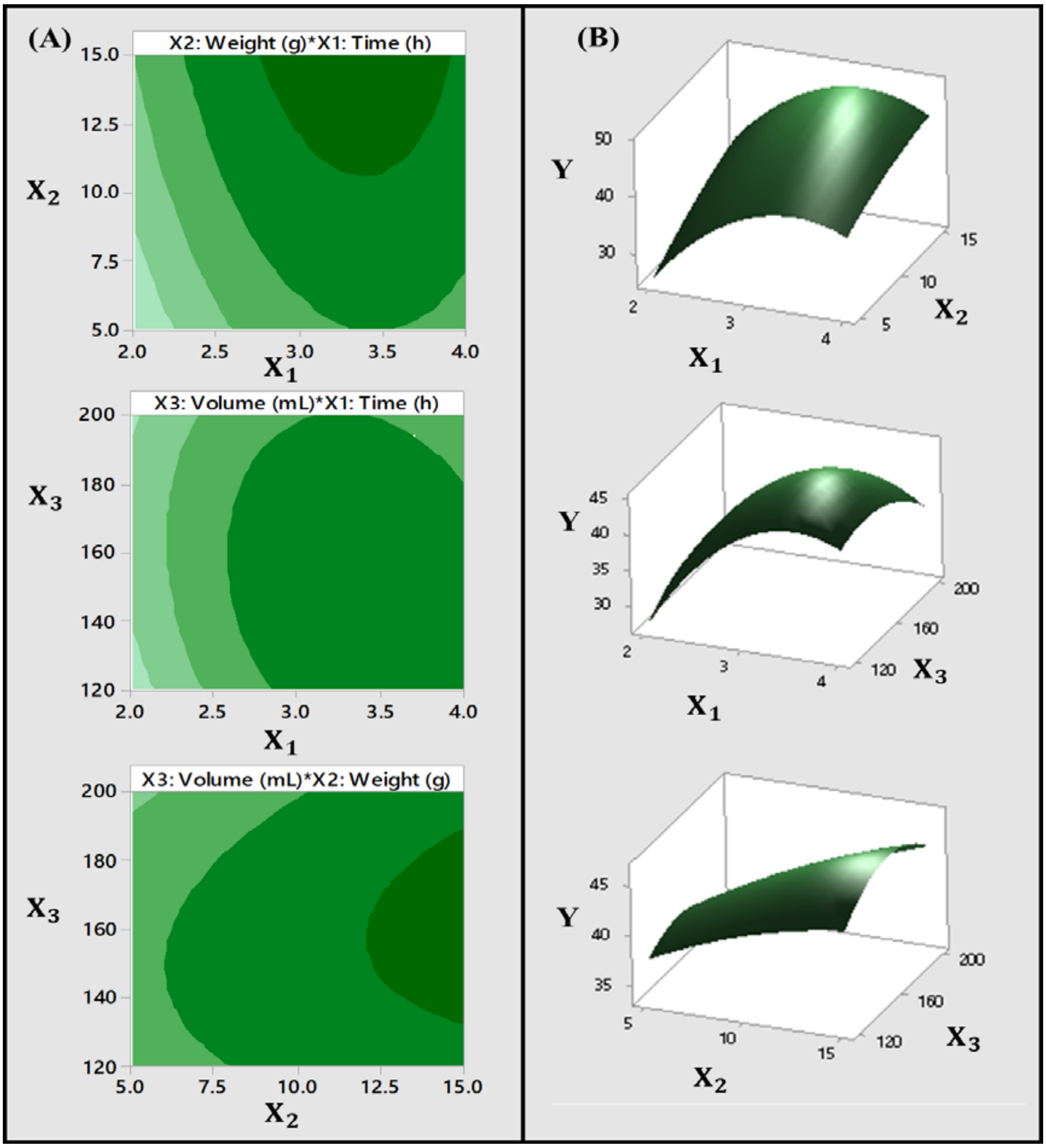

Figure 1. Contour lines (A) and surface plot (B) for cocoa butter extraction optimization using RS

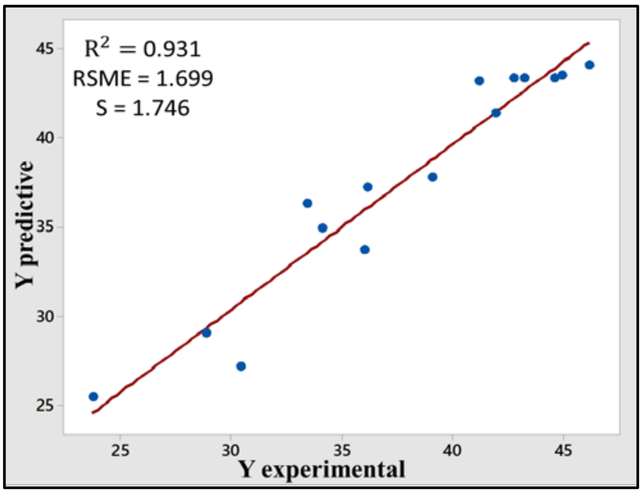

Figure 2. Correlation of predicted and experimental RSM values for cocoa butter extraction in soxhlet

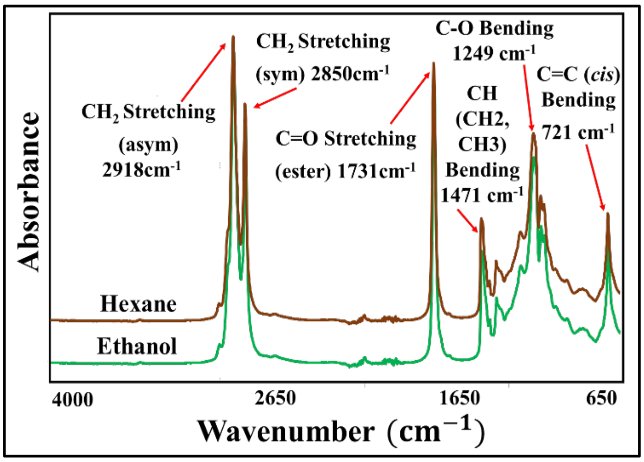

Figure 3. Mid-infrared spectrum (MIR) of cocoa butter extracted with different solvents

3.4. Analysis of Fatty Acids in Cocoa Butter

Regarding the fatty acid composition (Table 2), it was determined that there was no significant difference between the fatty acid contents of the cocoa butter extracted with the different treatments (hexane and ethanol) at 95% reliability ($p<0.05$). In general, cocoa butter was mainly composed of saturated fatty acids (SFA) and within this family stearic acid was the predominant one (35.47 - 36.29%), another representative saturated acid was oleic acid (31.15 - 32.08%) and to a lesser extent arachidic acid (<1.4%). On the other hand, from the family of monounsaturated fatty acids (MUFA) only oleic acid was quantified (31.2 - 32.1%), and from the family of polyunsaturated fatty acids (PUFAS), linoleic acid was found in a low proportion (2.27 - 2.43%).

4. Discussion

Regarding the characterization of cocoa nibs, the moisture content corresponds to that of dried and fermented beans whose values are less than 7% as in the case of cocoa CCN-51 (Castro Naranjal Collection 51) with 6.72% and ICS-6 (Imperial Collage Selection 6) with 6.76% [17], those roasted beans can reach measurement below 3% [18]. The Trinidadian cocoa bean from Cameroon presents similar characteristics to those presented in this study, for example, Tonfack et al. (2018) [19] indicates the presence of moisture 5.9%, carbohydrates 27.7%, ash 7.3% and crude protein 17.9%. Likewise, Adeyeye et al. (2010) [20] reported values the Nigerian Trinidadian cocoa beans with crude protein of 15.2% in the case of fermented cocoa and 13.6% for unfermented cocoa, these values were not far from the data presented in this study. Regarding the butter content in cocoa beans from Peru, Chire-Fajardo et al. (2019) [17] has reported 40.67% for CCN-51 and 42.73% for ICS-6, these values were slightly lower than the cocoa content obtained in the present investigation. Ostrowska-Ligeza et al. (2021) [21] also characterized the chemical composition of roasted and unroasted cocoa beans from Peru (Criollo) and Ecuador (Forastero), indicating that for both types, fat was the main nutrient (> 40%), followed by carbohydrates (29-35%). and protein (14.1-14.8%).

The optimum cocoa butter content from the soxhlet extraction in this study (47.317 g/100g) can be compared to cocoa butter extraction yields that have been realized by various methods using other solvents. Saldaña et al. (2002) [22] indicated that ~50% butter content can be obtained from cocoa beans using the soxhlet method and traditional solvents, and further indicated that all of this butter can be extracted using the supercritical fluid method using mixtures of CO2 and ethane (acceptable and non-polluting solvents for food products).

The extraction of cocoa fat may present other factors that could be relevant apart from those presented in this study, Asep et al. (2008) [23] determined that the yield of cocoa butter extraction using for example the supercritical fluid technology increased significantly with the reduction in particle size, likewise the highest butter yield was obtained using unfermented cocoa beans roasted for a time of 35 min and a temperature of 150°C.

The extract of cocoa butter with ethanol presented a dark pigmentation, to obtain pure butter the extract was centrifuged. However, due to the polarity of the ethanol solvent that dragged other components such as cocoa pigments, it is confirmed by Villa & Benalcázar (2015) [24] that indicates that ethanol being a good extraction solvent, its selectivity towards lipids is relatively low compared to other solvents, so that, in extractions with ethanol, other components such as sugars, pigments or amino acids can appear. Moreover, by means of a hexane-ethanol mixture, it is possible to extract more than 98% of the fatty acids present in the biomass.

The RSM predictive model developed in the present study is comparable to those developed by Monzón et al. (2021) [25], who obtained predictive models for phenolics in avocado seeds and peel, determining that the correlation between predicted and experimental values were significant ($p<0.05$) presenting a high correlation ($R^2 > 0.9907$) and a low RMSE for phenolics prediction (RMSE <0.9437 mg GAE/g). Agu et al. (2018) [26] extracted chiclayo (*Colocynthis vulgaris Shrad*) seed oil by developing predictive models with a coefficient of determination of $R^2 = 0.9962$ and a RSME = 0.0273%, significant ($p<0.05$).

The qualitative analysis of the vibrational spectra indicated that there are no differences between the cocoa butter obtained by using different solvent, especially between the bands detected at 2918 and 2850 cm^{-1} associated with the presence of lipids, these were similar the bands between 2924 and 2916 cm^{-1} indicating the asymmetric CH_2 stretching determined by Veselá et al. (2007) [27] and Mandrile et al. (2019) [28]. The band detected at 1731 cm^{-1} is associated with the C=O bond stretching motion typical of unconjugated triglyceride esters, carboxylic acids, aldehydes and ketones [29]. The band at 1471 cm^{-1} is associated with the presence of C-H bending vibrations in CH_2 and CH_3, and the band at 1249 cm^{-1} is characteristic of C-O stretching vibrations. Finally, the band centered at 721 cm^{-1} long-chain bending vibration in unsaturated fatty acids is also confirmed by the band at 717 cm^{-1} asymmetric in-plane or swinging C=C bending vibration reported by Rubio-Diaz & Rodriguez-Saona (2010) [30].

The fatty acid content of cocoa butter can be compared to that extracted by hydraulic pressure in the study by Indarti, E. (2007) [31] which reported 26.24% palmitic acid, 42.23% stearic acid and 26.53% oleic acid. Regarding the average palmitic acid content ~28% presented in Table 4, Ostrowska-Ligeza et al. (2021) [21] effectively identified that in Peruvian cocoa (criollo) the presence of palmitic acid is 28.03%, also indicated that this fatty acid is slightly lower than that of Ecuadorian cocoa (forastero) located at 30.02%. The differences in the fatty acid profile are explained mainly as an effect of geographical origin [32].

Table 4. Quantification of fatty acids in cocoa butter extracted with different solvents

Fatty acids	Solvents*	
	Hexane	Ethanol
Palmitic acid (C16:0)	28.635 ± 0.040	28.889 ± 0.146
Stearic acid (C18:0)	35.477 ± 0.164	35.584 ± 0.156
Oleic acid (C18:1)	32.080 ± 0.082	31.826 ± 0.377
Linoleic acid (C18:2)	2.411 ± 0.080	2.428 ± 0.085
Arachidic acid (C20:0)	1.397 ± 0.050	1.273 ± 0.058

* Fatty acids no presents significative differences ($p<0.05$).

5. Conclusion

The soxhlet extraction treatment using ethanol as the predominant solvent proved to be efficient in the extraction of cocoa nib butter. Optimization of the soxhlet extraction parameters resulted in a cocoa butter content of 47.317 g/100g. Therefore, this study provides as main contribution that the use of this solvent "ethanol", also generally recognized as safe (GRAS), can replace relatively easily at laboratory level the toxic, expensive and classical solvents such as hexane and petroleum ether. On the other hand, this study can be scaled up to studies of cocoa butter extraction at industrial level and provide possibilities of application at continuous and recirculation level.

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Conflict of Interests

Authors declare no conflict of interests.

Authors Contributions

AYP: Investigation, Methodology, Validation; CRJ: Investigation, Methodology, Validation; EA: Conceptualization, Supervision Project administration; GR: Writing – original draft, Writing - Review & Editing; ROLL: Data curation, Writing - Original Draft; RHP: Resources, Data curation; LPT: Visualitation, Writing - Original Draft; RHP: Resources, Data curation; LPT: Visualitation, Writing - Original Draft; BHS: Software, Validation, Formal analysis, EV: Methodology, Writing - Review & Editing, Supervision, Validation.

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