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Assessment of obtaining sunflower oil from enzymatic aqueous extraction using protease enzymes

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SUMMARY: The aim of this work was to maximize the enzymatic aqueous extraction (EAE) of sunflower seed oil using protease enzymes from the evaluation of various temperatures, pH and enzyme concentrations, using a Box-Behnken experimental design. The effect of a thermal pre-treatment of sunflower seeds on free oil yield (FOY) and oil quality was also determined. In the experimental range adopted, a lower temperature (40 °C) provided higher FOY values, as well as the intermediate pH (8.00) and maximum enzyme concentration (9% v/v). Thermal pre-treatment provided an increase in FOY in the initial extraction times (60 to 180 min) and decreased of the extraction time of 4 to 3 h to obtain the highest FOY value (~16%). The fatty acid composition of the oils obtained showed a predominance of oleic (~47.5%) and linoleic acids (~39.5%). The total phytosterol content in the samples was hardly affected by the heat pre-treatment of the seeds, while the fatty acid profile, tocopherol content and oxidative stability were not altered.

KEYWORDS: *Alcalase; Enzymatic extraction; Free oil yield;* Helianthus annuus *L.*

RESUMEN: ***Evaluación de la obtención de aceite de girasol a partir de extracción acuosa enzimática usando enzimas proteasa.*** El objetivo de este trabajo fue maximizar la extracción acuosa enzimática (EAE) de aceite de semillas de girasol utilizando la enzima proteasa a partir de la evaluación de las variables temperatura, pH y concentración de la enzima, utilizando un diseño experimental de Box-Behnken. Además, se determinó el efecto del pretratamiento térmico de las semillas de girasol sobre el rendimiento (RA) y la calidad del aceite. En el rango experimental adoptado, las temperaturas más bajas (40 °C) proporcionaron valores de RA más altos, así como el pH intermedio (8,00) y la concentración máxima de enzima (9% v/v). El pretratamiento térmico proporcionó un aumento del RA en los tiempos de extracción iniciales (60 a 180 min) y una disminución del tiempo de extracción de 4 a 3 h para obtener el valor de RA más alto (~ 16%). La composición en ácidos grasos de los aceites obtenidos mostró predominio de los ácidos oleico (~47,5%) y linoleico (~39,5%). El contenido total de fitosteroles en las muestras se vio poco afectado por el pretratamiento térmico de las semillas, mientras que el perfil de ácidos grasos, el contenido de tocoferoles y la estabilidad oxidativa no se vieron afectados.

PALABRAS CLAVE: *Alcalasa; Extracción enzimática;* Helianthus annuus *L.; Rendimiento de aceite.*

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1. INTRODUCTION

The sunflower (*Helianthus annuus* L.) is cultivated in all continents, and the plant is a dicotyledon, originating from the North American continent (Saydut *et al*., 2016). This crop is an oilseed which contains around 38 to 50% high quality oil and has a great capacity of adaptability in different soil and climate conditions (Castro and Leite, 2018). In 2018, the worldwide production was 50 million tons, and Ukraine is the largest producer in the world, follow by Russia and Argentina (FAO, 2020). Sunflower oil is mainly used for human consumption, such as edible oil, margarine and salad sauce (Sánchez-Muniz *et al*., 2016). Furthermore this oil is used in pharmaceutical, cosmetics and biodiesel production (Saydut *et al*., 2016).

The oil quality is associated with fatty acid profile. Sunflower oil stands out for being rich in these compounds, with a predominance of unsaturated fatty acids, mainly linoleic acid (60 to 70%), followed by oleic acid (20 to 30%) (Aquino *et al*., 2019). The consumption of vegetable oil with high quantities of linoleic and oleic acids can help to decrease low-density lipoproteins (LDL cholesterol), and consequently reduce the risk of heart disease (Sánchez-Muniz *et al*., 2016). The oil extracted from sunflower seeds is also composed of natural antioxidants, such as a-tocopherols, phytosterols, vitamins A, D and E, which aid in oxidative stability (Aquino *et al*., 2019; Chen *et al*., 2020), These components are in minor quantity and provide additional nutritional value.

In order to achieve less harmful processing to the environment, without the use of toxic and flammable solvents, vegetable oil extraction with petroleum-based solvent can be replaced by enzymatic aqueous extraction (EAE), a sustainable method which is considered a green process (Cheng *et al*., 2019). Enzymatic extraction is characterized by using water as a solvent and enzymes for hydrolysis of the cell wall, which is responsible for trapping oil in the oleaginous (Yusoff *et al*., 2015). Thus, it is necessary to break up the cell wall and membranes of the oilseed by enzymatic hydrolysis, to release of oil, which is in intracellular vacuoles (Liu *et al*., 2016). Enzymatic hydrolysis, in addition to breaking the wall cell, is effective in breaking down the molecular complex lipoprotein and lipopolishaccaride in simple molecules, releasing extra oil that would not be extracted by means of another method (Campbell *et al*., 2016; Yusoff *et al*., 2015).

In the primary plant cell wall, there is an insoluble micro-fibrillary phase consisting of cellulose and hemicellulose which provides support and constitutes its main structure where other components such as proteins and glycoproteins are incorporated and a non-cellulosic polymers phase, which consists of pectic polysaccharides (Broxterman and Schols, 2018). Due to the composition of the structure cell wall, the most commomly-used enzymes for the enzymatic aqueous extraction of vegetable oil are cellulose, hemicellulose, pectinase, protease and a-amylase (Liu *et al*., 2016). Extraction efficiency and oil quality depend on the application of one or a combination these enzymes (Yusoff *et al*., 2015).

Protease enzymes play a significant role in the cell's metabolism because these enzymes can digest long protein chains in shorter fragments through the hydrolysis of peptide bonds (Gong *et al*., 2017). Alcalase® is a bacterial alkaline protease produced by *Bacillus licheniformis* and has been considered by many researchers to be one of the best enzymes for protein hydrolysis (Memon *et al*., 2019). Proteases are one of the most important enzyme groups used commercially, constitute approximately 40% of world's enzyme market. They are widely used in the food, detergent, leather, pharmaceutical and biotechnology industries (Vijayaraghavan *et al*., 2014). In the extraction of vegetable oil, proteolytic enzymes hydrolyze oleosins, which are proteins that surround the body of oil in the oilseed, decreasing surface activity and promoting the release of the oil (Moura *et al*., 2008). Protease enzymes are used for oil extraction from sunflower (Ribeiro *et al*., 2016), *Camellia oleifera* (Meng *et al*., 2018), pecan nuts (Polmann *et al*., 2019) and pomegranate seeds (Goula *et al*., 2018).

The aim of this work was to maximize the enzymatic aqueous extraction of sunflower oil using protease enzymes and to evaluate the effects of the temperature (40 to 60 °C), pH (7.0 to 9.0) and enzyme concentration (1% to 9% (v/v)) on the free oil yield (FOY). In the condition of maximum FOY, the influence of thermal pre-treatment on the seeds and quality parameters of the oil were verified.

2. MATERIALS AND METHODS

2.1. Materials

Sunflower seeds were purchased at a local market in Umuarama (Paraná – Brasil). Alcalase® 2.4L FG (endo-protease that hydrolyzes most peptide bonds within a protein) with an activity of 2.4 $U \cdot mL^{-1}$ (Unit defined by the hydrolysis of casein to produce 1 mmole of tyrosine per minute at pH 7.5 and 37 °C), was provided by LNF Latino Americana. The reagents used to adjust the pH were sodium hydroxide (Nuclear, 95%) and Chloridric acid (Nuclear, 40%) and *n*-hexane was used to determine the non-lipid fraction in the free oil (Panreac, Castellar del Vallès, Barcelona). The solvents ethanol (95%, Anhydrol, Diadema, São Paulo, Brazil) and *n*-hexane (Panreac, Castellar del Vallès, Barcelona) were used in the oil recovery tests. The fatty acid profile was determined using methanol (≤ 99.9%, Panreac, Castellar del Vallès, Barcelona), sodium hydroxide (≤ 97%, Anidrol, Diadema, São Paulo, Brazil), a boron trifluoride-methanol solution (BF_3, B1252, Sigma-Aldrich, St. Louis, MO, USA) and heptane (Neon, Suzano, São Paulo, Brazil). The contents of phytosterols, tocopherols and free fatty acids were determined using *N,O*-Bis (trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA, 15238, Sigma-Aldrich, St. Louis, MO, USA), heptane (Neon, Suzano, São Paulo, Brazil) and 5-α-Cholestane (≥ 97.0%, C8003, Sigma-Aldrich, St. Louis, MO, USA) and methyl heptadecanoate (≥ 99.0%, 51633, Sigma-Aldrich, St. Louis, MO, USA) as internal standards. Acylglycerols were quantified by external standardization using monoolein (purity ≥ 99%), 1,3-diolein (purity ≥ 99%) and glyceryl trioleate (purity ≥ 99%), purchased from Sigma-Aldrich (Saint Louis, Missouri, United States).

2.2. Sunflower seeds preparation

Sunflower seeds with moisture 4.3% ± 0.20 (determined in an oven at 105 °C) were crushed using a household blender and then the particles obtained were classified (set of Tyler-type sieves, Bertel) and a fraction with an average diameter of 0.725 mm was used in the extractions.

In the experiments which evaluated thermal pre-treatment effect of the seeds, before crushing and granulometric classification, the methodology described by Tian *et al*. (2019) was adopted. Thus, whole seeds were immersed in distilled water in the ratio of 1:3 (w/v). After 3 hours of immersion at room temperature, the excess water was removed and the seeds (150 g) were spread in a thin layer under a metal sieve. The sieve was put in an oven with air circulation (Marconi, Model MA035) at 120 °C for 60 minutes.

2.3. Enzymatic aqueous extraction

The maximization of enzymatic oil extraction from sunflower seeds with the enzyme Alcalase® 2.4L FG was carried out using the Box-Behnken experimental design with three factors, three levels and four central points. The values of the three variables evaluated were: temperature (A) 40, 50 and 60 °C; pH (B) 7.0, 8.0, and 9.0 and enzyme concentration (C) 1, 5 and 9% (v/v). The three values for the variables corresponded to the levels: -1 (low), 0 (central point) e +1 (high), respectively. The values adopted for the independent variables took into account the Novozyme (2019) indications, which report temperature and pH range for optimal activity of the Alcalase® 2.4L FG enzyme at 30 to 65 °C and pH 7.0 to 9.0, respectively. Regarding the enzyme concentration evaluated (in relation to the extraction medium volume), it was based on the enzymatic aqueous oil extractions performed by Jiang *et al*. (2010), Ribeiro *et al*. (2016) and Meng *et al*. (2018).

The enzymatic extraction was carried out in Erlenmeyer flasks (125 mL) the crushed sunflower seeds (10 g) and distilled water (40 mL), in the mass ratio of 1:5 (g of seeds/g of water), proportions used according to the study of Aquino *et al*. (2019). In sequence, the pH was adjusted according to the condition to be evaluated with NaOH solution (1 $mol \cdot L^{-1}$) and enzyme added in the concentration of the test. Flasks were put in an orbital shaker (Marconi, model MA 830/A) at 180 rpm, for 5 hours with controlled temperature according to the experimental design.

After extraction, the free oil was recovered using the steps and conditions described by Aquino *et al*. (2019). Therefore, the suspensions had pH adjusted to 5.0 and the flasks were incubated for 1 hour under shaking 180 rpm at 25 °C. Then, the samples were stored overnight in a refrigerator (Consul, 340) at 4 °C. After that, the samples were centrifuged twice at 2700 rpm for 15 minutes at room temperature and four phases were formed (solid, aqueous, emulsion and free oil). The free

oil in the upper phase was transferred to a petri dish and kept in an oven until reaching constant weight. To assess the free oil content in the upper phase, the determination of the non-lipid fraction in the sample was performed as described by Rodriguez *et al.* (2021), obtaining 4.7% ± 0.71 of non-lipid compounds from this phase. The free oil yield (FOY) was calculated by Equation 1:

$$\text{Free Oil Yield (\%)} = \frac{\text{Weight of recovered free oil (g)}}{\text{Weight of sunflower seed (g)}} \text{x100} \quad \text{(Eq. 1)}$$

The analysis of variance (ANOVA) was used to evaluate the effects of independent variables on the dependent variable (free oil yield). The experimental data were adjusted to a second-order polynomial mode, using the Statistica® 8.0 software. The generalized model used is expressed in Equation 2:

$$Y = \beta_0 + \Sigma_{i=1}^{3} \beta_i X_i + \Sigma_{i=1}^{3} \beta_{ii} X_i^2 + \Sigma_{i=1}^{2} \Sigma_{j=i+1}^{3} \beta_{ij} X_i X_j \quad \text{(Eq. 2)}$$

Where β_0, β_i, β_{ii} and β_{ij} are the regression coefficients (β_0 = constant term; β_i = linear term; β_{ii} = quadratic term; β_{ij} = linear interaction term) and Y is the response variable (free oil yield - FOY) observed in the experiments. X_i and X_j are independent variables: temperature, pH and enzyme concentration.

The experimental conditions provided the maximum FOY in the evaluated experimental range as determined by Equation 2. In this condition, verification experiments (triplicate) were carried out to evaluate the predictive capacity of Equation 2 and the experimental results were submitted to the Student's t-test (Excel®, 2016) to estimate differences between the experimental values and the predictions. In this same experimental condition, experiments were carried out in triplicate to verify the efficiency of the solvent recovery of the oil from the phases. 5 mL of solvent (ethanol or *n*-hexane) were added in the step where four phases were formed, and the flasks were shaken to ensure homogenization and in the sequence centrifuged at 2700 rpm for 15 minutes. This procedure was performed three times for each solvent. The oil with solvent was transferred to a petri dish, and kept in an oven until reaching constant weight.

The influence of the thermal pretreatment of sunflower seeds was evaluated in the experimental conditions which give maximum FOY, as obtained from Equation 2. The extractions (destructive) were performed in triplicate at the times 60, 120, 180, 240, 300 and 360 minutes for seeds with and without treatment.

2.4. Characterization of free oil

A gas chromatograph coupled to the mass spectrometer (GC-MS) (Shimadzu, model CG-2010 Plus, Tokyo, Japan) and equipped with an automatic injector (Shimadzu, model AOC-20i, Tokyo, Japan) was used to analyze the fatty acid profile and contents in phytosterols, tocopherols and free fatty acids.

For the fatty acid composition, the oil was previously prepared according to the procedure described by Stevanato and Silva (2019). 1.5 mL of a 0.5 mol·L^{-1} methanolic sodium hydroxide solution were placed in a test tube. The tube was shaken vigorously and heated in a thermostatic bath (Nova Ética, model 314/8, Piracicaba, São Paulo, Brazil) at 100 °C for 100 min. Subsequently, 2 mL of derivative agent BF_3 were added and the tube was subjected to heating again for 5 min. A 5-mL aliquot of heptane was added to the test tube and after phase separation the supernatant was collected and sent for analysis. The analytes were separated in a capillary column DB-Wax™ (Shimadzu, 30m × 0.25mm × 0.25 µm, Tokyo, Japan), using helium as carrier gas (1.0 mL·min^{-1}). The temperature of the injector, the ionic source and the interface were 250, 260 and 250 °C, respectively. The initial temperature of the column was 80 °C, which was elevated to 180 °C at a rate of 10 °C·min^{-1}, and then elevated again to 240 °C at 4 °C·min^{-1}, remaining constant for 2 min at this temperature. Fatty acid methyl esters (FAMEs) were identified using the NIST Spectrum Library Spectrum Library (version 2014). The FAME quantification was performed from the normative area of the chromatographic peaks, using the percentage of the relative area of each peak in relation to the sum of all peaks.

The contents of phytosterols, tocopherols and free fatty acids were determined using BSTFA/TMSC as derivatizing agent, following the method described by Stevanato and Silva (2019). A SH-Rtx-5MS™ capillary column (Shimadzu, 30m ×

0.25mm × 0.25 μm, Tokyo, Japan) was used to elute the compounds. The injection temperature was 280 °C. The oven was initially operated at 150 °C with an increase in temperature to 230 °C (10 °C·min^{-1}), then the temperature was increased again to 280 °C (15 °C·min^{-1}), and kept constant for 25 min. The identification was carried out as previously described and quantification was performed by internal standardization using 5-α-cholestane (5 mg·mL^{-1}) and methyl heptadecanoate (5 mg·mL^{-1}) as reference standards.

The acylglycerol composition was determined on a gas chromatograph (Shimadzu, GC-2010 Plus, Tokyo, Japan) equipped with flame ionization detector (FID), *on-column* injector (Shimadzu, Tokyo, Japan) and capillary column Zebron™ ZB-5HT inferno (Phenomenex, 10 m×0.32 mm×0.10 m, Torrance, CA, USA). The chromatographic conditions were previously described by Stevanato and Silva (2019). Chromatographic areas generated from the standard solutions of triolein (0.1 to 3.1 mg·mL^{-1}), diolein (0.03 to 2.5 mg·mL^{-1}), and monolein (0.05 to 2 mg·mL^{-1}) were plotted against the concentration to obtain the line equations ($R^2 > 0.99$).

The oxidative stability of the oil was determined using the Professional Rancimat Biodiesel equipment (Metrohm, model 823, Herisau, Switzerland). The samples (2.5 ± 0.003 g) were exposed to an air flow of 20 L·h^{-1} at a constant temperature of 130 °C. The secondary oxidation products were transferred to the measuring vessel containing 50 mL of distilled water. The induction period was automatically determined by the equipment, and measured from the increase in thermal conductivity of the distilled water.

2.5. Statistical analysis

To verify the influence of heat pre-treatment on sunflower seeds and oil quality, the results were evaluated by analysis of variance (ANOVA) and Tukey's test with a significance level of 5% (α=0.05), using the Statistica® 8.0 software. All treatments and analyses were performed at least in duplicate (n=4).

3. RESULTS AND DISCUSSION

3.1. Free oil yield (FOY)

The experimental condition and the free oil yield (FOY) obtained from each experimental condition, adopted in the experimental design, are presented in Table 1. Based on the results from this table, it can be verified that FOY varied from 7.02 to 15.59% and to identify the influence of each variable and its interactions on the response variable, the coded variable was adjusted to a second-order polynomial equation as expressed in Equation 3:

$$FOY\ (\%) = 7.82 - 1.39A + 0.36B + 1.70C + 3.24A^2 + 0.03B^2 + 0.32C^2 - 0.27AB - 0.93AC - 0.23BC \quad \text{(Eq. 3)}$$

Table 2 presents the ANOVA results which were used to validate the second-order polynomial model (Equation 3) adjusted to the experimental data, as well as to evaluate the influence of each variable on the response. The results showed that the model was significant ($p < 0.05$) only for the linear effects of the three variables and quadratics of the temperature variable (A). For the interactions among them, the model was significant only for the interaction of the varying temperature (A) and enzyme concentration (C). These results are shown by high values for F and low values for *p*.

Table 1. Experimental conditions applied and free oil yield (FOY) obtained in the experiment to assess the effects of the operating variables using a Box–Behnken design

Run	Variable[a]			FOY (%)
	A	B	C	
1	-1 (40)	-1 (7.0)	0 (5%)	11.61
2	1 (60)	-1 (7.0)	0 (5%)	9.64
3	-1 (40)	1 (9.0)	0 (5%)	13.07
4	1 (60)	1 (9.0)	0 (5%)	10.04
5	-1 (40)	0 (8.0)	-1 (1%)	10.21
6	1 (60)	0 (8.0)	-1 (1%)	9.03
7	-1 (40)	0 (8.0)	1 (9%)	15.59
8	1 (60)	0 (8.0)	1 (9%)	10.68
9	0 (50)	-1 (7.0)	-1 (1%)	6.04
10	0 (50)	-1 (7.0)	1 (9%)	9.76
11	0 (50)	1 (9.0)	-1 (1%)	7.02
12	0 (50)	1 (9.0)	1 (9%)	9.83
13	0 (50)	0 (8.0)	0 (5%)	8.06
14	0 (50)	0 (8.0)	0 (5%)	8.06
15	0 (50)	0 (8.0)	0 (5%)	7.61
16	0 (50)	0 (8.0)	0 (5%)	7.55

[a]A= Temperature; B= pH and C= Enzyme concentration.

TABLE 2. Analysis of variance (ANOVA) of the quadratic model for the maximization of free oil yield (FOY) from the enzymatic aqueous extraction of sunflower oil

Source	Sum of square	Degree of freedom	Medium square	F	p[a]
A (L)	15.374	1	15.374	198.624	0.0008
A (Q)	42.055	1	42.055	543.349	0.0002
B (L)	1.059	1	1.059	13.676	0.0343
B (Q)	0.003	1	0.003	0.039	0.8559
C (L)	22.984	1	22.984	296.954	0.0004
C (Q)	0.397	1	0.397	5.128	0.1085
A*B	0.281	1	0.281	3.629	0.1529
A*C	3.478	1	3.478	44.938	0.0068
B*C	0.207	1	0.207	2.675	0.2005
Lack of fit	0.262	3	0.087	1.127	0.4619
Pure error	0.232	3	0.077		
Total	86.332	15			

A= Temperature; B = pH; C = Enzyme concentration; L = Linear effect; Q = Quadratic effect; [a] Statistical significance ($p < 0.05$).

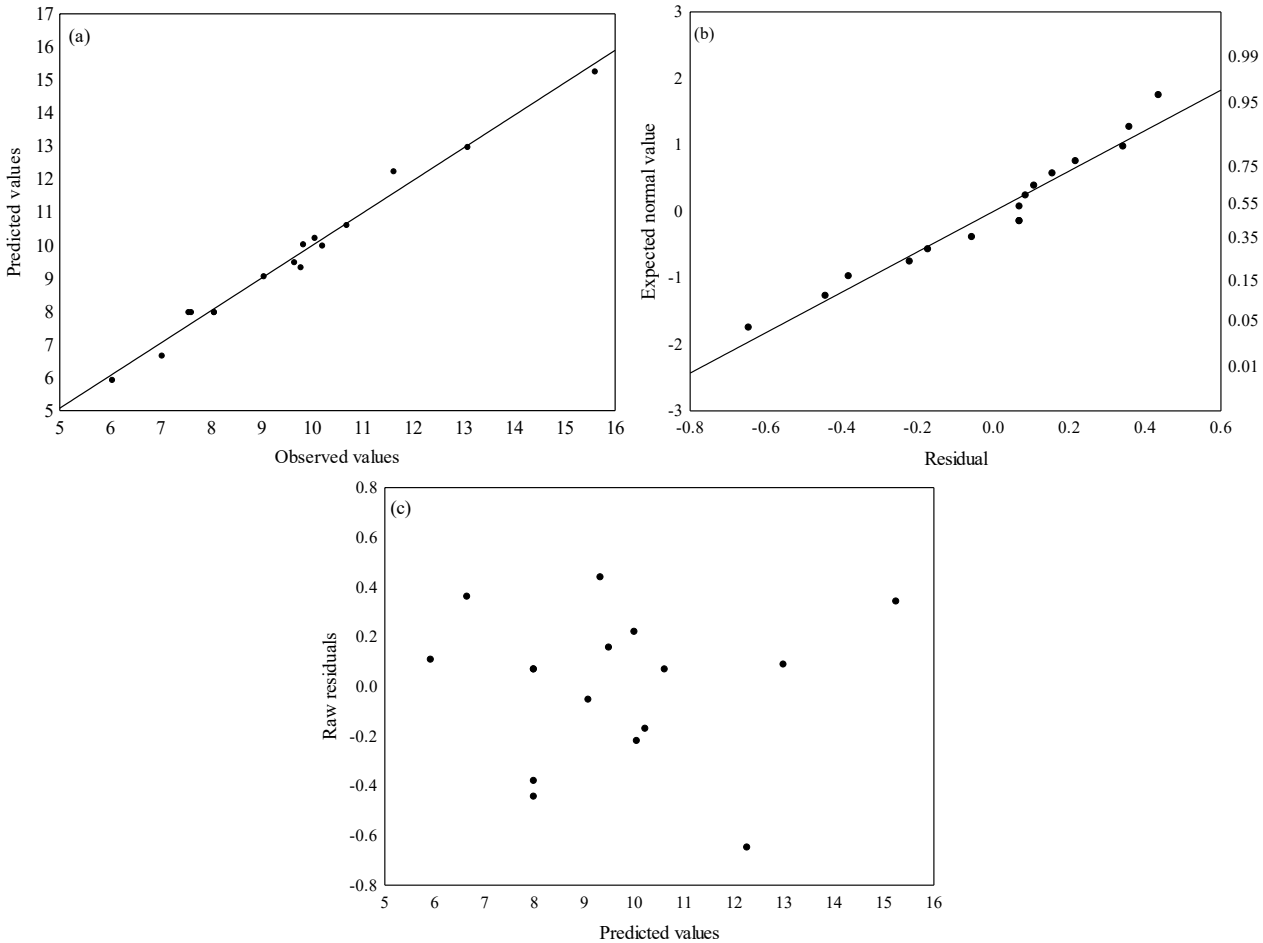

FIGURE 1. Diagnostic plots to verify the proposed model. (a) plot of predicted values versus observed values; (b) plot of normal probability of the residues; (c) raw residuals versus predicted values.

According to the ANOVA data, F_{calc} (123.28) was higher than F_{tab} (3.33); thus the polynomial model was valid in relation to the experimental data. The coefficient of determination (R^2) and the adjusted coefficient of determination (R^2Adj) of the model were 0.984 and 0.976, respectively, which indicates a high degree of correlation between the observed and predicted values.

In order to assess the adequacy of the predicted model, diagnostic graphs were generated, and are shown in Figure 1. Figure 1a shows good agreement between the experimental and predicted data, since the data are close to the straight line, which indicates a satisfactory fit. The graph of normal probability of the residues (Figure 1b) confirms the assumption of normality of the residues. This fact is characterized by the position of the points near the straight line, indicating that the errors are normally distributed. In the graph of raw residuals versus predicted values (Figure 1c), it can be seen that most of the residues were randomly distributed around zero, showing that there is no pattern of behavior between them and that the variance was constant. Thus, the information presented in Figure 1 confirms the adequacy of the model, as well as the validity of its predictions.

3.1.1. Effect of temperature

Temperature was the variable that had the greatest influence on the FOY and by maintaining the pH value and enzyme concentration parameters constant, the highest values for response variables were obtained with the lowest temperature (40 °C). This was evidenced by the results from runs 1, 3 and 7, which are shown in Table 1 as the highest FOY values. According Passos *et al*. (2009), enzymatic aqueous extraction is favored at 40 °C, a fact that allows energy saving and facilitates the preservation of enzymatic activity. In addition, the use of higher temperature increases energy costs (Cheng *et al*., 2019).

Enzymes normally have activities at temperatures between 35 and 60 °C and an increase in temperature results in protein denaturation (Yusoff *et al*., 2015). Consequently, this reduces the release of oil from oilseeds. However, it should be noted that within the optimal temperature range for enzymes, oleaginous matrix characteristics also have an influence at an appropriate temperature for the oil extraction process (Liu *et al*., 2016). The rate of enzymatic extraction is directly related to temperature, where high temperatures can increase extraction rate, but on the other hand they can darken the oil (Liu *et al*., 2016). The temperature influences oil quality, where a mild temperature does not deteriorate the oil due to the oxidation of polyunsaturated fatty acids and the development of rancidification (Ribeiro *et al*., 2016). Furthermore, high temperatures can also be the cause of the caramelization of the carbohydrates present in the extraction medium, which will reduce the quality and yield of the extracted oil (Yusoff *et al*., 2015).

In the enzymatic extraction of peanut oil using the Protizyme protease enzyme, Sharma *et al*. (2002) reported the highest oil yield at 40 °C. Li *et al*. (2011) evaluated five enzymes in the aqueous extraction process and the highest yield was obtained with the Alcalase® enzyme at 40 °C. According to Rui *et al*. (2009), the optimal temperature range for the hydrolysis of pectinase, cellulase and protease enzymes was between 40 to 55 °C.

3.1.2. Effect of pH

The increase in pH in the extraction medium favored the content of free oil. However, this increase was noted to be lower in magnitude than other parameters. Extraction efficiency can be maximized at the optimal pH; whereas each enzyme has an optimal specific value (Abdulkarim *et al*., 2005). The optimal pH should not be near the isoelectric point, which is 9.4 for Alcalase® 2.4L FG protease enzyme (Sigma-Aldrich, 2021), because in the specific isoelectric point the enzyme is insoluble and can make t oil extraction difficult (Kumar *et al*., 2017). The pH does not affect only enzyme activity, but also the separation of oil and protein. The enzymes can simultaneously solubilize and hydrolyze protein and break up polysaccharides, which facilitates oil extraction (Latif and Anwar, 2011).

When evaluating the enzymatic extraction of pine kernel oil with 2% (v/v) of Alcalase® at 50 °C, Li *et al*. (2011) obtained oil yield of 87 and 76% for extraction at pH 9.0 and 12.0, respectively. Meng *et al*. (2018) carried out the oil extraction of *Camellia oleifera* with Alcalase® enzyme and evaluated the effect of pH in the range of 8.5 to 9.5, obtaining the highest yield (93.5%) at pH 9.2. In obtaining pecan nut oil using aqueous extraction with the enzyme Alcalase®, Polmann et al. (2019) obtained an extraction yield of 65.3% at pH 8.0 and 52 °C.

3.1.3. Effect of enzyme concentration

The FOY was higher with the increase in enzyme concentration and this fact can be observed when comparing results achieved by varying the value for enzyme concentration and keeping temperature and pH values constant. For example, when comparing experimental runs 9 and 10, there was an increase of 62.6% in the FOY when enzyme concentration passed from 1 to 9% (v/v), respectively.

The enzyme amount is directly related to the substrate available for enzymatic hydrolysis and higher enzyme concentration increases the enzymatic interaction with substrate, which degrades the cell wall and releases the oil. Goula *et al*. (2018) reported that enzyme concentration affects hydrolysis and extraction yield. When extracting oil from pomegranate seeds, the greater the amount of enzyme used, the faster the extraction was and the higher the yield was. When higher enzyme concentrations of cellulase and protease (Peclyve V) enzymes, the oil yield was higher at 10%. However, there is a saturation point where the addition of more enzyme does not increase the yield, in addition to increasing processing costs and resulting in bitterness and darkening of the oil (Jiang *et al*., 2010; Latif and Anwar, 2011).

Siriwardhana *et al*. (2004) found that the extraction yield obtained from *Hizikia fusiformis* using Ultraflo® and Alcalase® 2.4 L FG protease enzymes increased when the enzyme concentration increased to 5%. In the enzymatic aqueous extraction of peanut oil using protease (Alcalase® 2.4 L FG) and cellulase (Cellulase AE80) enzymes, Jiang *et al*. (2010) reported that FOY increased with the increase in the amount of enzyme from 1 to 2%.

Although the enzyme represents a cost for the enzymatic aqueous extraction, Cheng *et al.* (2019), reported that the enzymatic extraction of soybean oil can be economically viable, because it requires less energy and the initial investment cost is lower when compared to the solvent extraction process. In addition, economic viability can be improved by recycling the enzyme and using the concept of biorefinery, where co-products, carbohydrates and proteins could be used as raw material for other processes (Sekhon *et al.*, 2018, Cheng *et al.*, 2019).

3.1.4. Interaction of variables

The contour graphics for the interaction between two independent variables were generated by keeping one variable at the central level (Figure 2). FOY increased with decreasing temperature and increasing pH value (Figure 2a) or enzyme concentration (Figure 2b). However, the effect of temperature was greater in combination with enzyme concentration than with pH, resulting in a more pronounced curve concavity (Figure 2b) and significant interaction. Figure 2c shows that the level curves of the variables pH and enzyme concentration did not show curvature. This linear behavior indicates that there were no considerable interactions between these independent variables and the FOY.

3.1.5. Maximization of FOY

To determine the set of variables that maximized of FOY from EAE, the desirability function was applied from Statistica® software, considering only the significant terms of Equation 3. The results showed that conditions were: temperature of 40 °C, pH 8.0 and enzyme concentration of 9% (v/v), which resulted in the theoretical FOY of 15.61%. To validate the efficiency of the predictive equation, the experiment was carried out under the conditions of maximum oil removal, in quintuplicate, and FOY obtained was 14.77% ±0.55. The efficiency of the model was verified by the Student´s *t*-test, which showed that there was no significant difference between real and predictive results.

Campbell *et al*. (2016) reported 39% sunflower oil at 50 °C, seed-to-water mass ratio of 1:10 and with the addition of 2% protease (Protex 7L) and 2% cellulase (Multifect CX 13L). The oil yield present in this work was determined with free oil and the article cited includes free and emulsified oil, which justifies the higher oil yield value than that presented in this work. Moradi and Rahimi (2019) extracted sunflower oil with a mixture of cellulase and pectinase enzymes and obtained free oil yield of 23.7% using 2% enzyme at 40 °C, pH 4.5 and seed-to-water mass ratio of 1:6. However, in order to separate the free oil it was washed with *n*-hexane, which may have contributed to obtaining higher oil mass. Thus, for comparative effects, experiments were performed in the condition of maximum FOY to verify the yield with the recovery of the emulsified

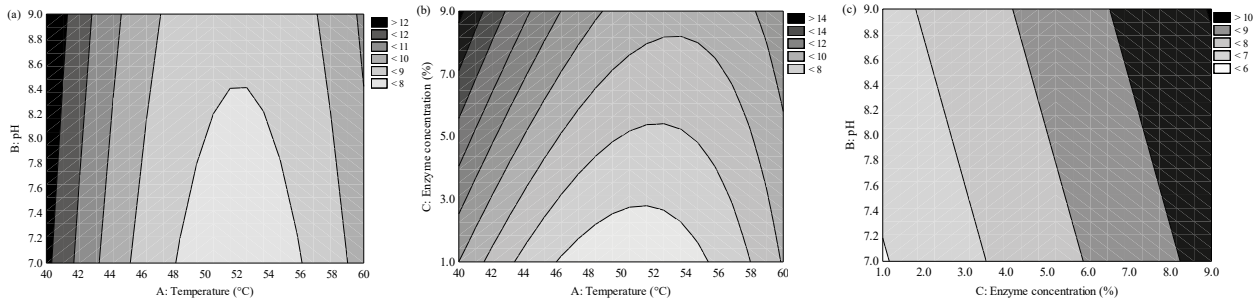

FIGURE 2. Contour plot of response surface showing the effects of binary interactions between independent variables on the free oil yield (FOY); (a) pH and temperature; (b) enzyme concentration and temperature; (c) pH and enzyme concentration.

oil, obtaining 19.04%±0.7 and 30.78%±0.79 of oil with the use of ethanol and *n*-hexane, respectively.

Ribeiro *et al*. (2016) obtained 36.6% of free oil in the enzymatic extraction with 10% of each enzyme, pectinase (Pectinex Ultra SPL), cellulase (Celluclast 1.5L) and protease (Alcalase® 2.4L FG), sunflower seed-to-water mass ratio of 1:6 at 55 °C and 8 hours of extraction. However, to obtain greater yield than that obtained in the present work, the concentration of the enzyme used by the authors was triple the amount in addition to the use of three different enzymes. Aquino *et al*. (2019) used Celluclast® 1.5 L enzyme and obtained 17.76% ± 0.46 of sunflower free oil in the enzymatic extraction. The authors used a temperature of 60 °C, seed-to-water mass ratio of 1:5 and 1% (v/v) enzyme. The value was close to that found in this study at 14.77% ± 0.55 using seeds of the same origin and lot. This difference can be justified by the action of the cellulase enzyme, which has different activity than the protease enzyme. The vegetable cell wall is composed of a higher amount of cellulose than protein (Szymanska-Chargot *et al*., 2015), so the action of cellulase enzyme in cellulose hydrolysis may have released a higher amount of oil. On the other hand, the protease enzyme can create protein hydrolysates which are better emulsifiers than native proteins. In this case the extracted oil is retained in the emulsion, decreasing FOY (Campbell *et al*., 2016).

3.1.6. Influence of thermal pre-treatment of sunflower seeds

Figure 3 presents the results for FOY obtained from extractions using seeds with and without thermal pre-treatment. Thermal pre-treatment influenced the FOY in the initial 180 minutes of extraction, thus the highest difference was observed after the first hour, with an increase of 71.34% in FOY. For

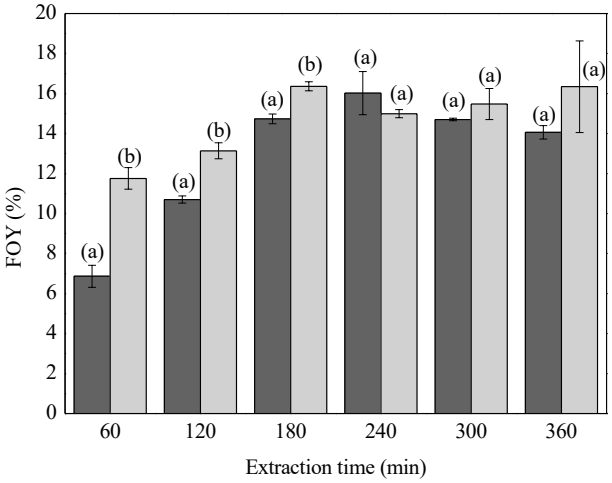

FIGURE 3. Free oil yield (FOY) from enzymatic aqueous extraction of oil from sunflower seeds after thermal treatment (light grey bars) and without thermal treatment (dark grey bar). Conditions: 40 °C, seed-to-water mass ratio of 1:5 (g/g), pH 8.0 and enzyme concentration of 9% (v/v). Data represent the means of duplicate analyses (n=3). Values with different superscript letters are significantly different ($p < 0.05$) for each extraction time. Differences were determined using the Tukey's test.

extractions of 2 and 3 hours, increases of 22.78% and 11.01% were obtained, respectively. After 240 minutes of extraction, no influence of heat treatment on FOY was observed. In this way, the process of enzymatic extraction with thermal pre-treated seeds reached equilibrium 1 hour before the process without thermal pre-treatment. This fact suggests that the oleosins around of a body oil and/or proteins are responsible for the emulsion stability in sunflower seeds and were de-naturated by the action of heat and humidity from the thermal treatment, which contributed to the increased FOY.

Li *et al*. (2016) treated thermally crude peanuts (150 °C for 20 minutes) and obtained an increase in the extraction oil yield and attributed the results to the

fact that temperature possibly affected the functional property of peanut proteins which are responsible for the stability of emulsion. Song *et al*. (2019) applied thermal treatment to peony seeds (110 °C and 0.48 MPa for 60 minutes) after immersed them in water at a seeds-water ratio of 1:5 (g/g) and obtained an enzymatic aqueous extraction of oil using pectinase enzyme. The treated seeds presented increased free oil yields of 77.13% to 89.45%. Furthermore, the microstructure of peony seeds with and without thermal treatment were analyzed through laser scanning microscopy, and it was possible to observe the rupture of oleic bodies and consequently the oil coalescence. Tian *et al*. (2019) applied a thermal pre-treatment (120 °C for 60 minutes) after immersion of colza seeds in water in a ratio of 1:3 (g/g) and related the increase in the yield to aqueous extraction. Thus, the authors concluded that the combination of humidity and higher temperature improved heat transfer and helped to irreversibly denature the layer of oleosin proteins that surround the oil bodies in the oleaginous seeds where oil coalescence occurs.

3.2. Oil characterization

The chemical composition of the oil obtained from sunflower seeds with and without heat pre-treatment obtained with 3 and 4 hours extraction time, respectively, is shown in Table 3. Oleic, linoleic, palmitic and stearic acids were the four main fatty acids present in the oil, with a predominance of oleic. According to Codex Alimentarius standards, sunflower oil obtained from EAE is classified as mid-oleic acid, whose content in this fatty acid is

Table 3. Effect of thermal pre-treatment on the chemical properties of sunflower oil obtained from enzymatic aqueous extraction

Property		Without thermal pre-treatment	With thermal pre-treatment
Fatty acids (%)	Capric	0.02±0.00[a]	0.02±0.00[a]
	Myristic	0.06±0.00[a]	0.07±0.00[a]
	Palmitic	5.72±0.07[a]	5.66±0.00[a]
	Palmitoleic	0.08±0.04[a]	0.08±0.00[a]
	Stearic	4.46±0.01[a]	4.41±0.02[a]
	Oleic	47.22±0.73[a]	47.70±0.18[a]
	Trans-Vaccenic	0.50±0.01[a]	0.49±0.01[a]
	Linoleic	39.67±0.73[a]	39.37±0.13[a]
	Arachidic	0.36±0.03[a]	0.36±0.01[a]
	Behenic	1.20±0.00[a]	1.18±0.02[a]
	Lignoceric	0.38±0.04[a]	0.40±0.01[a]
	Not identified	0.31±0.08[a]	0.25±0.01[a]
Phytosterols (mg per 100 g)	Campesterol	15.80±0.34[a]	15.99±0.14[a]
	Stigmasterol	21.46±0.60[a]	18.88±0.17[b]
	γ-Sitosterol	12.07±0.54[a]	8.72±0.01[b]
	β-Sitosterol	100.07±3.89[a]	90.08±1.13[b]
	Total phytosterols	149.41±5.33[a]	133.66±0.83[b]
Tocopherol (mg per 100 g)	α-Tocopherol	31.49±0.41[a]	34.55±1.32[a]
Free fatty acid (%)		0.57±0.08[a]	0.52±0.06[a]
Acylglycerols (%)	Triacylglycerols	86.86±0.65[a]	88.87±0.66[a]
	Diacylglycerols	3.36±0.03[a]	3.82±0.04[a]
	Monoacylglycerols	0.68±0.01[a]	0.64±0.01[a]
	Total acylglycerols	90.89±0.69[a]	93.33±0.71[a]
Induction period (h)		2.28±0.29[a]	1.95±0.04[a]

Means followed by the same lowercase letter (in each row) do not differ statistically ($p > 0.05$). Differences were determined using the Tukey's test (n=4).

in the range of 43.1-71.8% (Codex Alimentarius, 2019). The heat pre-treatment of the seeds did not influence the fatty acid profile of the oil, which showed high levels of monounsaturated fatty acids (~48%) and polyunsaturated fatty acids (~40%); while the saturated fatty acid content was low (~12%). The higher proportion of oleic acid than linoleic acid is an advantage to the quality of the oil. Smith *et al*. (2007) showed that sunflower oil with high oleic acid content has better oxidative and thermal stability than regular sunflower oil with high linoleic acid content (71.6%).

As shown in Table 3, four phytosterols (campesterol, stigmasterol, γ-sitosterol and β-sitosterol) and one tocopherol (α-tocopherol) were identified in sunflower oil. Among the phytosterols, β-sitosterol was the major component, representing on average 67.19% of the total composition. The high concentration of phytosterols in the oil can promote anti-lipid and hypolipidemic effects when ingested (Dai *et al*., 2013). In addition, phytosterols have antioxidant activity based on electron transfer and also have the ability to scavenge free radicals (Liu *et al*., 2019), attenuating lipid oxidation. The α-tocopherol present in the samples can also increase the oxidative stability of the oil due to its antioxidant capacity, which interrupts the chain reaction of free radicals (Liu *et al*., 2021). The heat pre-treatment slightly reduced (~10.54%) the total phytosterol content, due to the lower levels of stigmasterol, γ-sitosterol and β-sitosterol quantified in the oil. This effect can be attributed to the thermal degradation caused by heating during the pre-treatment. It is known that phytosterols undergo oxidation when subjected to heating, as reported by Chen *et al*. (2020) who studied the thermo-oxidative stability of soy germ phytosterols and reported that heating the oil to 120 °C for 60 min promoted a loss of ~8% in these phytosterols, which is in accordance with this study. However, α-tocopherol was not influenced by seed pre-treatment. The values obtained for phytosterol content were higher than those reported using the conventional method with n-hexane (Aquino *et al*., 2019) after 8 hours' extraction.

Sunflower oil had a low free fatty acid content (>0.6%), which indicates the absence of hydrolytic reactions responsible for causing rancidity and decomposing triglycerides (Goszkiewicz *et al*., 2020). Sunflower oil contains an average of ~92.11% total acylglycerols, which shows that aqueous enzymatic extraction showed high selectivity to this oil. The thermal pre-treatment did not modify the composition of acylglycerols, indicating that the heating time of the seeds was insufficient to hydrolyze the triacylglycerides into smaller components (MG and DAG).

The oil obtained in the present study had a longer period of induction compared to the studies by Ghosh *et al*. (2019) (0.56 h) and Ramos *et al*. (2020) (1.47 h), who evaluated the oxidative stability of sunflower oil at 130 °C. The high oxidative stability of sunflower oil can be attributed to the presence of active compounds, such as phytosterols and tocopherols. In addition, the mild conditions applied in the oil extraction of the oil can contribute to resistance to thermal oxidation. The oils obtained from seeds with and without heat treatment showed similar thermal stability. This result was expected, since susceptibility and oxidative resistance is mainly affected by the chemical composition of the oil and in this study the oil obtained from raw and pre-treated seeds showed similar compositions.

4. CONCLUSIONS

Temperature was the variable that had the greatest effect on the response variable, as the lower temperature favored the increase in free oil yield. In addition, the intermediate pH and the maximum level of enzyme concentration contributed to the increase in the response variable. Therefore, the conditions for maximum FOY (14.77% ± 0.55) from EAE using protease enzyme were 40 ºC, pH 8.0 and enzymatic concentration of 9% (v/v). The pre-treatment applied to sunflower seeds promoted an increase in FOY at the beginning of the extraction and decreased the extraction time by 1 hour when compared to enzymatic extraction with untreated seeds. Thus, FOY of 16.4%±0.8 was obtained in 3 hours of extraction. Therefore, the mild temperature, the short extraction time of 3 hours and the addition of just one enzyme, present the advantage of this extraction process, which provides a lower cost in relation to the enzyme and energy expenditure. The oil obtained by aqueous enzymatic extraction showed oleic and linoleic acid as major fatty acids. B-sitosterol was the main phytosterol present in the oil; while γ-tocopherol was the only tocopherol found. Still, the oils showed high resistance to thermo-oxidative degradation. The chemical composition of the oil was not affected by the pre-heat treatment of the seeds.

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