



Phospholipase cocktail: A new degumming technique for crude soybean oil

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ABSTRACT

Enzymatic degumming (EDG) is an emerging alternative process for decreasing the phosphorus content, increasing the oil yield, and preserving the oil quality. Purifine® 3G is a cocktail of phospholipases composed of phospholipase A₂ (PLA₂), phospholipase C (PLC), and phosphatidylinositol phospholipase C (PI-PLC). In this study, Purifine® 3G was applied to crude soybean oil, and the optimum degumming conditions (enzyme concentration, temperature, and water dosage) were determined using a central composite rotatable design (CCRD). The contents of diacylglycerols (DAGs) and free fatty acids (FFAs) in the studied system considerably increased at temperatures below 64 °C and enzyme concentrations above 100 mg/kg, while the phosphorus content decreased with increasing water amount and enzyme concentration. In particular, EDG with 200 mg/kg of Purifine® 3G conducted for 120 min at a temperature of 60 °C and water concentration of 3% (w/w) lowered the residual phosphorus content to 8.9 mg/kg and increased the FFA and DAG concentrations by 0.17% and 0.72%, respectively. Meanwhile, EDG retained the tocopherol content in crude soybean oil, maintaining its quality. Hence, Purifine® 3G increases the neutral oil yield (FFA and DAG), decreases the phosphorus content, and preserves the oil quality, which make it a commercially viable degumming agent.

1. Introduction

Soybeans represent the most commonly produced oilseeds in Brazil. Its soybean planted area is expected to expand by 38.5 million hectares during the 2020/21 season, which will increase the soybean production by 131.5 million metric tons (MMT). The forecast for soybean exports in 2020/21 was 85 MMT, and the high demand for soybean oil is mainly driven by its domestic consumption (Ustinova, 2020). Crude soybean oil obtained by oilseed processing must be refined before consumption to remove unwanted components such as free fatty acids (FFAs), metal traces, and phospholipids with minimal losses of useful compounds, including acylglycerols, free and esterified sterols, tocopherols, and tocotrienols. According to Sampaio et al. (2015), the refinement of edible oils can be performed by either chemical or physical processing. In general, physical refining is preferable due to its environmental benefits and sustainability.

Degumming is the first and most important step of the refinement process that separates the majority of phospholipids and gums from oils, which is imperative for the production of high-quality oils (Jiang, Chang, Jin, & Wang, 2015a). To ensure high phospholipid removal efficiency, an oil with a phosphorus content of less than 10 mg/kg should be obtained (Jahani, Alizadeh, Pirozifard, & Qudsevali, 2008). Phospholipids constitute 0.3–0.6% of soybean seeds or 1.5–3.0% of crude soybean oil (Liu & Ma, 2011). Conventional techniques used for the removal of phospholipids include water degumming and acid degumming. Water degumming is used to remove hydratable phospholipids, while acid degumming removes non-hydratable phospholipids through the addition of acids such as phosphoric and citric ones (Passos et al., 2019).

Enzymatic degumming is an emerging process for the removal of phospholipids from crude oils that utilizes enzymes called phospholipases. Phospholipase A₁ (PLA₁) and phospholipase A₂ (PLA₂) remove fatty acids (FAs) from positions 1 and 2 of phospholipids with respect to

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Abbreviations

ANOVA	analysis of variance
CCRD	central composite rotatable design
DAG	diacylglycerol
EDG	enzymatic degumming
FA	fatty acid
FFA	free fatty acid
MMT	million metric ton
PA	phosphatic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI-PLC	phosphatidylinositol phospholipase C
PLA ₂	phospholipase A2
PLC	phospholipase C

glycerol moieties, respectively. Phospholipase C (PLC) hydrolyzes the bond between the acylglycerol and phosphate groups to liberate diacylglycerols (DAGs) (Jiang, Chang, Jin, & Wang, 2015b; Qu et al., 2016; Sampaio, Zyaykina, Uitterhaegen, Greyt, & Verhé, 2019). To facilitate the enzymatic degumming process, chemical conditioning is typically performed in the previous step. As formulated by Sampaio et al. (2015), the purpose of chemical conditioning is to increase the hydratability of non-hydratable phospholipids and determine the optimal pH value corresponding to the maximum enzyme activity.

Purifine® 3G is an enzyme cocktail composed of phospholipase C (PLC), phosphatidylinositol – specific phospholipase C (PI-PLC), and a minor amount of PLA₂, which effectively converts phospholipids into predominantly diglycerides (DAGs), phosphates, FFAs, and lysophospholipids (Nikolaeva et al., 2020). While each phospholipase generates a specific product, the cocktail not only increases the FFA and DAG concentrations, but also decreases the phosphorus content in a one-step process.

Very few previous studies have focused on increasing the neutral oil yield, decreasing the phosphorus content, and preserving the tocopherol content during degumming vegetable oils with the Purifine® 3G cocktail. Nikolaeva et al. (2020) examined the gum mesostructures formed in crude soybean oil after water degumming and enzymatic degumming with the Purifine® 3G cocktail; however they did not optimize the process parameters. Sein, Hitchman, and Dayton (2019) evaluated the degumming process using the Purifine 3G cocktail from a molecular point of view. Guerrand (2017) discussed various enzyme applications and the use of Purifine 3G for the production of edible oils; nevertheless, the author did not mention its applicability to a specific vegetable oil. Therefore, this study aimed to investigate the effects of different process conditions, such as temperature, water dosage, and enzyme (Purifine® 3G) concentration, on the enzymatic degumming of crude soybean oil.

2. Material and methods

2.1. Oil and enzyme

Crude soybean oil was kindly provided by Cargill (Uberlandia-MG/Brazil). The cocktail Purifine® 3G (PLC + PI-PLC + PLA₂) with an activity of 16900 PLCU/g was supplied by DSM Food Specialties (Delft, the Netherlands). PLA₂ enzyme was produced by a selected strain of *Aspergillus niger*; PLC enzyme was produced by a selected strain of *Pichia pastoris*; and PI-PLC enzyme was produced by a selected strain of *Pseudomonas fluorescense*.

2.2. Physicochemical analysis

P, Mg, Fe, and Ca elemental concentrations were obtained by

inductively coupled plasma – atomic emission spectroscopy according to Method Ca 20–99 (American Oil Chemists' Society, 2012a). The pH of the gums was measured directly with a pH electrode inserted into the gum fraction. FFA content was determined by titration according to the official Ca 5a-40 method of the American Oil Chemists' Society (American Oil Chemists' Society, 2012b).

DAG content was determined according to the AOCS official method Cd 11b-91 (American Oil Chemists' Society, 2012c). Approximately 0.05 g of the analyzed sample was dissolved in 100 µL of tetradecane and 300 µL of N,O-bis(trimethylsilyl)trifluoroacetamide, and the resulting mixture was homogenized and heated to 70 °C for 20 min. After that, 50 µL of the derivatized sample was diluted with hexane (1 mL) and injected into a gas chromatograph (Agilent Technologies, model 7890A) equipped with a DB-5HT capillary column (15 m × 0.32 mm i.d.; film thickness: 0.10 µm). Analysis was performed under the following conditions: oven temperature ramp from 50 to 200 °C (15 °C/min), from 200 to 290 °C (3 °C/min; held for 10 min), and from 290 to 360 °C (10 °C/min; held for 15 min); flame ionization detector (380 °C); and He carrier gas. DAGs were identified using a diolein standard.

Tocopherol content was measured as described by Ansolin, Souza, Meirelles, and Batista (2017). The utilized solvents included HPLC grade methanol and isopropanol (J. T. Backer, USA), while Milli-Q water and HPLC grade ammonium hydroxide solution (28–30%) (J. T. Backer, USA) were used to prepare a mobile phase. Tocopherol standards (α, β, γ, and δ) were utilized for tocol quantification (Merck, USA). The mobile phase was composed of a methanol: water: ammonia mixture (99:1:0.1, v/v/v, phase A) and pure isopropanol (phase B). Analysis was performed on a Waters Acquity SQD/UPLC System (USA) equipped with a quaternary pump, an automatic injector, a column oven, a photodiode array detector, and a single quadrupole mass spectrometer with electrospray ionization operated in the negative mode. Chromatographic separation was achieved at 25 °C using an Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm, Waters, USA). The gradient program was 0–6.00 min 100% A (0.2 mL/min), 6.01–9.00 min 100% B (0.15 mL/min), and 9.01–15.00 min 100% A (0.2 mL/min). All samples were analyzed in duplicate.

2.3. Degumming efficiency

Degumming efficiency was calculated using the formula proposed by Lamas, Constenla, and Raab (2016), which was based on the decrease in phosphorus content:

$$Efficiency(\%) = \frac{P_i - P_r}{P_i} \times 100 \quad (1)$$

P_i: the total phosphorus content in crude oil (mg/kg);

P_r: the residual phosphorus content in the degummed oil (mg/kg)

2.4. Theoretical phospholipid content and increases in DAG and FFA concentrations

The content of phospholipids was determined from the conversion of phosphorus to phospholipids calculated using the ratio of the phosphorus atomic weight (P = 31) to the estimated molecular weight of the phospholipids (PL), which was equal to approximately 25 (Galhardo & Dayton, 2021):

$$PL(\%) = \frac{25 \times P(\text{mg/kg})}{10000} \quad (2)$$

The increases in the FFA and DAG concentrations were obtained by the method described in the work of Galhardo and Dayton (2021) with some modifications. The maximum theoretical increase in the FFA content after enzymatic degumming was computed from the phospholipid content in crude oil multiplied by the ratio of the molecular mass of FFA (282 g/mol) to the average molecular mass of phospholipids

(phosphatidylcholine (PC): 758 g/mol; phosphatidylethanolamine (PE): 692 g/mol; phosphatidylinositol (PI): 887 g/mol; phosphatidic acid (PA): 699 g/mol), which was equal to approximately 759 g/mol. Here, the authors assumed that all FAs remained in the oil. The estimated FFA concentration increase is expressed by the following equation:

$$FFA (\%) = PL \times \frac{282}{759} \quad (3)$$

where FFA is the relative amount of FFAs formed during the enzymatic reaction (%), and PL is the total phospholipid content in crude oil (%).

The maximum theoretical DAG concentration increase was calculated as the content of selected phospholipids present in crude oil (PC, PE, and PI) multiplied by the ratio of the molecular mass of DAG (617 g/mol) to the average molecular mass of these phospholipids (PC = 758 g/mol; PE = 692 g/mol; PI = 887 g/mol), which was equal to approximately 779 g/mol. In this case, the authors assumed the reaction efficiency of 85% due to the partial reaction of PE. The estimated DAG concentration increase is expressed by the following equation:

$$DAG = (PC + PE + PI) \times \frac{617}{779} \times 0.85 \quad (4)$$

where DAG is the amount of DAGs formed during the enzymatic reaction (%), and (PC + PE + PI) is the total amount of phospholipids hydrolyzed by PLC (%).

2.5. Water degumming

Water degumming was performed as described previously by [Sampaio et al. \(2015\)](#). Crude soybean oil (300 g) was first heated to 80 °C followed by water addition (3% w/w), after which the resulting mixture was homogenized by mechanical stirring (350 rpm) for 15 min. The produced gums were separated by centrifugation (2000×g for 15 min).

2.6. Chemical conditioning

Crude soybean oil (300 g) was heated to 80 °C followed by the addition of 30% aqueous solution of citric acid at a concentration of 900 mg/kg. The resulting system was high-shear mixed for 1 min at a speed of 16000 rpm and subsequently agitated for 15 min at 350 rpm. After that, an aqueous solution of sodium hydroxide (14%) was added to the reaction mixture at a concentration of 500 mg/kg. The oil was subjected to high shear mixing (1 min/16000 rpm) to disperse the caustic solution. The produced gums were separated from the degummed oil by centrifugation (2000×g for 15 min) ([Sampaio et al., 2015](#)).

2.7. Enzymatic degumming

Enzymatic degumming experiments were performed using 300 g of crude soybean oil. In the first step, the solution pH was adjusted to 5.5 by chemical conditioning. Subsequently, the oil was heated to 80 °C for 15 min under stirring at 350 rpm, after which the temperature of the oil mixture was decreased to the values established by a central composite rotatable design (CCRD). The amounts of added Purifine® 3G and water were also determined according to the CCRD. The obtained mixture was homogenized under high-shear mixing (16000 rpm) for 1 min and then maintained at a required temperature under mixing (350 rpm) for 120 min. The reaction was stopped by heating the oil to 85 °C for 15 min at 350 rpm. Thereafter, the oil mixture was centrifuged (2000×g for 15 min) to separate the degummed oil from the gums.

2.8. Experimental design and data analysis

Response surface methodology was adopted to optimize the enzymatic degumming of crude soybean oil. To obtain the minimum phosphorus, maximum FFA, and maximum DAG contents, a central rotatable

design (CCRD – 2³) was used. Enzyme dosage (X₁), temperature (X₂), and water dosage (X₃) were treated as independent variables. A total set of 17 experiments (= 2^k + 2, k + 3 repetitions at the central point), where k is the number of independent variables with five different levels (= 1.68), is provided in [Table 1](#). The relationship between the coded and actual values used for statistical calculations is described by the following equation:

$$X_i = \frac{A_i - A_0}{\Delta A} \quad (5)$$

where X_i is the coded value of the variable, A_i is the actual value of the variable, A₀ is the actual value of A_i at the central point, and ΔA is the step change of the variable (the obtained design matrix is also listed in [Table 1](#)). The phosphorus, DAG, and FFA contents were treated as responses. The model for each independent variable was generated using the following quadratic equation:

$$Y = B_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (6)$$

where Y is the predicted response, β₀ is the constant, β_i is the linear coefficient, β_{ii} is the squared coefficient, β_{ij} is the cross-product coefficient, X_i is the independent variable (uncoded value), and i and j are the levels of significance of the terms.

The quality of the design was estimated by computing the determination coefficient (R²), while the analysis of the variance (ANOVA) was performed to evaluate the statistical significance of the proposed model by calculating the regression values and mean squared residual error. The graphical representation of Eq. (6), known as the response surface, was applied to determine the interactions between the independent variables and their effects on each response. The obtained data were analyzed using the Statistica 5.0 software package (StatSoft Inc., United States). Statistically significant differences were observed at the p < 0.1 level. The Tukey test was performed to analyze the samples obtained after aqueous degumming and chemical conditioning as well as crude oil and tocopherol samples. Here, significant differences were observed at p ≤ 0.05.

3. Results and discussion

3.1. Soybean oil characterization

The concentrations of FFA, DAG, phospholipids, and minerals in crude soybean oil, the degummed water, and the chemically conditioned oil are listed in [Table 2](#). The FFA contents in the degummed water and chemically conditioned oil were lower than that in crude soybean oil as both processes removed acidic compounds. The DAG concentration was reduced by a higher value (56%) during water degumming than that obtained after chemical conditioning (41%). According to [Hitchman \(2009\)](#), this phenomenon was caused by the formation of an emulsion due to the presence of intact phospholipids (such as PC, which was the strongest emulsifier), which increased the oil content in the gums and, therefore, the oil loss. The estimated phospholipid contents in crude soybean oil, the degummed water, and the chemically conditioned oil were 1.52%, 0.11%, and 0.04%, respectively. The mineral content in soybean oil decreased after both processes. First, water degumming removed the hydrated forms of phospholipids (PC, PI, and partially PE),

Table 1
Independent variables and their levels used for the CCRD-2³ during the enzymatic degumming of crude soybean oil.

Variables	Symbols	Levels				
Enzyme (Purifine® 3G) (mg/kg)	X ₁	-1.68	-1	0	1	1.68
Temperature (°C)	X ₂	30	64.4	115	165.6	200
Water dosage (% w/w)	X ₃	50	54	60	66	70
		1.0	2.2	3.0	4.2	5.0

decreasing the phosphorus content by 93%. Afterwards, acid conditioning removed the non-hydratable forms of phospholipids (PA and partially PE) that were bound to divalent ions such as Ca, Mg, and Fe, resulting in a 97% reduction in the phosphorus content. Similar results were obtained by Sampaio et al. (2019) who performed the enzymatic degumming of crude corn oil by traditional processes.

3.2. Fitting models

The experimental runs were performed using the independent variables and their ranges specified in Table 1. The FFA (Y_1), DAG (Y_2), and phosphorus (Y_3) contents were determined for each condition, and the obtained results are presented in Table 3. The data were analyzed using the Statistica 5.0, software, which generated different models, model coefficients, R^2 values, F-values, and significant probabilities. Using these parameters, the significance of each experimental variable was obtained. For simplicity purposes, the non-significant terms were removed from each model. However, the statistically non-significant linear terms were retained when the respective quadratic effects were statistically significant according to Jahani et al. (2008). Using this approach, the representativeness of a selected model was improved, the importance of the term in its linear and quadratic form, and possible interactions were determined. According to the obtained regression models, the parameters X_1 , X_1^2 , X_2 , X_2^2 , and X_1X_3 had significant effects on the FFA content; the parameters X_1 , X_2 , X_3 , X_3^2 , and X_1X_2 were significant for the DAG content; and the parameters X_1 , X_3 , and X_3^2 produced significant effects on the phosphorus content. The second-order polynomial equations containing the coded factors obtained from the CCRD model for FFA (%), DAG (%), and phosphorus (mg/kg) content are listed below:

$$\text{FFA content (\%C18 : 1)} = 1.08 + 0.086X_1 - 0.044X_1^2 + 0.037X_2 - 0.058X_2^2 - 0.044X_1X_3 \quad (9)$$

$$\text{DAG content (\%)} = 1.38 + 0.51X_1 - 0.18X_2 + 0.022X_3 - 0.16X_3^2 + 0.21X_1X_2 \quad (10)$$

$$\text{Phosphorus content (mg / kg)} = 12.56 - 11.00X_1 - 34.52X_3 + 38.25X_3^2 \quad (11)$$

where X_1 , X_2 , and X_3 are the coded variables for the enzyme concentration, temperature, and water dosage, respectively.

As indicated by the obtained statistical models, the enzyme concentration produced the strongest effects on the FFA (Eq. (9)) and DAG (Eq. (10)) contents followed by temperature. Enzyme activity can be affected by several factors, including enzyme concentration, temperature, reaction time, and solution pH. According to De Greyt (2013), enzyme concentration depends on the enzyme type and phospholipid content in the oil. Phospholipases are enzymes that cleave the bonds between the glycerol backbone and FAs, DAGs, and phosphate ester. The enzymes used in the present study existed in the form of a cocktail known as Purifine® 3G. According to Gupta (2017, pp. 60–76), this cocktail is specific to all four common phospholipids (PC, PE, PA, and PI). It contains PLC that interacts with PC and PE and PI-PLC that reacts with PI to produce DAG and phosphate ester. The other enzyme present in Purifine® 3G is PLA₂, which is selective for PC, PE, and PA. It cleaves the FA from position 2 of the glycerol backbone to produce FFA and lysophospholipids. Note that enzyme activity is high within a narrow range of temperatures, where the increase in temperature can increase the reaction rate; however, the temperatures higher than the optimal value can cause a partial denaturation of the enzyme accompanied by

Table 2

Characterization of crude soybean oil, water degummed and chemical conditioned oil.

Analyses	Crude soybean oil	Water Degumming	Chemical Conditioning
FFA (%C18:1)	1.24 ± 0.04	1.01 ± 0.06	1.00 ± 0.10
DAG (%)	1.67 ± 0.07	0.73 ± 0.04	0.98 ± 0.02
Phospholipids ^a (%)	1.52	0.11	0.04
Minerals content (mg/kg)			
P	608 ± 33.0	45 ± 0.4	19 ± 1.0
Fe	11.7 ± 0.1	0.34 ± 0.01	ND < 0.1
Ca	52 ± 3.0	23.8 ± 0.4	2.06 ± 0.08
Mg	58 ± 3.0	10.6 ± 0.2	1.44 ± 0.05

^a Phospholipids estimated by Equation (2).

the loss of its hydrolytic activity.

The phosphorus content (Eq. (11)), the most important parameter, is strongly affected by water addition followed by enzyme concentration. According to Sampaio et al. (2015), the main portion of the phospholipids present in crude soybean oil is hydratable and thus form micellar structures with water, which are insoluble in oil due to their polar properties and can be easily removed by centrifugation. In this case, the role of the enzyme is to hydrolyze the phospholipids into FFA or DAG molecules, thereby avoiding their removal in the intact form, which can significantly contribute to the oil loss.

Table 4 lists the ANOVA results. According to Rodrigues and Iemma (2014), parameters with a 90% significance can be considered significant due to the variability of bioprocesses involving enzymes and microorganisms. The R^2 values obtained for the FFA, DAG, and phosphorus contents using the mathematical model were 70.3%, 92.3%, and 87%,

respectively. Although the R^2 of the FFA content (70.3%) was not very high, $F_{\text{calc. (regression/residuals)}} > F_{\text{tab}}$, indicating a linear regression. Furthermore, $F_{\text{calc. (lack of fit/pure error)}} < F_{\text{tab}}$, suggesting that the

Table 3

Independent, dependent variables, and results for each condition of CCRD 2³.

Trials	Independent variables			Dependent variables		
	X_1^a	X_2^a	X_3^a	Y_1	Y_2	Y_3
1	-1.00	-1.00	-1.00	1.00	1.26	37.8
2	-1.00	-1.00	1.00	1.00	1.32	15.3
3	-1.00	1.00	-1.00	0.94	0.92	52.7
4	-1.00	1.00	1.00	1.07	0.95	35.6
5	1.00	-1.00	-1.00	1.01	1.66	30.0
6	1.00	-1.00	1.00	1.01	1.60	9.30
7	1.00	1.00	-1.00	1.08	1.63	35.1
8	1.00	1.00	1.00	1.07	1.65	15.5
9	-1.68	0.00	0.00	0.90	0.92	27.2
10	1.68	0.00	0.00	1.17	1.70	8.90
11	0.00	-1.68	0.00	0.98	1.44	21.0
12	0.00	1.68	0.00	1.05	1.15	13.0
13	0.00	0.00	-1.68	1.10	1.02	129.0
14	0.00	0.00	1.68	1.07	1.08	11.4
15	0.00	0.00	0.00	1.07	1.34	21.0
16	0.00	0.00	0.00	1.10	1.35	28.7
17	0.00	0.00	0.00	1.10	1.47	16.0

* X_1 - Enzyme concentration (mg/kg); X_2 - Temperature (°C); X_3 - Water dosage (%w/w).

** Y_1 - FFA content (as % C18:1); Y_2 - DAG content (%) and Y_3 - Phosphorus content (mg/kg)/.

^a For detailed information of the process conditions see Table 1.

Table 4
ANOVA results for the responses: FFA, DAG and Phosphorus content.

Independent variable	Variation Source	Sum of Squares	Degrees of Freedom	Mean Square	F _{calc.}	F _{tab.}	p-value
FFA content (%C18:1)	Regression	0.05	5	0.01	5.25	2.45	0.00
	Residuals	0.02	11	0.00			
	Lack of Fit	0.02	9	0.00	7.60	9.38	0.12
	Pure Error	0.00	2	0.00			
	Total	0.07	16				
	R ²	70.3%					
DAG content (%)	Regression	1.20	5	0.24	26.25	2.45	0.00
	Residuals	0.10	11	0.01			
	Lack of Fit	0.09	9	0.01	1.92	9.38	0.40
	Pure Error	0.01	2	0.01			
	Total	1.30	16				
	R ²	92.3%					
Phosphorus content (mg/kg)	Regression	9398.70	3	3132.90	24.94	2.56	0.00
	Residuals	1632.91	13	125.61			
	Lack of Fit	1551.05	11	141.00	3.45	9.39	0.24
	Pure Error	81.86	2	40.93			
	Total	11031.60	16				
	R ²	87.0%					

*Significance at $p < 0.1$.

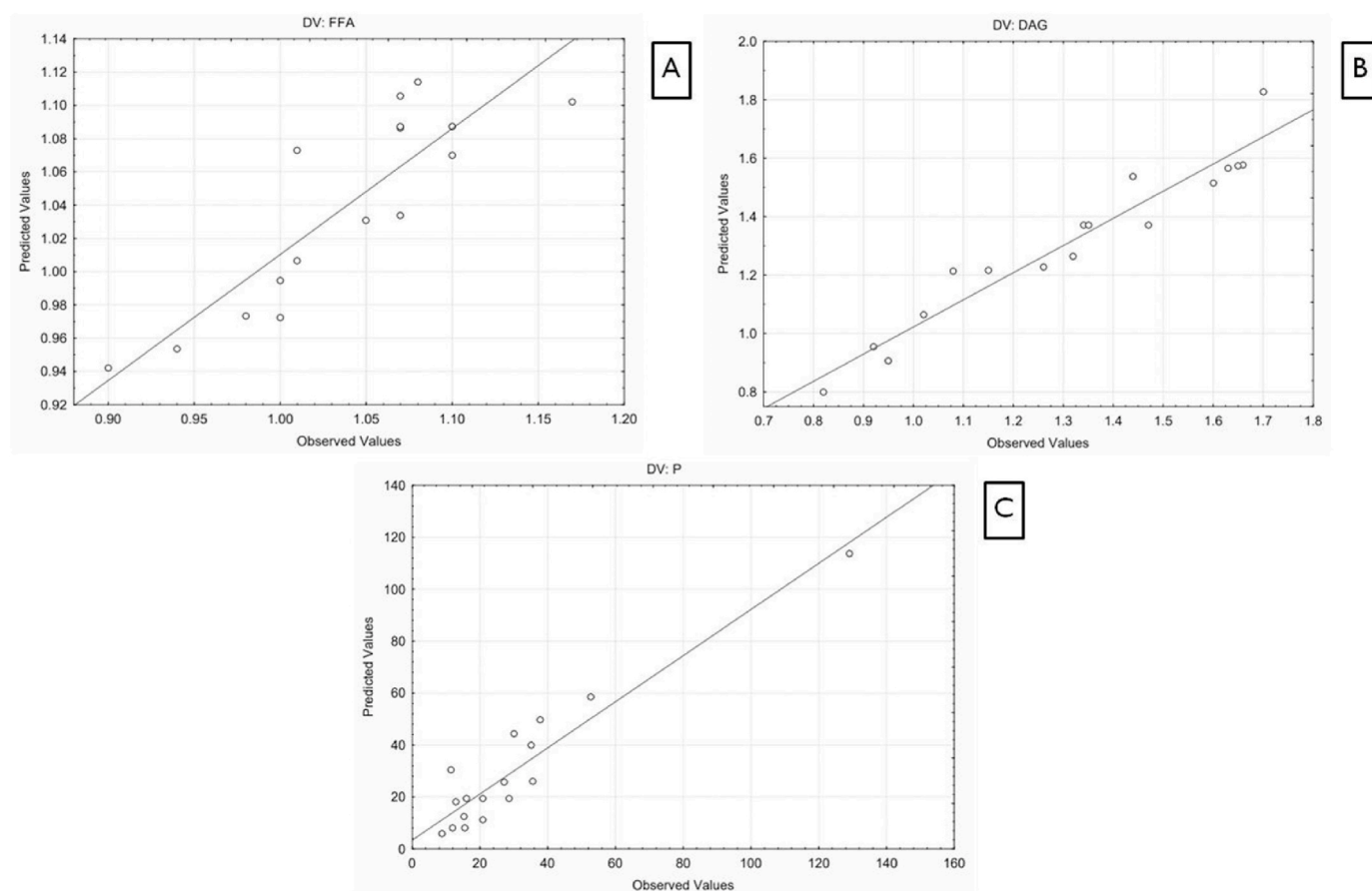


Fig. 1. Experimental and predicted values for A) FFA content, B) DAG content and C) Phosphorus content.

generated equation can adequately describe the analyzed data. According to [Rodrigues and Iemma \(2014\)](#), a suitable model must satisfy both the $F_{\text{calc. (regression/residuals)}} > F_{\text{tab}}$ and $F_{\text{calc. (lack of fit/pure error)}} < F_{\text{tab}}$ requirements for all responses.

As shown in [Fig. 1A](#), the FFA content exhibits low absolute variations (ranging from 0.9% to 1.27%), which may increase the difference between the experimental and predicted values. In [Fig. 1C](#), the water contents between 3 and 5% correspond to the phosphorus contents of 8.9–35.6 mg/kg, and with the water content of 1% produced a

phosphorus content of 129 mg/kg. These results indicate that the higher is the water content, the larger is the amount of hydratable phospholipids removed from the oil after centrifugation ([Costa, Almeida, Alvim-Ferraz, & Dias, 2018](#)).

3.3. Analysis of generated response surfaces

By using the obtained models, it was possible to draw surfaces that described the influences of enzyme concentration, temperature, and

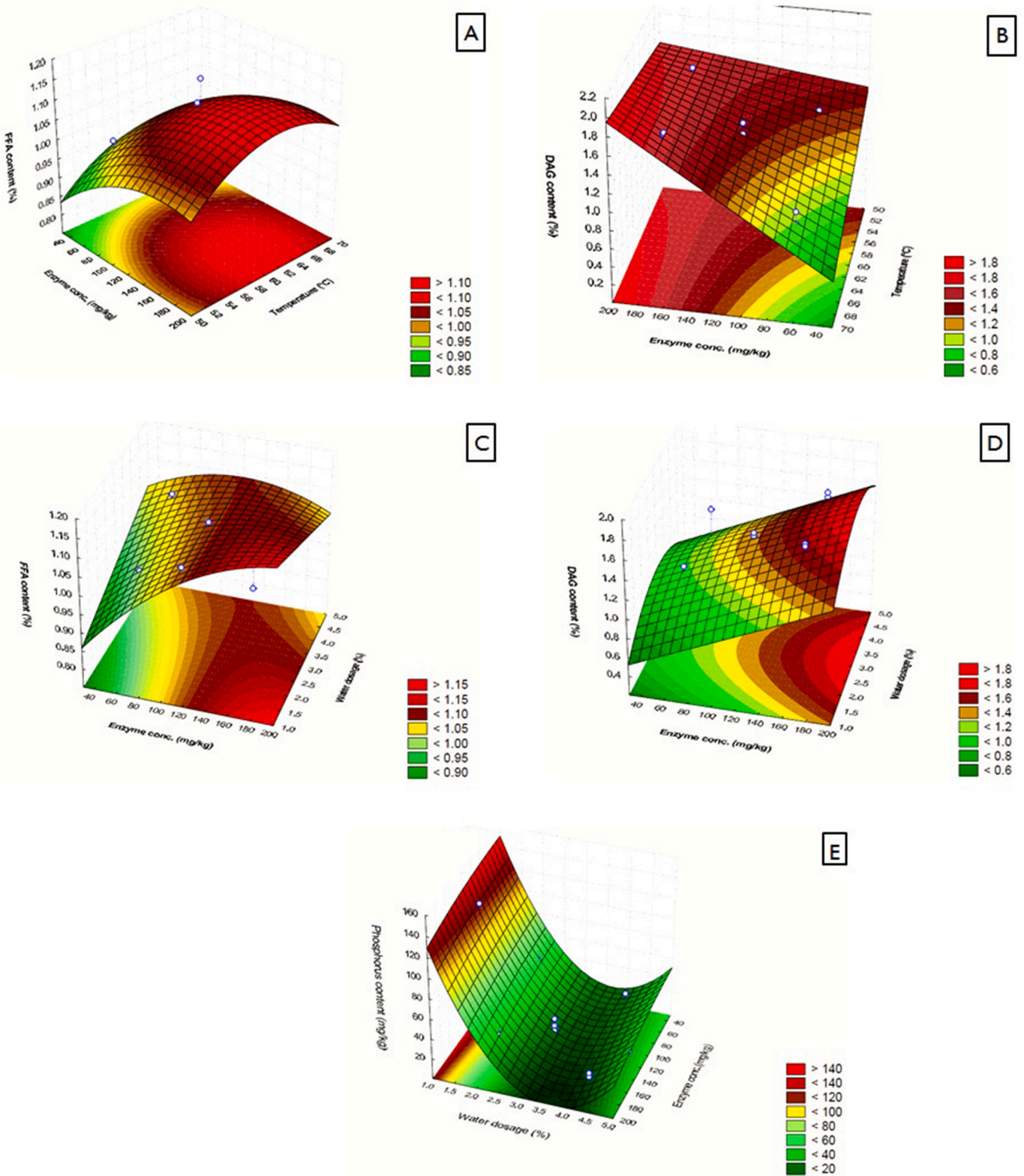


Fig. 2. Response surface of interaction among A) Temperature (°C) and Enzyme conc. (mg/kg) for FFA content; B) Temperature (°C) and Enzyme conc. (mg/kg) for DAG content; C) Water dosage (%) and Enzyme conc. (mg/kg) for FFA content; D) Water dosage (%) and Enzyme conc. (mg/kg) for DAG content; and E) Water dosage (%) and Enzyme (mg/kg) for Phosphorus content.

water dosage on the FFA, DAG, and phosphorus contents. It is well known that the FFA group can be removed by PLA₂ from position 2 of a phospholipid molecule with respect to glycerol during the enzymatic degumming process. Furthermore, PLC hydrolyzes the bond between acylglycerol and the phosphate group to release DAGs. In both cases, the enzymes generate compounds that are present in the oil, thus increasing the oil yield.

Fig. 2A and B shows that increasing the enzyme concentration (>100 mg/kg) and reaction temperature (<64 °C) increases the FFA and DAG contents, confirming the existence of synergistic effects between the variables. According to Eq. (3), an FFA increase of 0.56% was expected only for the PLA₂ component. However, because the cocktail had an unspecified small amount of PLA₂, a higher FFA content was detected in trial 10, which corresponded to only 31% of the total amount. Lamas et al. (2016) performed the enzymatic degumming of sunflower oil using MAXAPAL (PLA₂) as a result, the system acidity increased by 1.05 (mg KOH/g) due to the phospholipase action.

To calculate the theoretical DAG increase, the phospholipid composition of vegetable oil should be considered. For crude soybean oil, the ranges of the PC, PE, and PI contents relative to the total phospholipids content are equal to 25–33%, 19–31%, and 10–18%, respectively (Dayton & Galhardo, 2014; Jiang et al., 2015b; Sampaio et al., 2015). Thus, by applying these values to the total phospholipid content in crude soybean oil (1.52%), a range of 0.82–1.24% was obtained for the mixture of PC, PE, and PI. This was utilized to calculate the maximum theoretical DAG increase via Eq. (4), which varied between 0.55 and 0.83%. After comparing the results of trial 10 with the chemical conditioning data (1.00% FFA), a 0.17% increase in the FFA content and 0.72% increase in the DAG content were observed, indicating that almost all phospholipids (98.8%) have been converted. Nikolaeva et al. (2020) used Purifine® 3G and PLA₂ from DSM to perform the enzymatic degumming of crude soybean oil. For Purifine® 3G, the authors obtained 2% (w/w) of FFA and for PLA₂ –7% (w/w) of FFA, indicating that the fraction of PLA₂ in the cocktail strongly influenced the FFA concentration. Sampaio et al. (2019) performed the enzymatic degumming of crude corn oil using Purifine® PLC; as a result, the DAG content increased by 0.54% at a temperature of 60 °C and enzyme concentration of 200 mg/kg.

Fig. 2C and D depict the effects of enzyme concentration and water dosage on the FFA and DAG contents. They show that decreasing these

two parameters reduces both the FFA and DAG concentrations because the enzyme requires a certain amount of water for its activation and the hydrolysis of phospholipids. Jahani et al. (2008) studied the enzymatic degumming of rice bran oil using PLA₁ and noticed that the FFA content was reduced after decreasing the amount of water in the reaction mixture. Regarding the DAG content, Jiang, Chang, Jin, and Wang (2014) observed that higher water dosages increased the DAG content (expressed in terms of the oil recovery efficiency) during soybean gum deoiling by PLC.

Fig. 2E shows that the phosphorus content decreases with increasing enzyme concentration and water dosage because the enzyme promotes the hydrolysis of phospholipids and water directly contributes to the removal of hydratable phospholipids. The chemical conditioning process was characterized by the degumming efficiency of 97%, and trials 6 and 10 exhibited a degumming efficiency of 98.5% (Eq. (1)) with P < 10 mg/kg, which was required for physical refining. According to De Greyt and Kellens (2005), efficient removal of phospholipids during vegetable oil refining is essential for the production of high-quality oils because residual phospholipids can cause oil darkening and produce off-flavors.

In trial 10 (Table 3) in comparison to the water degumming and chemical conditioning processes (Table 2), increased the FFA content by 0.16% and 0.17% and the DAG content by 0.97% and 0.72%, respectively. The phosphorus content decreases observed during enzymatic degumming (trial 10), water degumming, and chemical conditioning were equal to 98.5%, 92.6%, and 96.8%, respectively. The Purifine 3G exceeded those of traditional degumming methods.

3.4. Tocopherol profiles obtained for different soybean oil samples

Edible oils are major natural sources of tocopherols and tocotrienols known as tocols. These compounds are among the most important lipid-soluble antioxidants present in foods as well as in human and animal tissues. According to Shahidi and De Camargo (2016), the most abundant types of tocopherols in soybean oil include α-tocopherol and γ-tocopherol. The concentration of these tocopherols in crude vegetable oils vary considerably due to climatic and agronomic factors, fruit quality, seed origin, and the utilized oil extraction system (Cert, Moreda, & Pérez-Camino, 2000). Hence, the influences of enzyme concentration, reaction temperature, and water dosage on the enzymatic degumming of soybean oil were studied in this work. The effects of the degumming

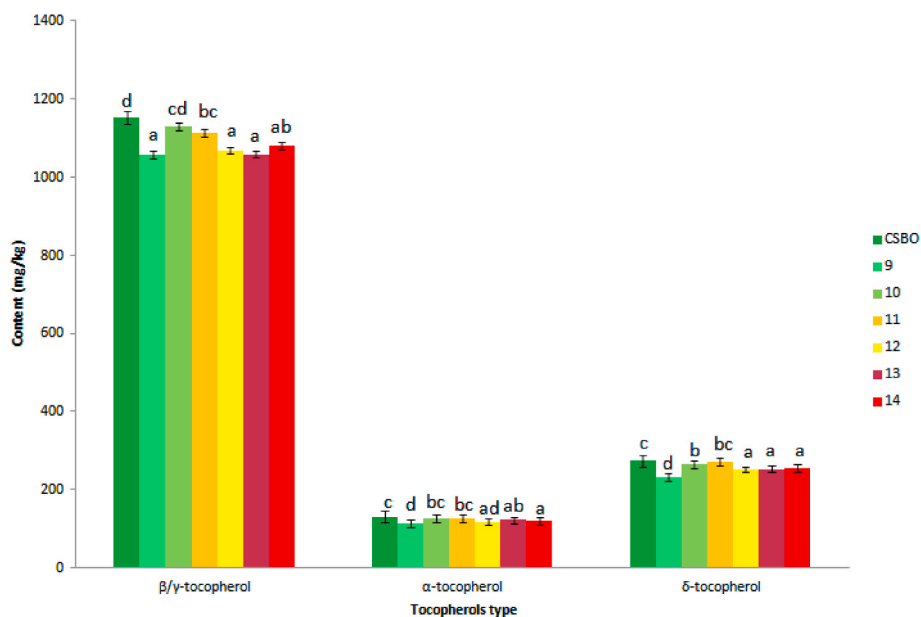


Fig. 3. Tocopherol profile for different soybean oil samples: CSBO (crude soybean oil), trials 9, 10, 11, 12, 13 and 14. Different letters in the same tocopherols type indicate significant differences ($p < 0.05$).

process on the tocopherol profiles obtained for different soybean oil samples are shown in Fig. 3. The concentrations of all tocopherol homologues were significantly different ($p < 0.05$) from those in crude soybean oil due to the process conditions. In trials 9 and 10, increasing the enzyme concentration from 30 to 200 mg/kg retained 6.3%, 10.9%, and 11.4% of β/γ -tocopherol, α -tocopherol, and δ -tocopherol species, respectively, owing to the stronger enzyme action and lower degree of oil entrainment. A comparison of trials 11 and 12 revealed that increasing the reaction temperature from 50 to 70 °C reduced the β/γ -tocopherol, α -tocopherol, and δ -tocopherol concentrations by 4.0%, 6.0%, and 6.7%, respectively, which was likely caused by the hydrolysis and/or oxidation reactions. In trials 13 and 14, when the water concentration increased from 1% to 5%, the concentration of all tocopherol species varied between 1.2% and 2.1%. Therefore, the amount of water slightly affected the tocopherol concentration.

4. Conclusion

The enzyme concentration, temperature, and water content strongly influenced the FFA and DAG contents, and the phosphorus content significantly decreased after degumming. By optimizing the process conditions, it was possible to maximize the FFA and DAG concentrations and minimize the phosphorus content in the degummed oil ($P < 10$ mg/kg). In addition, the enzymatic degumming process conducted at higher enzyme (200 mg/kg) and water (5%) concentrations and low temperature of 50 °C resulted in tocopherol concentrations that significantly differed from those in crude soybean oil ($p < 0.05$), indicating a high degree of tocopherol retention. Therefore, the application of the Purifine® 3G cocktail simultaneously increased the FFA and DAG contents and decreased the phosphorus content in soybean oil. In addition, the degumming alternative preserved the original tocopherol content in the oil, indicating its potential applicability for the oil industry. The combination enzymatic degumming with enzymatic transesterification using soft processes and non-refined inexpensive vegetable oils would be a promising way for biodiesel production, for this approach may noticeably reduce the reaction time (less steps), energy consumption (low temperature) and increase productivity (DAG and FFA increase), which represents an important topic for future studies.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRedit authorship contribution statement

Rafaela Menezes dos Passos: Writing – original draft, Conceptualization, Methodology, and, Formal analysis. **Rúbia Mariana da Silva:** Methodology, and, Formal analysis. **Paula Virginia de Almeida Pontes:** Methodology, and, Formal analysis. **Marcelo Antônio Morgano:** Methodology, and, Formal analysis. **Antônio J.A. Meirelles:** Supervision. **Christian V. Stevens:** Writing – review & editing. **Marcela Cravo Ferreira:** Writing – review & editing. **Klicia Araujo Sampaio:** Supervision, Conceptualization, Writing – review & editing, and, Funding acquisition.

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