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Response Surface Methodological Approach for Optimizing *Theobroma cacao* L. Oil Extraction

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Abstract: *Theobroma cacao* L. (Cocoa) is an agricultural product that is economically valuable worldwide; it is rich in bioactive compounds such as phenolic compounds and flavonoids. These compounds are known for their anti-inflammatory, anticarcinogenic, antimicrobial, antiulcer, and immune-modulating properties. Cocoa powder and cocoa butter are the major cocoa seed products, and cocoa seed oil (CSO) is the least-studied cocoa seed product. CSO is used in several industries; therefore, optimizing the extraction of high-quality CSO is essential. We used response surface methodology (RSM) to optimize the restriction dies, temperature, and sieve size to achieve a high yield and quality of CSO. The quality of the CSO was assessed according to total phenolic content (TPC), acid, and peroxide values, fatty acid content, and nitric oxide free radical scavenging activity. The highest yield (actual value: 46.10%; predicted value: 45.82%) was observed with the following restriction parameters: die size: 0.8 cm, temperature: 40 °C, and sieve size > 1.4 mm. The 2FI model for CSO extraction, the pressing time, the reduced quadratic model for acid value, the reduced cubic model for peroxide value, and the TPC showed that the model was significant. Our study primarily reported the impact of sieve size, restriction die, and temperature on CSO yield, acid, peroxide values, TPC of the CSO, and the influence of pressing time on the quantity and quality of the CSO. The high yield of CSO was of relatively lower quality. The temperature affected the yield, acid, peroxide values, TPC, and the nitric oxide free radical scavenging activity. In comparison, the fatty acid composition of the CSO was not affected by the processing temperature or sieve size. The results indicated that the extraction conditions must be chosen based on the application of the extracted oil. Further studies are warranted to confirm the results and further analyze other influential parameters during CSO extraction.

Keywords: *Theobroma cacao* L.; cocoa; cocoa seed oil; response surface methodology; total phenolic content

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1. Introduction

Cocoa (*Theobroma cacao* L.) is an economically important crop and the most valuable agricultural product worldwide [1]. Cocoa seeds are rich in phenolic compounds and flavonoids [2] and contain several bioactive compounds, such as procyanidins, anthocyanins, flavone and flavonol glycosides, epicatechin, gallocatechin, epigallocatechin, etc. [3]. Cocoa polyphenols are reputed to have anti-inflammatory, anticarcinogenic, antimicrobial, antiulcer, and immune-modulating properties. Cocoa antioxidants have a protective effect against cardiovascular diseases [3–5].

Cocoa powder and cocoa butter are the major cocoa seed products with several common usages, especially in food industries [6]. Cocoa seeds consist of 50–55% cocoa butter. High-quality cocoa butter is used in food, cosmetic, and pharmaceutical products [7]. Cocoa butter is widely used as an emollient in cosmetic formulations. The lipid of cocoa oil alleviates dry skin, improves skin elasticity, and provides protection against inflammation [8–10]. Thus, cocoa butter is used in dermatological formulations to protect the skin from damage and prevent photoaging. It is reported that cocoa butter in skin cream formulations has an antiestrogenic effect [11].

Cocoa seed oil (CSO) is the least-studied cocoa seed product [6]. The CSO quickly solidifies at room temperature due to the high saturated fatty acid content, hindering further processing and affecting the quality and marketability [12]. Liquefied cocoa seed fat is used in several industries, which use a heating process to maintain the liquid state of the CSO, and this can affect the quality of the oil. Thus, an optimized condition to achieve high-quality CSO is needed [13].

Factors such as particle size, temperature, solvents, pressure, fermentation, and preprocessing time affect cocoa seed extraction [7]. Physical extraction methods such as screws and hydraulic presses are ineffective for the extraction of cocoa butter because they require high temperatures, which affect the heat-sensitive compounds in the oil. Solvent-based extraction methods, in particular using hexane, extract the oils from several sources but solvent use may affect the quality of the final products [3]. Recently, feasible extraction methods, such as ultrasonic-assisted, supercritical fluid solvent, and microwave-assisted extraction methods, have been reported as obtaining high-quality oils [3,14,15]. Extraction methods affect the content of the extracts. In particular, the quality and quantity of the phenolic compounds, flavonoids, and their bioactivities in cocoa processing are greatly affected by the extraction procedures. Solvent-free extraction methods are preferable to guarantee the safety of the products [16].

Anthocyanins and phytosterols have been extracted from cocoa peel using microwave-assisted extraction. The microwave power, temperature, particle size, extraction time, and solvent-to-sample ratio influenced the final product [17,18]. Factors such as temperature, acidity, and sample-to-solvent ratio, affected the quality of the cocoa husk extract achieved using heat-assisted aqueous extraction [19]. Collectively, temperature, particle size, radiation strength and duration, and sample-to-solvent ratio affect the quality of the extract.

Response surface methodology (RSM) and central composite design (CCD) are statistical and mathematical tools for designing experiments and optimizing variables [20]. RSM reduces the number of experimental trials and has been effectively used for the optimization of several parameters in natural product extractions [21], fermentation conditions [22,23], and other conditional optimization processes in several fields [24,25]. The extraction of pectin from cocoa pod husks using enzymatic extraction was optimized using CCD [26]. The extraction of phenolic compounds from the cocoa shell using the green aqueous extraction method was also demonstrated. A temperature of 100 °C for 90 min, and 0.02 g of cocoa shell in 1 L of water was the optimum condition for extraction of the phenolic compounds. Protocatechuic acid, procyanidin B2, (−)-epicatechin, and (+)-catechin were found in the extract in significant quantities [19].

The objective of the study was to extract CSO using the screw press method and characterize the CSO. The variables used in our experimental design were temperature, restriction dies, and sieve size. CCD was used to optimize the process variables to achieve high yield and high-quality CSO. The quality of the oil was assessed by measuring the acid value, peroxide value, total phenolic content, nitric oxide scavenging activity, and fatty acid content. The results of this study may provide optimized conditions for obtaining high-quality CSO using physical extraction methods. Physical extraction methods produce safe, chemical-free oils, which could be used in the food, pharmaceutical, and cosmetic industries. This study provides optimized conditions that may enable small-scale entrepreneurs to produce superior CSO.

2. Materials and Methods

2.1. Materials

The cocoa seeds were purchased from a community enterprise engaged in the processing of agricultural products and medicinal plants in Kor Luang Village, Lamphun, Thailand. We also purchased the following materials for use in this study: 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (Sigma-Aldrich, Oakville, Canada); 2,2-diphenyl-1-picrylhydrazyl (±)-6-hydroxy-2,5,7,8-tetramethyl (Sigma-Aldrich, Taufkirchen, Germany); Folin–Ciocalteu reagent, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate (Loba Chemie, Mumbai, Maharashtra, India); naphthyl ethylenediamine dihydrochloride (Himedia, Maharashtra, India); sodium carbonate (RCI Labscan, Bangkok, Thailand); gallic acid, phosphoric acid, and methanol (Merck, Darmstadt, Germany); acetic acid, hexanes, sodium thiosulfate pentahydrate, sodium chloride, potassium iodide, ethanol, and potassium hydroxide (RCI Labscan, Bangkok, Thailand).

2.2. Extraction and Variables

The cocoa seeds (I.M.1 variety) were blended and then sieved through >1.4 mm and <1.4 mm sieves. The CSO was extracted using a screw press (FEA-101ss-M-H-Tc-2015, Energy Friend Ltd., Thailand) with selected factors. RSM and CCD were used to optimize the conditions for CSO extraction. The restriction dies, temperature, and sieve size were selected as variable factors to achieve a high yield of CSO. The evaluated results were oil yield, pressing time, and oil quality in terms of acid value, peroxide value, total phenolic content, nitric oxide free radical scavenging activity, and fatty acid content.

2.3. RSM and CCD

RSM and CCD were used to optimize the conditions for achieving the maximum cocoa oil yield and quality. The statistical software package Design Expert, version 10.0 (Stat-Ease Inc., Minneapolis, MN, USA) was used. The basic functions and statistical analyses of RSM and its application in designing experiments have been reported previously [14]. The yield and quality of CSO were the desired results after pressing. The restriction dies (0.4, 0.6, and 0.8 cm), temperature (40, 60, and 80 °C), and sieve size (>1.4 mm and <1.4 mm) were selected as variable factors based on preliminary experiments and the previous literature. A total of 22 independent experiments with 16 combinations and 6 center point replicates were carried out to extract the CSO (Table 1).

Table 1. Details of the standard runs (STD), restriction die range, temperature, and sieve size variables.

STD	Restriction Die (cm)	Temperature (°C)	Sieve Size
1	0.4	40	Level 1 (>1.4 mm)
2	0.8	40	Level 1 (>1.4 mm)
3	0.4	80	Level 1 (>1.4 mm)
4	0.8	80	Level 1 (>1.4 mm)
5	0.4	60	Level 1 (>1.4 mm)
6	0.8	60	Level 1 (>1.4 mm)
7	0.6	40	Level 1 (>1.4 mm)
8	0.6	80	Level 1 (>1.4 mm)
9	0.6	60	Level 1 (>1.4 mm)
10	0.6	60	Level 1 (>1.4 mm)
11	0.6	60	Level 1 (>1.4 mm)
12	0.4	40	Level 2 (<1.4 mm)
13	0.8	40	Level 2 (<1.4 mm)
14	0.4	80	Level 2 (<1.4 mm)
15	0.8	80	Level 2 (<1.4 mm)
16	0.4	60	Level 2 (<1.4 mm)
17	0.8	60	Level 2 (<1.4 mm)

Table 1. *Cont.*

STD	Restriction Die (cm)	Temperature (°C)	Sieve Size
18	0.6	40	Level 2 (<1.4 mm)
19	0.6	80	Level 2 (<1.4 mm)
20	0.6	60	Level 2 (<1.4 mm)
21	0.6	60	Level 2 (<1.4 mm)
22	0.6	60	Level 2 (<1.4 mm)

The following equation (Equation (1)) was used in the CCD model:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{123}X_1X_2X_3 + \beta_{112}X_1X_1X_2 + \beta_{113}X_1X_1X_3 + \beta_{122}X_1X_2X_2 + \beta_{133}X_1X_3X_3 + \beta_{223}X_2X_2X_3 + \beta_{233}X_2X_3X_3 + \beta_{111}X_1^3 + \beta_{222}X_2^3 + \beta_{333}X_3^3 \quad (1)$$

2.4. Extraction and Determination of Total Phenolic Content

The phenolic compounds in the CSO were extracted [27]. Briefly, 2.5 g of CSO was dissolved in 5 mL hexane, and 3 mL of methanol/water (60: 40, vol/vol) was added and vortexed for 2 min. After that, the samples were centrifuged at 3500 rpm for 10 min, and the hexane phase was collected and re-extracted with 3 mL of methanol/water solution. After 2 extraction procedures, the methanolic phase was collected for further study.

The total phenolic content (TPC) of the methanolic phase of CSO was determined using the Folin–Ciocalteu colorimetric method. Briefly, 80 µL of standard (gallic acid) or CSO, 20 µL Folin–Ciocalteu reagent, and 100 µL 7.5% w/v Na_2CO_3 were mixed and incubated at room temperature (RT) for 30 min and measured at 765 nm using a microplate reader (SpectraMax® M3 Multi-mode microplate reader). The TPC of the CSO was denoted as µg gallic acid equivalent (GAE) per gram of the oil [28]. The working standard of the gallic acid was prepared by diluting stock solution to a concentration of 1.25–25 µg/mL.

2.5. Determination of CSO Acid Value

The acid value of the CSO was determined according to United States Pharmacopeia 37 as detailed previously [29]. Briefly, 50 mL of 95% ethanol with 0.5 mL of 1% phenolphthalein was titrated against 0.1 M KOH until the neutral solution was obtained. Then, 2 g of CSO was added and titrated until a pink color was obtained. The same preparation without an oil sample served as a blank. The acid value was represented as mg KOH equivalent/g of oil (Equation (2)):

$$\text{mg KOH/g oil} = V \times 5.61/W \quad (2)$$

where V and W denote the volume of KOH (mL) and weight of oil (g), respectively, and 5.61 is a constant value (equivalence of mass of 0.1 M KOH).

2.6. Determination of Peroxide Value of CSO

The peroxide value of the CSO was determined according to United States Pharmacopeia 37 as detailed previously [29]. Briefly, 20 mL of acetic acid: chloroform (3: 2) solution, 0.5 mL of saturated potassium iodide solution, and 1 g of CSO sample were mixed, and the mixture was boiled for 30 sec. Then, 25 mL of distilled water and 20 mL of 5% potassium iodide solution were added and titrated against 0.002 N sodium thiosulfate containing 2–3 drops of starch as an indicator, until the blue color disappeared. The same preparation without CSO served as a blank. The peroxide value was denoted as milliequivalent of oxygen/kg of CSO (mEq/Kg) (Equation (3)):

$$\text{peroxide value} = \text{mEq/kg} = 2 \times (A - B)/\text{CSO sample (g)} \quad (3)$$

where A and B denote the volume of sodium thiosulfate (mL) in test and blank, respectively.

2.7. Determination of Fatty Acid Content

The fatty acid content of the CSO was also determined using gas chromatography with a flame ionization detector as detailed previously [29]. Briefly, 3 g of CSO sample were mixed with 9 mL of dichloromethane: methane (2:1 v/v). Then, the mixture was filtered through Whatman no. 1 filter paper after being vortexed for 1 h at 15 min intervals. Next, 0.1 M KCl was mixed with filtrate and centrifuged at 2000 rpm for 10 min. The upper layer of the solution was discarded. Then, 200 µL of samples were mixed with 2 mL of methanol: hexane (4:1 v/v) and 200 µL of acetyl chloride and heated at 100 °C with intermediate vortexing, and then cooled to room temperature. Then, 5 mL of 6% K_2CO_3 were mixed and centrifuged. The upper layer was collected for chromatography analysis (GC-2010 + AOC20i + s, Shimadzu, equipped with a DB-23 column (30 m × 0.25 nm) and flame ionization detector). The samples were injected via a split injector port (split ratio 50:1). The percentage of fatty acid was calculated (Equation (4)):

$$\text{The percentage of fatty acid (\%)} = \text{Area under the curve} \times 100/\text{Total area of all fatty acid} \quad (4)$$

2.8. Determination of Nitric Oxide Free Radical Scavenging Activity

Nitric oxide scavenging activity was evaluated in accordance with the method detailed previously [30]. Briefly, 120 µL of sodium nitroprusside in phosphate buffer pH 7.4 and 30 µL of the sample (the methanolic phase from 2.4) was mixed and incubated for 150 min at 37 °C. Then, 100 µL of Griess reagent (equal volume of 0.1% w/v naphthylethylenediamine dihydrochloride and 1% w/v sulfanilamide in 5% phosphoric acid) was mixed and incubated for 5 min at room temperature in the dark. After the incubation, the absorbance of the solution was measured at 540 nm. The results were expressed as µg curcumin equivalent (CE)/g of the oil and all the samples were tested in duplicate. Curcumin solution was prepared at a concentration of 10–120 µg/mL and was used as standard.

2.9. Statistical Analysis

Determinations of the quality of the CSO were carried out in duplicate. All values were expressed as mean ± standard deviation (SD). The difference between the group means was analyzed using one-way analysis of variance (ANOVA). The differences were considered significant at $p < 0.05$.

3. Results and Discussion

To our knowledge, the literature on CSO extraction and optimized conditions to achieve high yield and high-quality CSO is very limited. Several studies have detailed various cocoa butter extraction methods [3]. Based on thermodynamic and kinetic models, the oil yield increases as the solvent–oil seed ratio, temperature, and contact time increase [31–33]. Our designed experiments were performed to obtain the CSO. The quality of the CSO, in terms of acid value, peroxide value, and TPC, was assessed. The influence of pressing time was also determined. The outlier trials were removed from the analysis.

3.1. CSO Yield

The predicted yield of the center point (restriction die: 0.6 cm, temperature: 60 °C, and sieve size > 1.4 mm) standards (STDs) 9, 10, and 11 was 40.25%, whereas the actual yields were 45.99, 47.93, and 32.40%, respectively. Another predicted yield of the center point (restriction die: 0.6 cm, temperature: 60 °C, and sieve size < 1.4 mm) STDs 20, 21, and 22 was 40.95%, but the actual yields were 36.48, 39.62, and 40.84%, respectively. The highest yield was observed in STD 6 (restriction die: 0.8 cm, temperature: 60 °C, and sieve size >1.4 mm) with an actual value of 53.68% and a predicted value of 39.34% (Table 2). The analysis of the variance of the 2FI model for the CSO yield is presented in Table 3.

Table 2. The predicted and observed CSO yields, acid and peroxide values, total phenolic content, and pressing time.

STD	Yield (%)		Acid Value (mg KOH/g of Oil)		Peroxide Value (mEq/Kg of Oil)		Total Phenolic Acid (µg GAE/g of Oil)		Time (Min)	
	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	44.98	44.44	1.34	2.68	1.66	1.37	9.99	9.78	40.00	38.65
2	46.10	45.82	1.52	1.75	1.86	1.70	12.32	10.29	35.00	33.02
3	38.76	37.88	2.28	2.42	2.48	2.08	15.02	15.76	40.00	40.68
4	34.65	32.85	1.65	1.64	2.72	2.05	44.16	36.63	33.00	35.65
5	41.83	41.16	1.91	1.81	2.96	2.74	12.75	12.41	38.00	39.67
6	53.68	39.34	1.39	1.34	0.99	1.07	13.45	19.42	34.00	34.33
7	45.02	45.13	2.30	2.11	1.35	1.53	8.85	7.58	31.00	35.83
8	33.41	35.37	1.84	1.95	1.71	2.06	14.58	18.15	40.00	38.17
9	45.99	40.25	1.79	1.54	1.95	1.88	12.16	11.73	42.00	37.00
10	47.93	40.25	1.33	1.54	2.16	1.88	9.90	11.73	35.00	37.00
11	32.40	40.25	1.69	1.54	0.93	1.88	14.31	11.73	39.00	37.00
12	36.35	35.38	1.39	1.53	1.22	1.37	11.87	11.16	97.00	95.16
13	44.21	42.42	1.32	1.19	1.68	1.70	5.18	6.30	100.00	102.60
14	45.01	42.69	4.95	3.05	2.19	2.08	22.31	17.98	93.00	40.68
15	45.29	43.32	1.96	1.89	1.90	2.05	19.91	22.41	84.00	85.83
16	36.86	39.03	1.55	1.54	2.58	2.74	10.73	14.16	84.00	86.48
17	40.22	42.87	3.93	1.19	1.21	1.07	16.28	11.88	98.00	94.21
18	41.50	38.90	1.39	1.34	1.43	1.53	9.16	12.19	96.00	98.88
19	44.08	43.00	1.93	2.32	1.41	2.06	26.88	29.19	80.00	81.81
20	36.48	40.95	1.46	1.34	1.75	1.88	19.69	18.86	96.00	90.34
21	39.62	40.95	1.07	1.34	2.08	1.88	26.04	18.86	92.00	90.34
22	40.84	40.95	1.41	1.34	2.35	1.88	18.93	18.86	89.00	90.34

Table 3. Analysis of variance for the studied variables.

Responses	Models	Model (*p*-Value)	Lack of Fit (*p*-Value)	R^2	Adjusted R^2	Predicted R^2	Adequate Precision
Yield (%)	2FI	0.0472	0.9877	0.5782	0.3835	0.2255	6.2858
Acid value	Reduced quadratic	0.0008	0.9395	0.7495	0.6601	0.5050	10.3548
Peroxide value	Reduced cubic	0.0110	0.8405	0.5756	0.4430	0.2474	7.6494
TPC	Reduced cubic	0.0003	0.2398	0.8152	0.7228	0.5245	12.0579
Time	2FI	<0.0001	0.6063	0.9902	0.9861	0.9779	35.8860

The 2FI model for CSO extraction was significant ($p < 0.0472$) with adjusted R^2 (0.3835), predicted R^2 (0.2255), and nonsignificant lack of fit ($p = 0.9877$) values. The results indicated that the model equation was acceptable for predicting CSO extraction from cocoa seeds. The CCD-generated 2FI equation (Equations (5) and (6)) for CSO yield (%) was as follows:

$$\text{CSO Yield (\%)} = 43.2155 + 19.4588A - 0.0038B - 0.4004AB \text{ (For sieve size: >1.4 mm)} \quad (5)$$

$$\text{CSO Yield (\%)} = 14.6360 + 33.6030A + 0.3428B - 0.4004AB \text{ (For sieve size: <1.4 mm)} \quad (6)$$

where A = restriction die (0.4–0.8 cm) and B = temperature (40–80 °C).

A previous report stated that processing temperature affected the yield of virgin rapeseed oil and its sensory and aroma profile. A higher temperature showed more favorable effects in terms of the nutritional values of the oil [34]. Temperature plays a major role in cold-press oil extraction, i.e., increased roasting temperature produces nutritionally rich *Pistacia terebinthus* oil. Similarly, temperature was the influencing factor in the extraction of almond, walnut, peanut, and grape seed oils [35].

In this study, as the temperature increased, the yield of CSO decreased when we used a larger size restriction die. However, the yield was not affected at high temperatures when we used a bigger die size. The results showed that the size of the restriction die has an effect on yield depending on the extraction temperature (Figure 1).

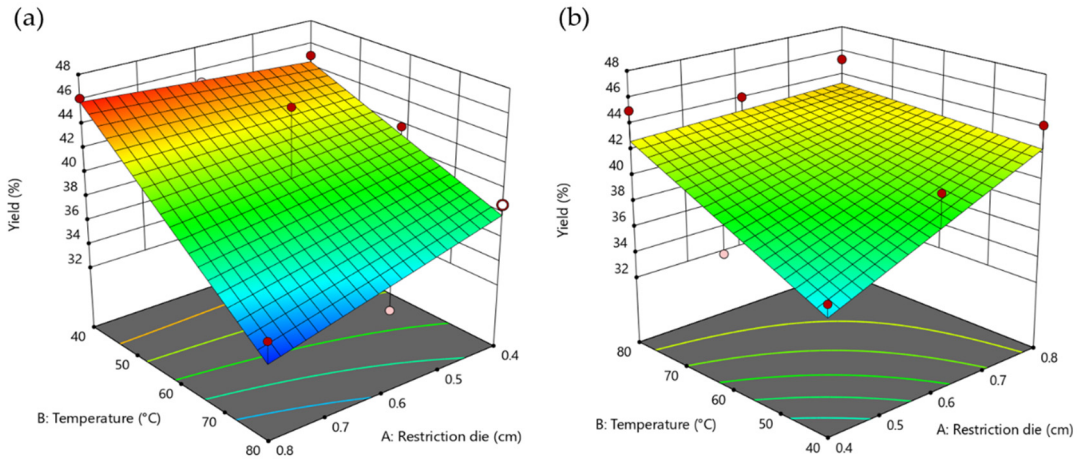

Figure 1. Response surface plot showing the effect of temperature and restriction die size on yield of CSO. (**a**) Sieve size: >1.4 mm, (**b**) Sieve size: <1.4 mm.

3.2. Acid Values of CSO

The predicted acid value of the CSO of the center point (restriction die: 0.6 cm, temperature: 60 °C, and sieve size > 1.4 mm) STDs 9, 10, and 11 was 1.54 mg KOH/g of oil, whereas the actual acid values of the oil samples were 1.79, 1.33, and 1.69 mg KOH/g of oil, respectively. Another predicted acid value of the center point (restriction die: 0.6 cm, temperature: 60 °C, and sieve size < 1.4 mm) STDs 20, 21, and 22 was 1.34 mg KOH/g of oil, but the actual acid values were 1.46, 1.07, and 1.41 mg KOH/g of oil, respectively (Table 2). The lowest acid value was observed in STD 21 (restriction die: 0.6 cm, temperature: 60 °C, and sieve size < 1.4 mm), with an actual value of 1.07 mg KOH/g of oil compared with the predicted value of 1.34 mg KOH/g of oil (Table 2). An analysis of the variance of the reduced quadratic model for the acid value was carried out. The reduced quadratic model for acid value was significant (p = 0.0008) with adjusted R^2 (0.6601), predicted R^2 (0.5050), and non-significant lack of fit (p = 0.9395) values. The results indicated that the reduced quadratic model equation was used for predicting the acid values of the CSO samples. The CCD-generated reduced quadratic equation for acid values (mg KOH/g of oil) was as follows (Equations (7) and (8)):

$$\text{Acid value (mg KOH per g of oil)} = \frac{1}{\sqrt[1.12]{-1.2176 + 0.5115A + 0.0499B - 0.0004B^2}} \text{ (For sieve size : } > 1.4 \text{ mm)} \quad (7)$$

$$\text{Acid value (mg KOH per g of oil)} = \frac{1}{\sqrt[1.12]{-0.5546 + 0.5115A + 0.0406B - 0.0004B^2}} \text{ (For sieve size : } < 1.4 \text{ mm)} \quad (8)$$

where A = restriction die (0.4–0.8 mm) and B = temperature (40–80 °C).

The reduced quadratic model equation was statistically significant for acid value prediction. Sieve size significantly affected the acid value. The correlation between extraction temperature and the acid value was positive, which indicates that the quality of the CSO depends on the extraction temperature. The acid values of CSO were also influenced by restriction die size (Figure 2).

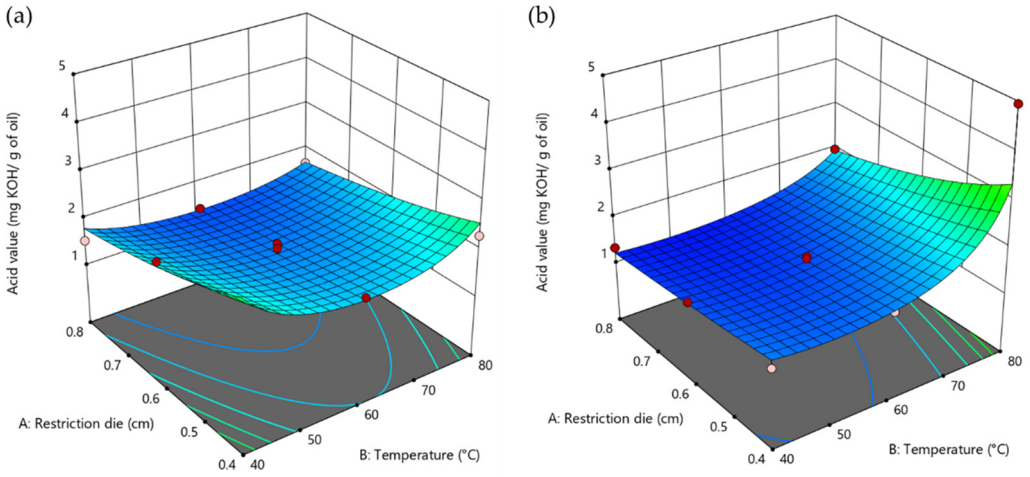

Figure 2. Response surface plot showing the effect of temperature and restriction die size on the acid value of CSO. (**a**) Sieve size: >1.4 mm, (**b**) Sieve size: <1.4 mm.

3.3. Peroxide Value of CSO

Similarly, the actual peroxide value of the CSO differed from the predicted values of center point STDs 9, 10, and 11 (restriction die: 0.6 cm, temperature: 60 °C, and sieve size > 1.4 mm), and 20, 21, and 22 (restriction die: 0.6 cm, temperature: 60 °C, and sieve size < 1.4 mm). The lowest actual peroxide value (0.93 mEq/Kg of oil) was observed in STD 11 (restriction die: 0.6 cm, temperature: 60 °C, and sieve size > 1.4 mm), but the lowest predicted peroxide value (1.07 mEq/Kg of oil) was noted in STDs 6 and 17 (restriction die: 0.8 cm, temperature: 60 °C, and sieve size < 1.4 mm) (Table 2).

Analysis of the variance of the reduced cubic model for the peroxide value showed that the model was significant (p = 0.0110) with adjusted R^2 (0.4430), predicted R^2 (0.2474), and non-significant lack of fit (p = 0.8405) values (Table 3). The results indicated that the reduced cubic model equation was acceptable for predicting the peroxide values of the CSO samples. The CCD-generated reduced cubic equation for peroxide values (mEq/Kg of oil) was as follows (Equation (9)):

$$\text{Peroxide value (mEq per kg of oil)} = \sqrt[0.89]{-20.1043 + 33.3301A + 0.7606B - 1.2121AB - 0.0061B^2 + 0.0099AB^2} + 0.6 \quad (9)$$

where A = restriction die (0.4–0.8 mm) and B = temperature (40–80 °C).

Sieve size does not have a significant effect on peroxide value prediction. The restriction dies and temperature significantly influenced the peroxide value of CSO. Increased die size and temperature produced higher peroxide values (Figure 3).

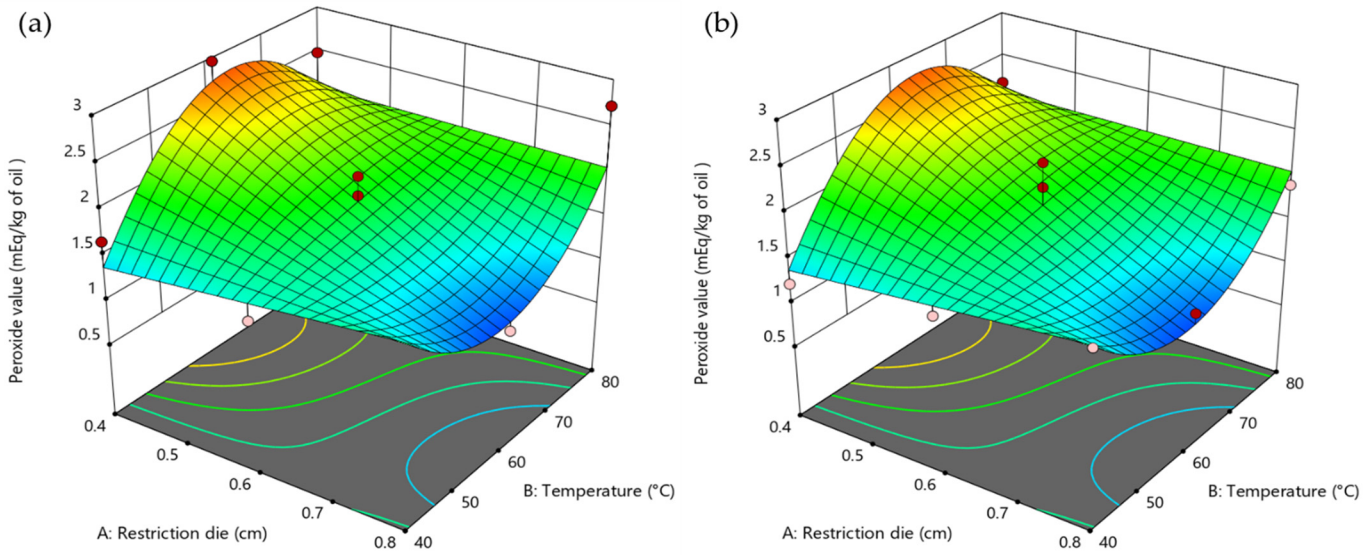

Figure 3. Response surface plot showing the effect of temperature and restriction die size on the peroxide value of CSO. (**a**) Sieve size: >1.4 mm, (**b**) Sieve size: <1.4 mm. Sieve size had no influence on the peroxide value of the CSO.

3.4. TPC of CSO

The predicted TPC of the CSO of center point STDs 9, 10, and 11 (restriction die: 0.6 cm, temperature: 60 °C, and sieve size > 1.4 mm) was 11.73 µg GAE/g of oil, whereas the actual TPC of the samples were 12.16, 9.90, and 14.31 µg GAE/g of oil, respectively. Other center point STDs 20, 21, and 22 (restriction die: 0.6 cm, temperature: 60 °C, and sieve size < 1.4 mm) showed a predicted TPC of 18.86 µg GAE/g of oil, whereas the actual values were 19.69, 26.04, and 18.93 µg GAE/g of oil, respectively. A high TPC of 44.16 µg GAE/g of oil was observed in STD 4 (Table 2).

Analysis of the variance of the reduced cubic model for TPC showed that the model was significant (p = 0.0003) with adjusted R^2 (0.7228), predicted R^2 (0.5245), and nonsignificant lack of fit (p = 0.2398) values (Table 1). The results indicated that the reduced cubic model equation was acceptable for predicting the TPC of the CSO samples (Figure 4). The CCD-generated reduced cubic equation for TPC (µg GAE/g of oil) was as follows (Equations (10) and (11)):

$$\text{Total phenolic content } (\mu\text{g GAE/g oil}) = 10^{2.0791-4.4564A\ -0.0034B\ +0.0215AB\ +3.0426A^2} \text{ (For sieve size } > \text{ 1.4 mm)} \quad (10)$$

$$\text{Total phenolic content } (\mu\text{g GAE/g oil}) = 10^{0.1322-3.3970A\ -0.0034B\ +0.0215AB-4.0654A^2} \text{ (For sieve size } < \text{ 1.4 mm)} \quad (11)$$

where A = restriction die (0.4–0.8 mm) and B = temperature (40–80 °C).

The TPC of the CSO was greatly affected by the extraction temperature (Figure 4). High TPC (44.16 µg GAE/g of oil) was noted in the CSO samples extracted with the conditions of restriction die: 0.8 cm, temperature: 80 °C, and sieve size > 14 mm. The higher the extraction temperature, the higher the TPC values. Sieve size and restriction die had no influence on the TPC of the CSO samples. However, the impact of restriction dies on TPC was influenced by the sieve size (Figure 4). Similarly, virgin rapeseed oil extracted at higher temperatures has greater nutritional value [34]. Although thermal processes used in the roasting of cocoa seeds may degrade the phenolic compounds, the overall antioxidant properties of the roasted cocoa seeds can be maintained or enhanced by the formation of new antioxidants, such as the Maillard reaction products [36,37].

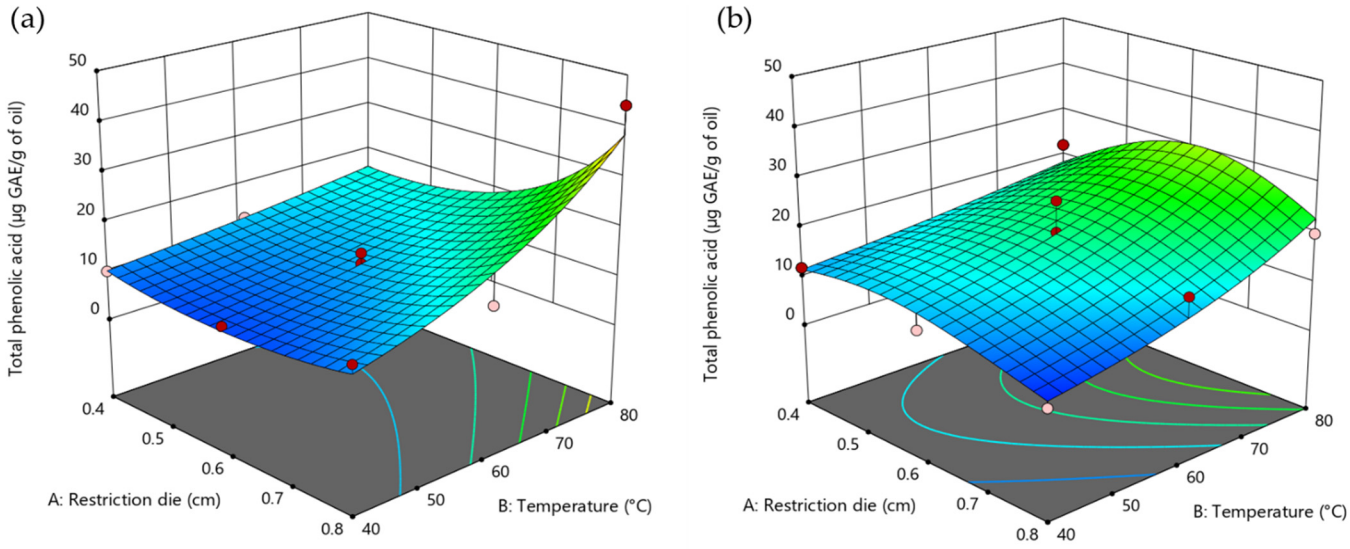

Figure 4. Response surface plot showing the effect of temperature and restriction die size on total phenolic content of CSO. (**a**) Sieve size: >1.4 mm, (**b**) Sieve size: <1.4 mm.

3.5. Pressing Time

The pressing time was noted in all STDs. The predicted center point STDs 9, 10, and 11 (restriction die: 0.6 cm, temperature: 60 °C, and sieve size > 1.4 mm) showed a pressing time of 37.00 min, whereas the actual values were observed as 42.00, 35.00, and 39.00 min, respectively. Other center point STDs 20, 21, and 22 (restriction die: 0.6 cm, temperature: 60 °C, and sieve size < 1.4 mm) showed a predicted pressing time of 90.34 min, but the actual values were found to be 96.00, 92.00, and 89.00 min, respectively (Table 2).

Analysis of variance of the 2FI model for the pressing time showed that the model was significant ($p < 0.0001$) with adjusted R^2 (0.9861), predicted R^2 (0.9779), and non-significant lack of fit ($p = 0.6063$) values (Table 3). The results indicated that the 2FI model equation was acceptable for predicting the pressing time to produce CSO. The CCD-generated 2FI equation for pressing time (min) was as follows (Equations (12) and (13)):

$$\text{Time (min)} = 42.8613 - 15.6022A + 0.0356B + 0.0378AB \text{ (For sieve size > 1.4 mm)} \quad (12)$$

$$\text{Time (min)} = 105.7059 + 17.0728A - 0.4494B + 0.0378AB \text{ (For sieve size < 1.4 mm)} \quad (13)$$

The pressing time influenced the rubber seed oil yield, which increased with pressing time [38]. Pressing time is one factor that affects the yield and quality of the oil. When we used sieve level 1, the pressing time did not alter, whereas sieve level 2 showed significant fluctuations in the pressing time (Figure 5).

The maximum recovery of sesame oil was achieved with a restriction die size of 10 mm, seed moisture content of 8.03%, and pressing speed of 20 rpm using the screw-pressing method [39]. In our study, when the pressing time increased (100 min), the TPC (5.18 µg GAE/g of oil) decreased (Tables 2 and 3). The results indicated that if the cocoa seed was exposed to heat for a long time, even at a low temperature, the TPC content would be affected. Sieve size plays a critical role in pressing time. At the same temperature (40 °C) and restriction die size (0.8 cm), different sieve sizes had a dramatic effect on the pressing time (level 1: 35 min, level 2: 100 min) (Table 2).

The ANOVA analysis suggested that temperature and sieve size had a significant ($p = 0.0044$) impact on CSO yield. The temperature ($p = 0.0036$), sieve size ($p < 0.0001$), restriction die and sieve size ($p = 0.0079$), and temperature and sieve size ($p = 0.0004$) significantly influenced the pressing time.

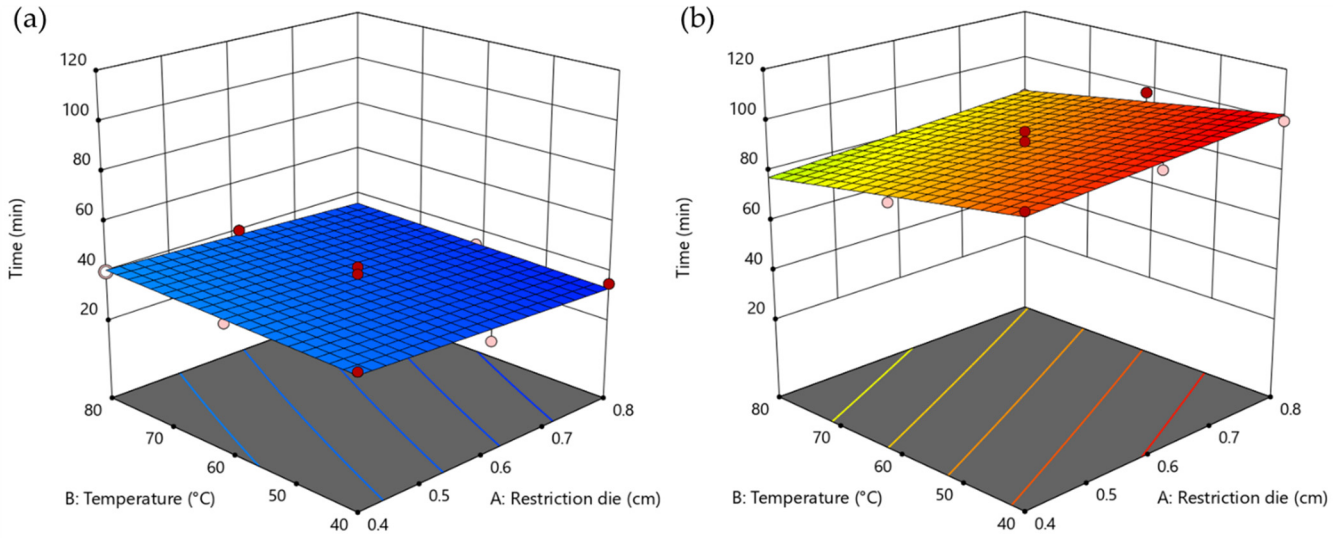

Figure 5. Response surface plot showing the effect of temperature and restriction die size on pressing time to extract the CSO. (**a**) Sieve size: >1.4 mm, (**b**) Sieve size: <1.4 mm.

The restriction die (p = 0.0055), temperature (p = 0.0248), sieve size (p = 0.0346), temperature and sieve size (p = 0.0065), and temperature^2 (p = 0.0023) significantly influenced the acid value.

The restriction die (p = 0.001), temperature (p = 0.0425), and the restriction die and temperature^2 (p = 0.0026) significantly influenced the peroxide value.

The temperature (p = 0.0001), sieve size (p = 0.0078), restriction die and temperature (p = 0.0363), restriction die and sieve size (p = 0.0428), and the restriction die^2 and sieve size (p = 0.007) significantly influenced the total phenolic content for CSO extraction (Table 4).

Table 4. Estimated Parameters for CSO extraction and other desirables.

Term	Estimated Parameters									
	Yield of CSO	***p*-Value**	**Time**	***p*-Value**	**Acid Value**	***p*-Value**	**Peroxide Value**	***p*-Value**	**TPC**	***p*-Value**
Restriction die (A)	0.5019	0.6443	0.6008	0.5781	0.1023	0.0055	−0.7268	0.0010	0.0295	0.3478
Temperature (B)	−1.41	0.1832	−3.68	0.0036	−0.0736	0.0248	0.2316	0.0425	0.1896	<0.0001
Sieve size (C)	0.3513	0.6632	26.67	<0.0001	0.0511	0.0346			0.1031	0.0078
Restriction die × Temperature (A × B)	−1.60	0.2164	0.1513	0.9119			−0.0803	0.5412	0.0861	0.0363
Restriction die × Sieve size (A × C)	1.41	0.2058	3.27	0.0079					−0.0676	0.0428
Temperature × Sieve size (B × C)	3.47	0.0044	−4.85	0.0004	−0.0935	0.0065				
Restriction die × Restriction die (A^2)									−0.0205	0.6565
Temperature × Temperature (B^2)					−0.1631	0.0023	−0.0726	0.6474		
Restriction die × Temperature^2 (A × B^2)							0.7947	0.0026		
Restriction die^2 × Sieve size (A^2 × C)									−0.1422	0.0070

3.6. Fatty Acid Composition

The quantitative analysis of the fatty acid content of representative samples was reported (Table 5). In all the samples, C18:0 was the predominant fatty acid (37.83–40.12%), followed by C18:1 n-9 cis and C16:0. The processing temperature did not affect the fatty acid composition of the CSO.

Table 5. The fatty acid content of representative CSO samples.

Fatty Acids	Fatty Acid Content (%)			
	Sieve Size (>1.4 mm)		Sieve Size (<1.4 mm)	
	Low Temp. (STD 2)	High Temp. (STD 6)	Low Temp. (STD 17)	High Temp. (STD 15)
Butyric acid (C4:0)	-	-	-	-
Caproic acid (C6:0)	-	-	-	-
Caprylic acid (C8:0)	-	-	-	-
Capric acid (C10:0)	-	-	-	-
Lauric acid (C12:0)	-	-	-	-
Myristic acid (C14:0)	0.05	0.05	0.05	0.06
Pentadecylic acid (C15:0)	0.02	0.01	0.01	0.02
Palmitic acid (C16:0)	22.63	22.74	22.96	22.24
Palmitoleic acid (C16:1)	0.19	0.17	0.18	0.24
Margaric acid (C17:0)	0.22	0.20	0.21	0.26
Heptadecenoic acid (C17:1)	0.01	0.01	0.01	0.01
Stearic acid (C18:0)	39.02	40.12	39.96	37.83
Elaidic acid (C18:1 n-9 trans)	-	-	-	-
Oleic acid (C18:1 n-9 cis)	33.23	32.51	32.43	34.07
Linolelaidic acid (C18:2 n-6 trans)	-	-	-	-
Linoleic acid (C18:2 n-6 cis)	3.08	2.84	2.89	3.54
γ-Linolenic acid (C18:3 n-6)	-	-	-	-
α-Linolenic acid (C18:3 n-3)	0.15	0.12	0.12	0.18
Arachidic acid (C20:0)	1.24	1.08	1.06	1.37
Eicosenoic acid (C20:1 n-9)	-	-	-	-
Eicosadieoic acid (C20:2 n-6)	-	-	-	-
Dihomogammalinolenic acid (C20:3 n-6)	-	-	-	-
Arachidonic acid (C20:4 n-6)	-	-	-	-
cis-11,14,17-Eicosatrienoic acid (C20:3 n-3)	-	-	-	-
Behenic acid (C22:0)	0.11	0.09	0.09	0.13
Erucic acid (C22:1 n-9)	-	-	-	-
Eicosapentaenoic acid (C20:5 n-3)	-	-	-	-
Tricosanoic acid (C23:0)	-	-	-	-
Lignoceric acid (C24:0)	0.04	0.03	0.03	0.05
Nervonic acid (C24:1)	-	-	-	-
Docosahexaenoic acid (C22:6 n-3)	-	-	-	-
Docosapentaenoic acid (C22:5)	-	-	-	-

Cocoa butter mainly consists of saturated fatty acids and about 30–35% unsaturated fatty acids [40]. The fatty acid profile of the cocoa seed varies based on the cultivars and geographical conditions. Differences in the lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), margaric acid (C17:0), ginkgolic acid (C17:1), and stearic acid (C18:0) content have been noted in different cocoa seed varieties [41]. Palmitic acid, stearic acid, oleic acid, and linoleic acid are the predominant fatty acids in most cocoa varieties. Żyżelewicz et al. reported that the roasting temperature did not affect the fatty acid content of the cocoa seed of the Forastero variety [42].

Palmitic and stearic acids are used in cosmetic preparations as emulsifiers. Stearic acid helps to reduce body fat, in particular abdominal fat [43]. Though palmitic and stearic acid fats do not affect serum lipids and lipoprotein, stearic acids effectively reduce the low-density lipoprotein-cholesterol level compared with palmitic acid [44]. Oleic acid has a role in the immune system, and it has been reported to have antitumor and anti-inflammatory effects [45,46].

Stearic acid, oleic acid, palmitic acid, and linoleic acids are the major constituents of the fatty acids present in CSO. Our results indicated that different sieve sizes and processing temperatures did not affect the quality of CSO in terms of its fatty acid content (Table 5).

3.7. Nitric Oxide (NO) Free Radical Scavenging Activity

The CSO extracted at low temperatures showed less NO scavenging activity, irrespective of the sieve size, while high-temperature samples exhibited relatively higher NO scavenging activity (Table 6). The NO scavenging activity was correlated with the TPC of the samples. The result represents the antioxidant properties of the CSO.

Table 6. The nitric oxide free radical scavenging activity of representative CSO samples.

Sieve Size (>1.4 mm)		Sieve Size (<1.4 mm)	
Low Temp. (STD 2)	**High Temp. (STD 6)**	**Low Temp. (STD 17)**	**High Temp. (STD 15)**
45.03 ± 0.13 [a]	147.36 ± 28.47 [b]	61.98 ± 0.51 [a]	128.46 ± 4.33 [b]

[a,b] indicates the significant changes among the samples.

3.8. Data Optimization

The design of a suitable CSO extraction was performed after predicting the optimal % yield, acid value, peroxide value, TPC, and pressing time by the CCD method. For sieve size > 1.4 mm, a good desirability index (desirability = 0.705) was achieved with restriction die 0.8 cm, temperature 46 °C. For sieve size < 1.4 mm, a good desirability index (desirability = 0.520) was achieved with restriction die 0.8 cm, temperature 76 °C. The desirability index is a multi-criteria optimization algorithm used when responses must be at the specified requirements, which are maximum % yield, minimum acid value, minimum peroxide value, maximum TPC, and minimum pressing time.

4. Conclusions

Our study described how sieve size, restriction die, and temperature influence the quantity and quality of CSO. High yields of CSO have relatively less quality in terms of the assessed parameters. Temperature affects the TPC, acid and peroxide values, NO scavenging activity, and yield of the CSO. The fatty acid composition of the CSO was not affected by processing temperature or sieve size. CSO quality may be influenced by other factors, such as seed variety, moisture content, extraction methods, etc. Thus, further studies are required to optimize the conditions to produce high-quality CSO in high quantities.

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