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ORIGINAL PAPER

Optimization of Enzymatic Hydrolysis of Sacha Inchi Oil using Conventional and Supercritical Carbon Dioxide Processes

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Abstract Sacha inchi (*Plukenetia volubilis*) oil has high polyunsaturated fatty acids content. The hydrolysis of this oil is an efficient way to obtain desirable free fatty acids (FFA). The optimization of parameters was carried out according to the maximum production of FFA using two enzymatic hydrolysis processes. The effect of enzyme concentration (5–40 % based on weight of oil), temperature (40–60 °C), and oil:water molar ratio (1:5–1:70) were studied for the conventional enzymatic hydrolysis process, while pressure (10–30 MPa) and oil:water molar ratio (1:5–1:30) were studied for the enzymatic hydrolysis in supercritical carbon dioxide (SC-CO_2) media. The hydrolysis in SC-CO_2 media resulted in higher production of FFA (77.98 % w/w) at 30 MPa and an oil:water molar ratio equal to 1:5 compared to the conventional process (68.40 ± 0.98 % w/w) at 60 °C, oil:water molar ratio equal to 1:70, and 26.17 % w/w, enzyme/oil. The only significant parameter on the production of FFA for conventional enzymatic hydrolysis was enzyme concentration, while for the hydrolysis in SC-CO_2 media both pressure and the molar ratio of oil:water were significant. Lipid class analyses showed that with both methods, FFA, monoglycerides, and diglycerides content in the final product increased compared to pure oil, while triglycerides content decreased. Fatty acid composition analysis showed that the content of fatty acids in the FFA form were similar to their triglyceride form.

Keywords Sacha inchi oil · Hydrolysis · Supercritical CO_2 · Lipozyme TL IM

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Introduction

Sacha inchi (*Plukenetia volubilis*) is an oilseed found in the Peruvian Amazon and various studies have been conducted to characterize and process its oil since 1992 [1–7]. The oil is obtained using different methods of extraction, including Soxhlet, cold pressing and supercritical carbon dioxide (SC-CO_2) [2, 4, 5]. The presence of unsaturated fatty acids in the oil makes it an excellent source of omega-3 and omega-6 essential fatty acids (linoleic and α-linolenic acids) and consequently a potential source to promote health benefits [8]. The studies performed to characterize the oil include physico-chemical properties, chemical composition, thermal properties, toxicological effects and phase behavior in SC-CO_2 [2–7].

Complete hydrolysis of vegetable oil is a reaction which consists of breaking the ester bond to produce valuable free fatty acids (FFA) and glycerol in the presence of water, while partial hydrolysis also provides monoglycerides (MG) and diglycerides (DG) [9]. Several applications are available for the reaction products. FFA is used for industrial production of soaps, detergents and high value-added products for the food industry [10], while MG and DG are used for emulsification purposes [11]. The use of enzymes in hydrolysis reactions has received considerable attention because of its advantages compared to the other two major types of hydrolysis (high pressure steam splitting and alkaline hydrolysis), such as low energy cost and preservation of polyunsaturated fatty acids [12]. Nevertheless, the main advantage of lipases is their specificity (positional, substrate or stereospecificity) [13]. In addition, immobilized enzymes are preferred for hydrolysis reactions rather than enzymes in solution form because they are easily separated from the product [12]. Immobilized enzymes can be also used several times [14]. They are

more thermally and chemically stable, and offer better control of the process and product quality [14, 15].

Conventional enzymatic hydrolysis is very simple to perform and it has been reported to hydrolyze oils, such as soybean oil [15, 16], black currant oil [17], castor oil [18], palm oil [19] and buriti oil [20]. The reaction consists of mixing the enzyme, oil and water under constant agitation using a mechanical stirrer [15, 18, 19] or a shaking water bath [10, 16, 17]. Then, the hydrolyzed oil is easily separated from the water and the enzyme by centrifugation [15, 17], extraction with a solvent [16, 17] or filtration [19]. For a good performance of immobilized enzymes during the reaction, some parameters should be controlled, such as pH, temperature, water content and substrate composition because each enzyme has its optimum value for each parameter in which they are more active [13]. In general, previous studies showed that the most important parameter for conventional hydrolysis is enzyme quantity. The optimum enzyme concentration depends on each system [10, 16, 17]. However, for some systems, temperature and water quantity also had an important effect on the degree of hydrolysis [10, 15, 18].

The use of a supercritical fluid like SC-CO_2 in enzymatic reactions has various advantages. The main advantage is that small changes in pressure and temperature result in different physical properties. In addition, SC-CO_2 has high diffusivity and low viscosity, and is easily removed from the final product [21–24]. Furthermore, the critical conditions of CO_2 (7.38 MPa and 31.1 °C) are in the range of optimal conditions to preserve enzyme activity [21–24]. Two excellent reviews report the extensive use of SC-CO_2 in enzymatic reactions, such as hydrolysis, ethanolysis, transesterification, esterification, acetylation, acylation, polymerization and *trans*-hydroxylation [21, 23]. However, few studies are reported in the hydrolysis of vegetable oils using enzymes in SC-CO_2 media, including parameters optimization of canola oil [11, 25, 26], blackcurrant oil [27, 28], sunflower oil [29], soy deodorized distillate [30] and conjugated linoleic acid-enriched anhydrous milk fat (CLA-enriched AMF) [31]. In general, these hydrolysis studies in SC-CO_2 media showed that pressure and water content are the parameters which most influence the degree of hydrolysis [11, 25, 26, 30], while the temperature effect is controversial. For example, the temperature showed no effect on the hydrolysis of canola oil, blackcurrant oil and CLA-enriched AMF, while temperature had a significant effect for triglycerides of a soy deodorized distillate.

According to Pereira and Meireles [32], in order to optimize a process two questions should be answered. First, identify the best operational conditions, and second, find out if the process results in the lowest cost of manufacturing (COM) of the desired product. In the past, supercritical fluid extraction was associated with high investments. But, recently estimation of the cost of manufacturing of several extracts using SC-CO_2 has shown to be a competitive alternative compared to other conventional extraction techniques [32]. Whereas the COM of supercritical fluid extraction has been reported for various extracts, no economic study of enzymatic hydrolysis reaction using a conventional process and using supercritical CO_2 media has been reported. A methodology to determine the COM of enzymatic reactions would be interesting to select the best process from an industrial perspective.

Response surface methodology (RSM) is a statistical approach which can be used to optimize parameters in a process with the advantage of reducing the number of experiments, chemicals, and time. The objective of this study was to optimize process parameters by RSM for the enzymatic hydrolysis of sacha inchi oil using the conventional and the SC-CO_2 processes. The parameters evaluated for the conventional process were temperature, enzyme concentration and oil:water molar ratio, while for the supercritical process were pressure and oil:water molar ratio. Lipid class (FFA, MG, DG, and TG) content in the final products was quantified by gas chromatography (GC) under optimized conditions for both methods. FFA obtained can be used for omega-3 and omega-6 enrichment of foods or the production of structural lipids.

Materials and Methods

Materials

Sacha inchi oil was purchased from Herbarella (Toronto, ON, Canada), while Lipozyme TL IM (lipase immobilized from *Thermomyces lanuginosus*) was kindly provided by Novozymes North America Inc. (Franklinton, NC, USA). The lipid standards were obtained from Nu-Chek Prep. Inc. (Elysian, MN, USA). Phenolphthalein, pyridine (≥99 %), benzene (ACS reagent, ≥99.0 %), petroleum ether (ACS reagent), trimethylsilyl (TMS)-diazomethane (2 M in hexane) and *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1 % trimethylchlorosilane (TMCS) solution were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Chloroform (HPLC grade-99.9 %), sodium hydroxide 0.1 N standard solution, and methanol (ACS reagent) were acquired from Fischer Scientific Ltd. (Nepean, ON, Canada). Hydrogen and nitrogen ultra-high purity (99.999 %), and carbon dioxide (99.8 % bone dry, water level <3 ppm) were purchased from Praxair Canada Inc. (Mississauga, ON, Canada).

Conventional Enzymatic Hydrolysis

The maximum amount of FFA produced was determined using RSM and a central composite face-centered (CCF) design. The effects of temperature (40–60 °C), enzyme concentration (5–40 % based on weight of oil), and oil:water ratio (1:5–1:70 mol/mol) were evaluated. Each parameter consisted of three levels. The experimental design, predicted and experimental values are shown in Table 1. The central point was carried out in triplicate. The polynomial equation was obtained by the adjustment of experimental values to a second order polynomial equation. Analysis of variance (ANOVA), polynomial equation, coefficient of determination, surface plot, and process conditions for maximum hydrolysis were obtained using Design-Expert 7.0 software (Stat-Ease Inc., Minneapolis, MN, USA).

Sacha inchi oil (10 g), water, and enzyme (Lipozyme TL IM—1,3 specific lipase) were mixed in a 250-mL Erlenmeyer flask. The temperature, amount of water and enzyme were changed according to the experimental design (Table 1). Nitrogen was flushed in the flask, which was closed immediately with parafilm. Nitrogen was flushed to avoid the presence of oxygen in the system, because this oil has a high content of unsaturated fatty acids and oxygen could oxidize the oil during the reaction time. A tape was used around the parafilm to hold nitrogen inside the system. Then, the flask was placed in a shaker water bath at 150 rpm for 6 h. After the hydrolysis reaction, the solution (oil, water, and enzyme) was centrifuged at 5500 rpm for 15 min. Then, the oil was recovered using a Pasteur pipette. The hydrolyzed oil was analyzed by titration, immediately after the reaction for the determination of %FFA. The samples were kept at −18 °C for further lipid class analysis.

Table 1 Face centered design with three process variables and experimental and predicted values for conventional enzymatic hydrolysis of sacha inchi oil

Run	Process variable			$\%FFA_{Exp}$ (w/w)	$\%FFA_{Pred}$ (w/w)
	x_1	x_2	x_3		
1	5	5	40	14.22	22.15
2*	22.5	37.5	50	59.02	55.12
3	22.5	5	50	63.55	59.71
4	5	5	60	17.84	20.03
5	22.5	37.5	40	51.75	54.12
6	5	37.5	50	42.76	24.60
7	40	37.5	50	33.19	47.85
8	22.5	70	50	66.75	67.08
9	40	5	40	66.58	58.51
10	22.5	37.5	60	62.54	56.67
11	40	5	60	61.90	63.68
12	5	70	40	46.16	45.25
13	40	70	40	49.41	48.09
14	40	70	60	62.37	55.32
15*	22.5	37.5	50	49.82	55.12
16	5	70	60	36.24	45.19
17*	22.5	37.5	50	49.51	55.12

Exp experimental, *Pred* predicted

* Central point 52.78 ± 4.41 %

x_1 enzyme concentration (% based on weight of oil), x_2 oil:water molar ratio, and x_3 temperature (°C)

Enzymatic Hydrolysis in SC-CO_2 Media

Similar to the conventional process, the RSM and the CCF design were used to maximize the production of FFA using Lipozyme TL IM (1,3-specific lipase) in SC-CO_2 media. The variables studied were pressure (10–30 MPa) and oil:water molar ratio (1:5–1:30). Enzyme load, temperature and CO_2 flow rate were kept constant as previous studies showed that temperature and CO_2 flow rate are the parameters with less effect on enzymatic hydrolysis of vegetable oils in SC-CO_2 media [11, 25–27, 31]. Although the degree of hydrolysis of canola oil was high with the increase of enzyme, it was not proportional [25]. For example, after 4 h of the on-line extraction-reaction, the molar fraction of FFA increased from ∼0.70 to ∼0.75 by increasing the enzyme load from 1 g to 5 g [25]. The experimental design, predicted and experimental values are shown in Table 2. The central point was carried out in triplicate.

A laboratory scale SC-CO_2 reaction unit (Fig. 1) described earlier in detail by Prado et al. [31] was used for enzymatic reactions. However, in this study, the tubing and

Table 2 Face centered design with two process variables and experimental and predicted values for enzymatic hydrolysis of sacha inchi oil in SC-CO_2 media

Run	Process variable		$\%FFA_{Exp}$	$\%FFA_{Pred}$
	x_1	x_2		
1	10	1:5	47.16	43.71
2	30	1:5	77.98	78.34
3	10	1:30	21.87	19.73
4	30	1:30	55.83	57.51
5	10	1:17.5	34.64	40.23
6*	20	1:17.5	62.43	67.74
7	20	1:5	67.34	70.43
8	20	1:30	47.56	48.02
9*	20	1:17.5	72.26	67.74
10	30	1:17.5	78.48	76.44
11*	20	1:17.5	72.07	67.74

Exp experimental, *Pred* predicted

* Central point 68.92 ± 4.59 %

x_1 pressure (MPa) and x_2 oil:water molar ratio

head pump installed before the oven were not heated because sacha inchi oil is liquid at ambient temperature. First, sacha inchi oil and water were mixed and the mixture was kept under continuous agitation on a plate magnetic stirrer. The mixture was pumped into the system using a piston pump (Gilson 305, Middleton, CT, USA) at a constant flow rate of 0.03 mL/min. Sacha inchi oil and water were mixed with SC-CO_2 before entering the reactor cell to ensure uniformity of the mixture. The reactor cell was filled with fresh immobilized enzyme and the remaining space was filled with stainless steel beads and glass wool. The pressure was controlled by a back pressure regulator (Tescom, Elk River, MN, USA) while the CO_2 flow rate was manually controlled with a micrometering valve (Autoclave Engineers, Erie, PA, USA). The outlet valves were heated at 90 °C to avoid the Joule–Thompson cooling effect. The samples were collected into a jacketed glass cold trap. The top layer of samples (recovered after phase separation at ambient conditions) was immediately analyzed for %FFA and then kept at −18 °C for further analysis.

The pressure and oil:water ratio were changed according to the experimental design shown in Table 2. Lipozyme TL IM was the enzyme utilized for these reactions. The parameters time (2 h), temperature (50 °C), enzyme load (1 g) and CO_2 flow rate (0.5 L/min measured at ambient conditions) were kept constant for all experiments. This temperature was selected because it is within the range of optimum temperature for enzyme activity. In addition, this temperature is the lowest for optimum enzyme activity, which provides less energy cost to the system.

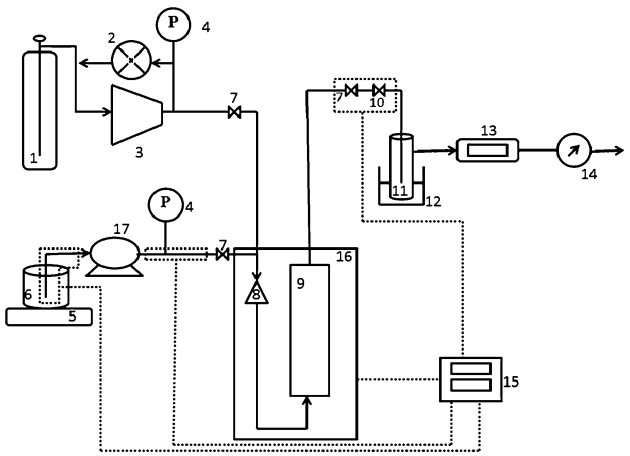

Fig. 1 SC-CO_2 reaction system. *1* CO_2 tank, *2* back pressure regulator, *3* compressor, *4* pressure gauge, *5* hot plate stirrer, *6* mixture of sacha inchi oil and water, *7* on/off valve, *8* mixer, *9* reaction cell, *10* micrometering valve, *11* sample collection tube, *12* cold water bath, *13* flow meter, *14* vent, *15* temperature controller, *16* oven, *17* pump

Analysis

Determination of the Acid Value

The procedure for determination of the acid value is described by Leitgeb and Knez [33]. The analysis was performed immediately after each reaction and the result (%FFA, weight of free fatty acids/weight of oil (w/w)) is expressed in terms of α-linolenic acid that is the most abundant fatty acid of sacha inchi oil.

Lipid Class Content

Lipid class content (TG, MG, DG, and FFA) was carried out using the silylation methodology described by Verleyen et al. [34] with some modifications previously reported by Prado et al. [31]. Standards of FFA, DG, MG and TG to calculate response factors and retention times were lauric acid, mono-, di- and tri-laurin; palmitic acid, mono-, di- and tri-palmitin, and arachidic acid, mono-, di- and tri-arachidin. The response factor for FFA, MG, and DG was 1.0 and for TG was 1.25 while retention times were 1.5–12.75, 12.75–19.00, 19.00–22.40, and 22.40–30.00 min for FFA, MG, DG, and TG, respectively.

Hydrolyzed sacha inchi oil (10 mg) was weighed in a screw-capped test tube and 0.5 mL of C17:0 in monoglyceride form diluted in pyridine with a known concentration (1.0 mg/mL) was added as an internal standard. Then, 100 µL of derivatizing and silylating agent (BSTFA containing 1 % TMCS solution) was added. Silylation was completed by placing the tube in an oven at 70 °C for 20 min. Finally, 2.0 mL of chloroform was added to the test tube and 1.0 mL of the solution was transferred to a GC vial. The GC instrument (Varian 3400, Palo Alto, CA, USA) was equipped with a cool on-column injection, a flame-ionization detector, an autosampler, and a 10 m in length × 0.32 mm ID stainless steel capillary column (MXT®-Biodiesel TG line, Restek Corporation, Belfonte, PA). The samples (1 µL) were injected using a hydrogen flow rate under head pressure equal to 48 kPa. The detector temperature was 340 °C. The injected samples were heated at 50 °C and held for 1 min, and then heated to 180 °C at 15 °C/min, increased 7 °C/min to 230 °C, and finally heated to 340 °C at 30 °C/min and held for 5 min [35]. The spectra were analyzed with the Galaxie software version V1.19.

Fatty Acid Composition

Starting Material The procedure for methylation of triglycerides was carried out according to the supplier [36]. Non-hydrolyzed sacha inchi oil (50 mg) was diluted in 0.5 mL of chloroform, where 50 µL of this solution was

transferred in a screw-capped test tube. Then, sodium methoxide 0.5 M was added (2 mL). The tube was heated at 80 °C for 20 min. Afterwards, water (2 mL) and hexane (2 mL) were added and anhydrous sodium sulfate was used to remove water from the top layer [36]. Finally, the solution was diluted in hexane to a concentration of 0.3 mg/mL for injection into the GC (Varian 3400, Palo Alto, CA, USA). Then, samples (1 μL) were injected into a fused-silica capillary column (SP-2560, 100 m length × 0.25 mm ID, Supelco Inc., Belfonte, PA, USA) with cool on-column injection. Hydrogen was used as the carrier gas at a flow rate of ~1 mL/min. The injector and detector temperatures were both 230 °C. The injection temperature at 70 °C was increased to 230 °C at 150 °C/min [37]. The chromatograms were analyzed with Galaxie software version V1.19 and the peaks were identified by comparison with a GC fatty acid methyl esters (FAME) standard (463-Nu Check Prep., Inc., Elysian, MN, USA).

Hydrolyzed Products Methylation of FFA in hydrolyzed sacha inchi oil was carried out according to the procedure described by Yurawecz et al. [38]. First, 20 mg of hydrolyzed sacha inchi oil was weighed in a screw-capped test tube. Then, a solution containing 20 % of methanol in benzene was added to the tube (1 mL) followed by the addition of 0.5 mL of TMS-diazomethane 2 M in hexane. TMS-diazomethane methylates only the FFA. The tubes were kept at room temperature with gentle shaking for 30 min. The excess of TMS-diazomethane was removed by adding ten drops of acetic acid. Afterwards, petroleum ether (5 mL) and water (5 mL) were added to each tube and anhydrous sodium sulfate was also used to remove the water from the top layer. Finally, the petroleum ether layer was diluted with hexane to a concentration of 0.3 mg/mL for GC injection. The GC equipment was described in the previous section.

Results and Discussion

Conventional Enzymatic Hydrolysis

The experimental values were correlated using a second order polynomial equation. The ANOVA presented in Table 3 shows that the model terms which have significance are x_1 and x_1^2 ($p < 0.05$), where x_1 is the enzyme concentration (percentage based on weight of oil). The terms with no significance (x_2 is oil:water molar ratio, and x_3 is the temperature) were removed from the model.

$$\%\text{FFA (w/w)} = 55.12 + 11.62x_1 - 18.90x_1^2$$

In Table 3, the p value of lack of fit for the model is $p = 0.1478$, which means that the model is good at predicting the %FFA for conventional enzymatic hydrolysis of sacha inchi oil. However, the model is not well fitted as the value of coefficient of determination ($R^2 = 0.7709$) is low. Therefore, the model cannot explain all behaviors during the hydrolysis process.

The maximum %FFA (69.38 %) for the conventional enzymatic hydrolysis of sacha inchi oil in the range evaluated was determined by the predicted equation at 60 °C, oil:water molar ratio equal to 1:70, and 26.17 % of enzyme (based on weight of oil). The experimental mean and standard deviation values for this condition were 68.40 ± 0.98 %, indicating a good prediction by the model.

Effect of Enzyme Concentration

According to ANOVA, the enzyme concentration is the only statistically significant parameter, which influences the hydrolysis of sacha inchi oil. The optimum enzyme concentration (26.17 % weight of oil) obtained was similar to the one reported with conventional enzymatic hydrolysis of soybean oil (25 % weight of oil) [16] which was also the parameter with the highest effect on the conventional enzymatic hydrolysis of soybean oil. Other oil which enzyme quantity was the parameter that most impacted the production of FFA was buriti oil (crude and refined). The optimized conditions were obtained at 31 °C, 21.6 U of lipolytic activity and oil:water ratio of 2.33 for crude buriti oil (64.8 % FFA), while for the refined oil (75.8 % FFA) was obtained at 45 °C, 31.2 U, and oil:water ratio of 1.80 [20].

Figure 2a shows the effect of enzyme concentration and the oil:water ratio at 60 °C, while Fig. 2b shows the effect of enzyme concentration and temperature at an oil:water molar ratio of 1:70. It can be observed that when a small amount of enzyme was used (Fig. 2a), the %FFA increased with high amounts of water. However, with large amounts of enzyme, the %FFA is practically constant with the increase of water at 60 °C. Figure 2b shows that although the production of FFA is favorable with high amounts of enzyme at 60 °C, it is practically constant after 22.5 % enzyme concentration, with an optimum concentration equal to 26.17 % (weight of oil). Hydrolysis of black currant seed oil also showed similar results where the degree of hydrolysis increased from 88.6 to 99.0 % when the enzyme concentration was increased from 20 to 100 mg/g oil [17]. However, the degree of hydrolysis remained constant when high amounts of enzyme were used. According to Wille and Wang [17], it might be because the reaction occurs in the interface between water and oil. Thus, with high enzyme concentrations, a smaller available interface between the oil and water is provided [17].

The optimum enzyme concentration (26.17 % weight of oil) in this conventional hydrolysis process is very high, which could potentially increase the COM.

Table 3 Analysis of variance (ANOVA) of response surface model to predict the %FFA for conventional enzymatic hydrolysis of sacha inchi oil

Factor	Sum of squares	Degrees of freedom	Mean square	F ratio	*p value*
Regression	3171.20	9	352.36	2.62	0.1091
x_1	1350.94	1	1350.94		0.0158
x_2	135.72	1	135.72		0.3488
x_3	16.31	1	16.31		0.7381
x_1x_2	561.80	1	561.80		0.0804
x_1x_3	26.57	1	26.57		0.6703
x_2x_3	2.10	1	2.10		0.9041
x_1^2	956.58	1	956.58		0.0322
x_2^2	183.67	1	183.67		0.2811
x_3^2	0.20	1	0.20		0.9702
Residual	942.53	7	134.65		
Lack of fit	884.14	5	176.83	6.06	0.1478
Pure error	58.39	2	29.20		
Total	4113.73	16			

x_1 enzyme concentration (% based on weight of oil), x_2 oil:water molar ratio, and x_3 temperature (°C)

Effect of Temperature

Temperature did not show any significant effect on the conventional enzymatic hydrolysis of sacha inchi oil. The same result was verified on the conventional hydrolysis of black currant seed oil, where changes in temperature from 20 to 40 °C provided small variations of the hydrolysis (2–5 %) [17]. The effect of temperature can be observed in Figs. 2b and c. Figure 2b shows that %FFA increases with the increase in temperature for high enzyme concentrations. In addition, in Fig. 2c, the increase in temperature from 40 to 60 °C results in a slight increase in %FFA by maintaining the water quantity constant. The optimum temperature for maximum degree of hydrolysis was 60 °C, which was expected as it is within the range of optimum Lipozyme TL IM activity (50–70 °C) [39].

Effect of Water

In this study, water content had no significant effect on the conventional enzymatic hydrolysis of sacha inchi oil. In general, the degree of hydrolysis tended to increase with the increase in water, achieving its optimum at a molar ratio of oil:water equal to 1:70. The opposite effect was found in the hydrolysis of soybean oil, where the degree of hydrolysis increased with a decrease of water content [15, 16]. According to Valivety et al. [40], the optimum amount of water should be determined for each specific system, as the enzyme activity depends on water distribution among all components. In addition, lipase also promotes esterification simultaneously when a small amount of water is provided. Thus, the optimization of water content should be studied to find the water quantity necessary to achieve equilibrium in a hydrolysis process [41].

Enzymatic Hydrolysis in SC-CO_2 Media

The experimental values were adjusted to a second order polynomial equation, where x_1 corresponds to pressure (MPa) and x_2 corresponds to the ratio of oil:water (mol/mol).

$$\%\text{FFA (w/w)} = 67.74 + 18.10x_1 - 11.20x_2 - 9.40x_1^2 - 8.51x_2^2$$

Pressure ($p < 0.001$) and water quantity ($p < 0.01$) are significant parameters for the production of FFA using enzymatic hydrolysis of sacha inchi oil in SC-CO_2 media. These results are verified in Table 4 with the ANOVA analysis. Pressure has a positive effect, while the increase in water content has a negative effect. However, the interaction between these two parameters do not have influence on the response ($p > 0.05$). Thus, this term was removed from the model. In addition, Table 4 shows that the p value of lack of fit ($p = 0.6236$) and the value of coefficient of determination ($R^2 = 0.9613$) indicate a very good fit of the model. The comparison of predicted and experimental values also indicated that the model was well fitted, as the relationship was linear.

The model was able to predict the condition to achieve maximum production of FFA (79.67 % w/w) at 29.4 MPa and molar ratio of oil:water equal to 1:9.8. The experimental value of this condition was 74.90 ± 1.27 % (w/w), which was in agreement with the predicted value.

Effect of Water and Pressure

The effect of water and pressure is shown in Fig. 3. The pressure has a positive effect on the production of FFA, as the amount of FFA increased with the increase in pressure. In contrast, the increase in water leads to a decrease in the amount of FFA. As stated by Habulin et al. [23], the production of FFA tends to increase with high pressures as the oil is more soluble with the increase in CO_2 density (achieved at high pressures). The maximum hydrolysis of canola oil (97 % of TG conversion using Lipozyme RM IM at 24 MPa, 35 °C, 0.5 L/min CO_2 flow rate, and 0.002 mL/min water flow rate) and CLA-enriched AMF (86.79 % w/w of FFA using Lipozyme TL IM at 23 MPa, 1:5 fat:water molar ratio and 55 °C) were also achieved with small amounts of water [26, 31]. Water is essential for enzyme activity as it influences the enzyme structure via non-covalent binding and disruption of hydrogen bonds [23]. Water also influences reagent diffusion that could influence the reaction

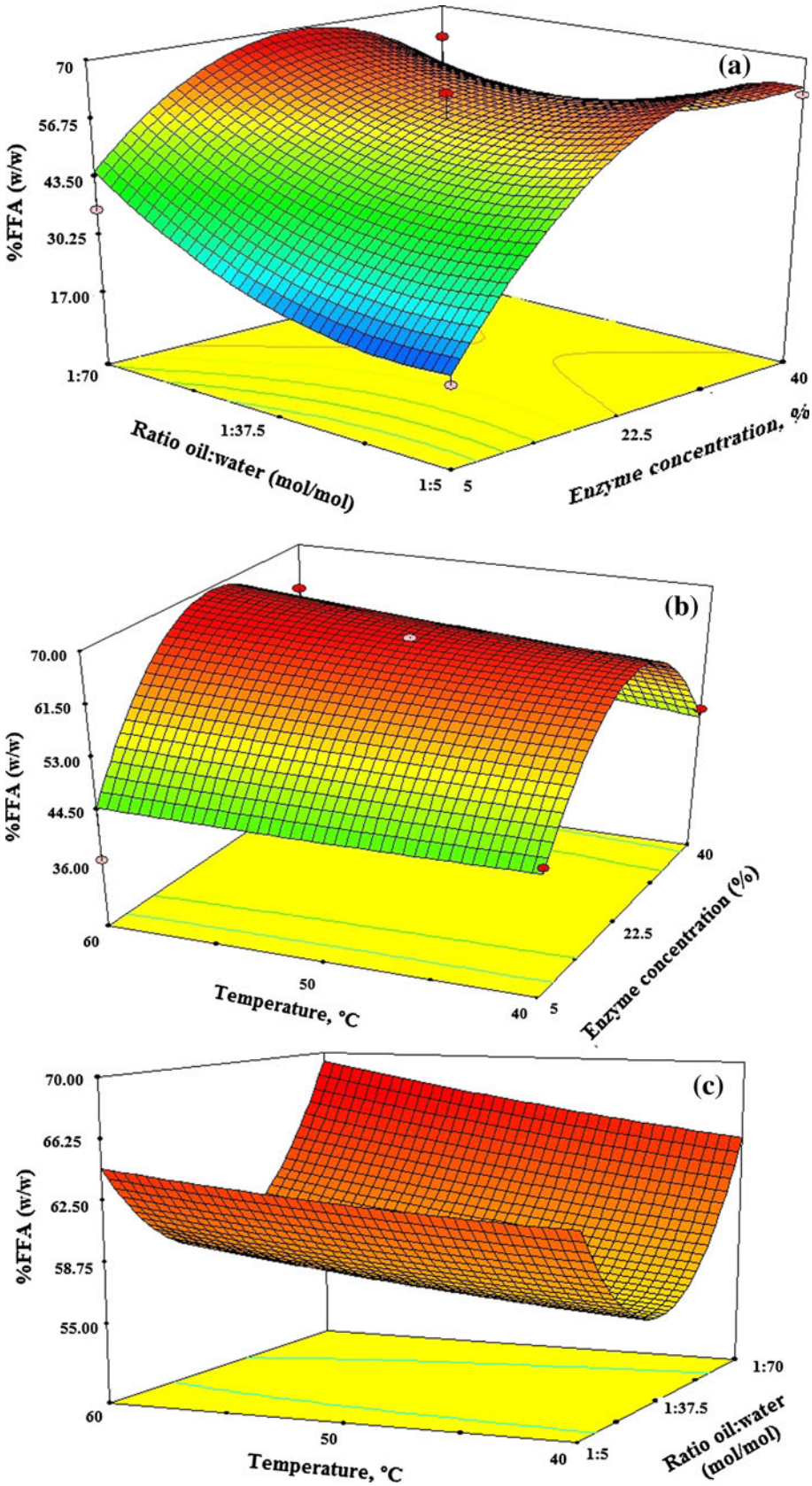

Fig. 2 Surface plots of parameters optimization of conventional enzymatic hydrolysis of sacha inchi oil: **a** Effect of the oil:water molar ratio and enzyme concentration at 60 °C, **b** Effect of temperature and enzyme concentration at an oil:water molar ratio of 1:70, and **c** Effect of temperature and oil:water molar ratio at 26.17 % enzyme concentration (weight of oil)

Table 4 Analysis of variance (ANOVA) of response surface model to predict the %FFA for enzymatic hydrolysis of sacha inchi oil in SC-CO_2 media

Factor	Sum of squares	Degrees of freedom	Mean square	F ratio	*p value*
Regression	3277.04	5	655.41	24.82	0.0015
x_1	1966.38	1	1966.38		0.0003
x_2	753.09	1	753.09		0.0031
x_1x_2	2.46	1	2.46		0.7723
x_1^2	223.95	1	223.95		0.0333
x_2^2	183.56	1	183.56		0.0462
Residual	132.02	5	26.41		
Lack of fit	68.82	3	22.94	0.73	0.6236
Pure error	63.20	2	31.60		
Total	3409.07	10			

x_1 pressure (MPa) and x_2 oil:water molar ratio

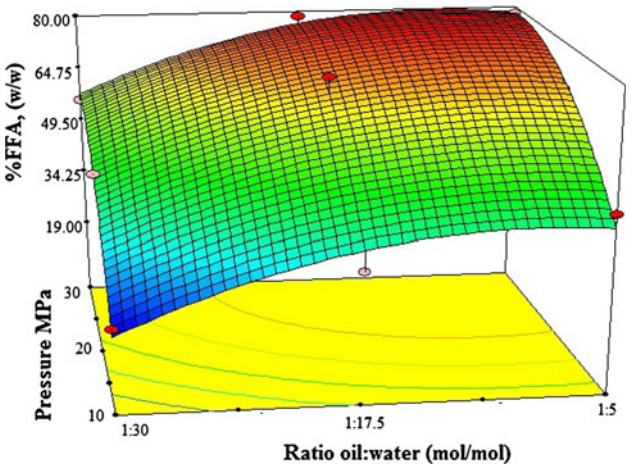

Fig. 3 Effect of the oil:water molar ratio and pressure at 50 °C and 0.8 L/min CO_2 flow rate for enzymatic hydrolysis of sacha inchi oil in SC-CO_2 media

equilibrium [23]. However, the increase in water creates a barrier, which might limit the oil access to the active sites of the enzyme [22]. The excess of water for hydrolysis of sacha inchi oil in SC-CO_2 media had a negative effect, by creating a barrier around the enzyme and limiting the oil access to the active sites of the enzyme. By changing the molar ratio of oil:water between 1:5 and 1:17.5, the FFA production was similar in the range of pressure studied (between 20 and 30 MPa). The condition predicted by the model for maximum production of FFA was at 29.4 MPa/oil:water molar ratio of 1:9.8. The predicted value was 79.67 % (w/w) and the experimental value was 74.90 ± 1.27 % (w/w). However, the conditions at 30 MPa/oil:water molar ratio of 1:5 and 30 at MPa/oil:water molar ratio of 1:17.5 were very similar to the predicted values: 77.98 and 78.48 % (w/w), respectively. Thus, the condition for maximum enzymatic hydrolysis of sacha inchi oil in SC-CO_2 media might be considered to be 30 MPa and an oil:water molar ratio of 1:5 (77.98 % w/w), as the use of water is reduced, leading to a decrease in the COM with a similar result.

Enzymatic hydrolysis of canola oil in SC-CO_2 provided a low conversion of TG (63–67 %) [11]. The authors suggest that this result is attributed to the concentration of substrates in SC-CO_2. Because the concentration of substrates was below their saturation limit in SC-CO_2, it could have caused the reaction rate to drop accordingly. In addition, SC-CO_2 excess may dry off the water around the enzyme, causing inactivation of the enzyme [11]. In this study, the amount of sacha inchi oil and water introduced to the system were 29.39 mg/g CO_2 and 3.05 mg/g CO_2, respectively. The solubility of water at 30 MPa and 50 °C (the same of this study) is reported as ~ 3.41 mg/g CO_2 [42], while the solubility of sacha inchi oil at 30 MPa and 50 °C (58.26 mg/g CO_2) was calculated using the same model and the same parameters provided by Prado et al. [6]. These results are consistent with the observation of Rezaei and Temelli [11] that substrates close to their saturation limit are desired. In this study, the %FFA was quite low (77.98 % w/w—which corresponds to a conversion of TG equal to 73.96 %) as the concentrations of substrates were below their saturation limit. In contrast, when the amount of substrates introduced into to the system were both above their saturation limit on the enzymatic hydrolysis of CLA-enriched AMF in SC-CO_2, the %FFA achieved was higher (86.79 ± 7.28 % w/w), but it was still less than 90 % [31]. Although this result suggests that the %FFA was higher when the concentration of substrates was above their saturation limit, further detailed evaluation should be done to verify whether the hydrolysis of sacha inchi oil would increase by providing substrates quantity close to their saturation limit. However, this hypothesis cannot be evaluated as an isolated factor. Heterogeneity of the substrates (sacha inchi oil, canola oil, and anhydrous milk fat) should also be considered, as many lipases are specific to fatty acids substrates [13]. For example, some lipases are long-chain specific and others are medium or short-chain specific [13]. In the same study of enzymatic hydrolysis of CLA-enriched AMF in SC-CO_2, phase behavior prediction of the system showed that it was composed of the solid enzyme, liquid phase (fat, water and CO_2), SC-CO_2, and the distribution of reactants and products between the phases. The presence of a liquid phase was suggested because the introduction of fat and oil was above their saturation limits. In contrast, in this study, the phase behavior prediction indicates that the presence of a liquid phase does not exist, as the amount of substrates introduced were below their saturation limit. However, the phase behavior prediction of these systems was estimated

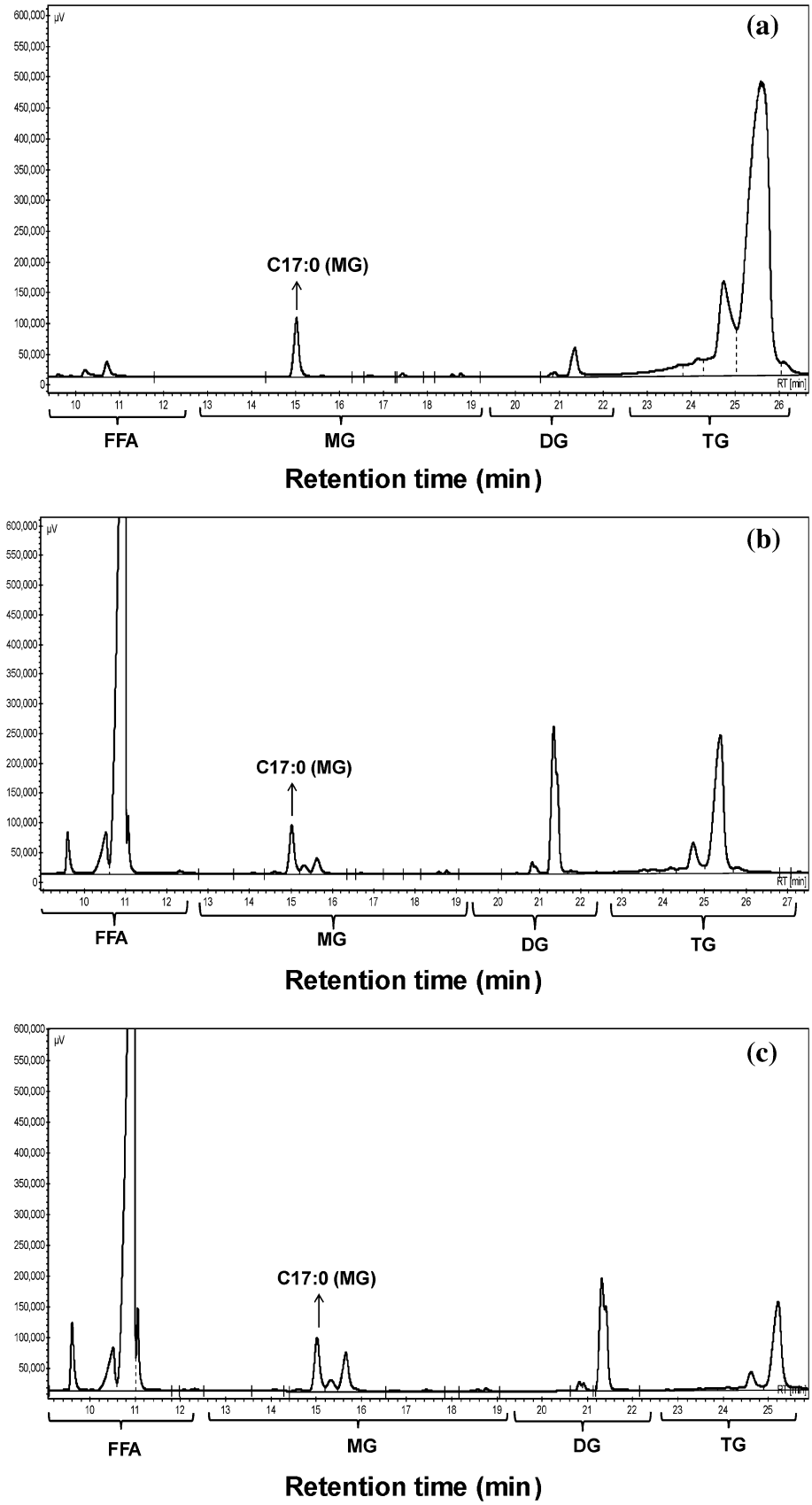

Fig. 4 Chromatograms of: **a** pure sacha inchi oil, **b** conventional hydrolyzed sacha inchi oil at 60 °C, an oil:water ratio of 1:70 (mol/mol), and 26.17 % enzyme (weight of oil), and **c** hydrolyzed sacha inchi oil in SC-CO_2 media at 30 MPa, an oil:water ratio of 1:5 (mol/mol), 0.5 L/min CO_2 flow rate, and 1 g of enzyme

Table 5 Lipid class content of pure and hydrolyzed sacha inchi oil

	%FFA (w/w)	%MG (w/w)	%DG (w/w)	%TG (w/w)
Pure oil	1.27	0.44	1.98	96.07
Conventional hydrolyzed oil[a]	66.22[c]	2.44[c]	12.60[c]	21.67[c]
Hydrolyzed oil in SC-CO_2 media[b]	71.01[d]	5.00[d]	10.98[c]	14.01[d]

[a] 60 °C, ratio of oil:water 1:70 (mol/mol), and 26.17 % of enzyme (weight of oil)

[b] 30 MPa, ratio of oil:water 1:5 (mol/mol), 50 °C, 0.5 L/min CO_2 flow rate, and 1 g of enzyme

[c,d] Values within columns followed by the same superscript letter are not significantly different at $p > 0.05$

Table 6 Fatty acid composition of starting oil and hydrolyzed products

	C16:0 (% area)	C18:0 (% area)	C18:1 (% area)	C18:2 (% area)	C18:3 (% area)
Pure oil[a]	4.13	2.87	8.59	35.24	49.17
FFA-conventional process[b]	3.13[c]	2.43[c]	8.94[c]	34.99[c]	50.51[c]
FFA-process in SC-CO_2 media[b]	4.43[d]	3.72[d]	9.23[d]	35.12[d]	47.50[d]

[a] FA composition in TG form of non-hydrolyzed oil obtained with the methylation method using a sodium methoxide reagent

[b] FA composition in FFA form of hydrolyzed products obtained with the methylation method using the TMS-diazomethane reagent. Conventional hydrolysis process: 60 °C, ratio of oil:water 1:70 (mol/mol), and 26.17 % of enzyme (weight of oil). Enzymatic hydrolysis in SC-CO_2 media: 30 MPa, ratio of oil:water 1:5 (mol/mol), 50 °C, 0.5 L/min CO_2 flow rate, and 1 g of enzyme

[c,d] Values within columns followed by the same superscript letter are not significantly different at $p > 0.05$

based on binary system data available in the literature [6]. Ternary systems can provide different solubility data. For example, water could be more soluble in SC-CO_2 than in SC-CO_2 saturated with oil. In addition, phase behavior prediction was estimated under optimum conditions. Different conditions would provide different solubilities of products and reactants.

Lipid Class Content

Figure 4 shows the chromatogram of pure oil and hydrolyzed oil under optimum conditions to produce maximum FFA using conventional and SC-CO_2 processes. A decrease in TG peaks and an increase in FFA, MG, and DG peaks in hydrolyzed oils are clearly observed compared to pure oil. Table 5 shows the quantification of each lipid. As expected, a drop in TG content was achieved in hydrolyzed oils, while an increase in FFA, MG, and DG was obtained. The presence of intermediate products (MG and DG) and TG indicates that the oil was not completely hydrolyzed. These results confirm that enzymatic hydrolysis in SC-CO_2 media achieved higher hydrolysis (higher amount of FFA and lower content of TG) than the conventional hydrolysis process.

The amount of DG was higher than MG in both processes (Table 5). Since the enzyme used in this study is 1,3-specific, it was expected to have more 2-MG. One possible explanation is that some enzymes have stereospecificity, thus, positions 1 and 3 are hydrolyzed at different rates [13].

Fatty Acid Composition

Table 6 shows the results of fatty acid composition analysis expressed as percentages of area. It can be observed that all fatty acids content in the hydrolyzed samples have a similar content compared to non-hydrolyzed oil. Because Lipozyme TL IM is a 1,3-specific lipase, this result would suggest that in sacha inchi oil there is no positional specificity and the fatty acids are distributed randomly in the glycerol backbone. However, it is known that during the reaction, acyl migration can occur. Thus, fatty acids at the *sn*-2 position can migrate to *sn*-1 or *sn*-3 positions [13]. Therefore, if migration occurred during the hydrolysis, fatty acids located at the *sn*-2 position were further hydrolyzed by 1,3-specific lipase. However, this result is based on percentages of area and more detailed studies should be performed to confirm this preliminary observation.

Conclusions

The highest amount of FFA (w/w) produced by conventional enzymatic hydrolysis was about 10 % lower than the amount obtained using enzymatic hydrolysis in SC-CO_2 media. Moreover, conventional hydrolysis required more time to hydrolyze the oil (6 h against 2 h for the SC-CO_2 process). However, these times were fixed to optimize the parameters and the time necessary for each process should be determined with a kinetic curve. In addition, the amount

of water and enzyme necessary to achieve reaction equilibrium for the conventional hydrolysis was 14 and 4 times higher than those for hydrolysis in SC-CO_2 media, respectively, leading to an increase in cost of manufacturing. The optimum enzyme concentration for conventional hydrolysis (26.17 % weight of oil) is extremely high, which would make this process unviable. Lipid class analysis showed that the hydrolysis was not completed using both conventional and SC-CO_2 processes. Although the enzymatic hydrolysis of sacha inchi oil in SC-CO_2 media shows various advantages over the conventional process, other factors might be considered in the selection of the process, such as capital cost through an economic study.

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