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Enzymatic Esterification of Oleic Acid with Aliphatic Alcohols for the Biodiesel Production by *Candida antarctica* Lipase

Isac G. Rosset · Maria Cecília H. T. Cavalheiro · Elisabete M. Assaf · André L. M. Porto

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Abstract Biodiesel can be obtained by esterification reactions of free fatty acids with enzymatic catalysts (lipases). In this study, the immobilized *Candida antarctica* lipase was employed in enzymatic esterifications of oleic acid with aliphatic alcohols (methanol, ethanol, *n*-propanol, *n*-butanol). Some features that influence the enzymatic esterification reaction, such as amount of biocatalysts, reaction time, hydration level and biocatalyst turnover were evaluated. The products were determined by GC-FID and ^{1}H NMR analyses and these analytical methods were compared. The enzymatic catalyst (*C. antarctica* lipase) was efficient providing high yields of biodiesel (above 90 %) in less than 24 h to ethanol, *n*-propanol and *n*-butanol, whereas for methanol, the enzyme was inactive after ten cycles of reaction. Two new quantitative easy methods were also developed to quantify esters produced by ^{1}H NMR based on the α-CH_2 protons of oleic acid and esters. The quantification method used in the enzymatic reactions by ^{1}H NMR showed effective with small differences in comparison with GC-FID analyses.

Keywords Biocatalysis · Hydrolases · CALB · NMR quantification

1 Introduction

The utilization of biodiesel in diesel motors is very attractive, in view of the environmental aspects and be a renewable energy source. It can be obtained by either the triacylglyceride transesterification present in vegetable oils and animals fats or esterification of free fatty acids [1].

Alkaline catalysis is most widely used in industrial processes of transesterification and sodium hydroxide is the most utilized catalyst because it is cheaper than enzymes or heterogeneous catalysts. However, in many cases, this type of catalysis results in saponification reactions, consuming the catalyst, decreasing the efficiency of the process and hampering the biodiesel purification and glycerol separation [2]. Acid catalysts are usually utilized when the content of fatty acids is high, but they have showed some inconveniences, like corrosiveness, fewer yields than alkaline catalysis, slow reactions and need of high temperatures [3, 4].

An alternative to these processes is the use of enzymes, more exactly, lipases for the biodiesel production. There has been a huge interest in the utilization of lipases as biocatalysts to convert vegetable oil and animal fatty into biodiesel. Their utilization for the biodiesel production is advantageous, as different solvents (hydrophilic and hydrophobic) can be utilized, the enzymes are versatile, robust and many lipases show considerable activity to catalyze not only transesterification reactions of oil and fats [5], but also fatty acid esterification, which is not possible when using conventional alkaline catalysts (Fig. 1), if the enzyme is immobilized and does not lose activity, it can be reused [6–11]; relative thermostability and tolerance to short chain alcohols [12–16].

The main advantages in the biodiesel production are that the enzyme shows high chemoselectivity and mild conditions

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I. G. Rosset · M. C. H. T. Cavalheiro · E. M. Assaf · A. L. M. Porto (✉)
Instituto de Química de São Carlos, Universidade de São Paulo, Av. João Dagnone, 1100, Santa Angelina, CP 780, São Carlos, SP CEP 13563-120, Brazil
e-mail: almporto@iqsc.usp.br

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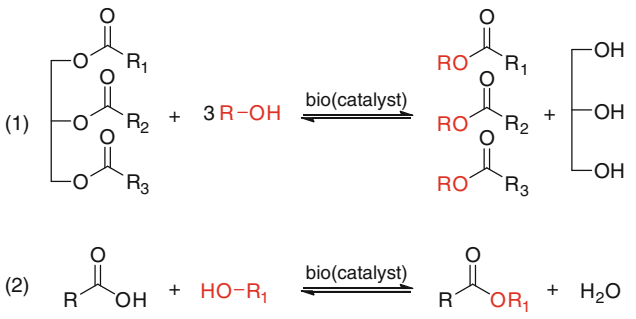

Fig. 1 (**1**) Transesterification and (**2**) esterification of triacylglycerides and fatty acids

(consumes less energy in the process-temperatures around 35 °C) [17]. As triglycerides and fatty acids are natural substrates for lipases, and these accept a structural diversity of substrates, which is advantageous because it allows the use of different sources of raw materials. Free fatty acids are the key parameters to determine the viability of the transesterification process of vegetable oil into biodiesel. They are industrially obtained from triacylglyceride hydrolysis and when oil has higher acidity (>1.0 %), it hampers the efficiency of the biodiesel conversion if this oil is used for transesterification [18]. Therefore, the researches on the enzymatic processes of fatty acids are of great importance to the optimization of biodiesel production employing biocatalysts.

Residual fatty acids are undesirable in biodiesel, i.e., the transesterification and esterification processes must be complete so that these components do not cause any operational damage, like deposits in the engine, filter obstruction or fuel deterioration [19]. Therefore, the quality control of biodiesel is very important for its commercialization [20]. Gas Chromatography (GC) is the main method to quantify esters of fatty acids produced, but the Nuclear Magnetic Resonance (NMR) has come to prominence, mainly because this technique does not require sample derivatization [21]. Several studies have shown the quantification of biodiesel is possible by employing NMR analysis [22]. Recently, we published a study showing the efficiency of NMR in the biodiesel quantification from soybean oil with enzymatic catalysis [23]. However, the literature lacks studies of the NMR quantification of products generated by esterification reactions from fatty acids.

In this study, we performed the enzymatic esterification of oleic acid with *Candida antarctica* lipase with several aliphatic alcohols. The quantification by GC-Flame Ionization Detector (GC-FID) and the development of two simple methodologies by ^{1}H NMR to quantify the esterification reactions for the biodiesel production were also developed.

2 Experimental

2.1 Materials

Methanol, ethanol, *n*-propanol and *n*-butanol (HPLC grade) were purchased from Tedia (USA), and *n*-hexane and ethyl acetate (PA grade) were obtained from Quimis (Brazil) and used without further purification. Immobilized lipase type B from *C. antarctica* (CALB; Novozym 435®) was kindly donated by Novo Nordisk Bioindustrial Ltda (Araucária, PR, Brazil). The GC-FID analysis was carried out in a model GC 2010 Shimadzu gas chromatograph equipped with an AOC 20i auto injector, a FID and a SelectTM Biodiesel for Glycerides UltiMetal column (15 m × 0.32 mm). The chromatographic conditions employed were carrier gas nitrogen at 60 kPa, split ratio = 1:20, injection volume = 1.0 µL, injector temperature = 270 °C, detector temperature = 350 °C, oven temperature program initially at 100 °C and increased at 15 °C/min to 320 °C, remaining at 320 °C for 6.8 min and total time of analysis = 23.5 min. Tricaprine (Aldrich) was used as internal standard. A Brucker (Germany) AC200 spectrometer was used for ^{1}H and ^{13}C NMR spectroscopy, operating at 200 and 50 MHz, respectively, with TMS as the internal standard. The NMR experiments were conducted and the chemical shifts (δ) were expressed in ppm and the coupling constants (*J*) in Hz. The multiplicities were presented as **s** (singlet), d (duplet), t (triplet), q (quartet) and m (multiplet). The orbital shaker used was Tecnal (Brazil) model TE-421. The reaction was monitored by TLC employing aluminum-backed precoated silica gel 60 F254 layers (Whatman, UK) with a 98:02 *n*-hexane:ethyl acetate like eluent.

2.2 Standards of Esters

First, the esters of oleic acids were prepared by acid catalysis to be employed as standards in GC-FID and ^{1}H NMR. The oleic acid (1 eq., 9.51 mmol, 3.0 mL), alcohol (6.0 mL), 20 mL of toluene and concentrated sulfuric acid (1.0 % w/w in relation to oleic acid) were added in a two-neck flask with a Dean–Stark system. The system remained under reflux for 15 h and the reaction was monitored by TLC. The mixture was then diluted with a saturated solution of $NaHCO_3$ (3 × 20 mL) and the remaining alcohol was removed by low pressure. The products were dried with anhydrous $MgSO_4$ and purified by *flash* chromatography with silica gel as the stationary phase and *n*-hexane:ethyl acetate (98:02) as the effluent. The products were characterized by IR, ^{1}H and ^{13}C NMR analyses.

2.3 Enzymatic Preparation of Esters from Oleic Acid

For the enzymatic esterification reactions, the following parameters were evaluated: amount of biocatalyst, reaction

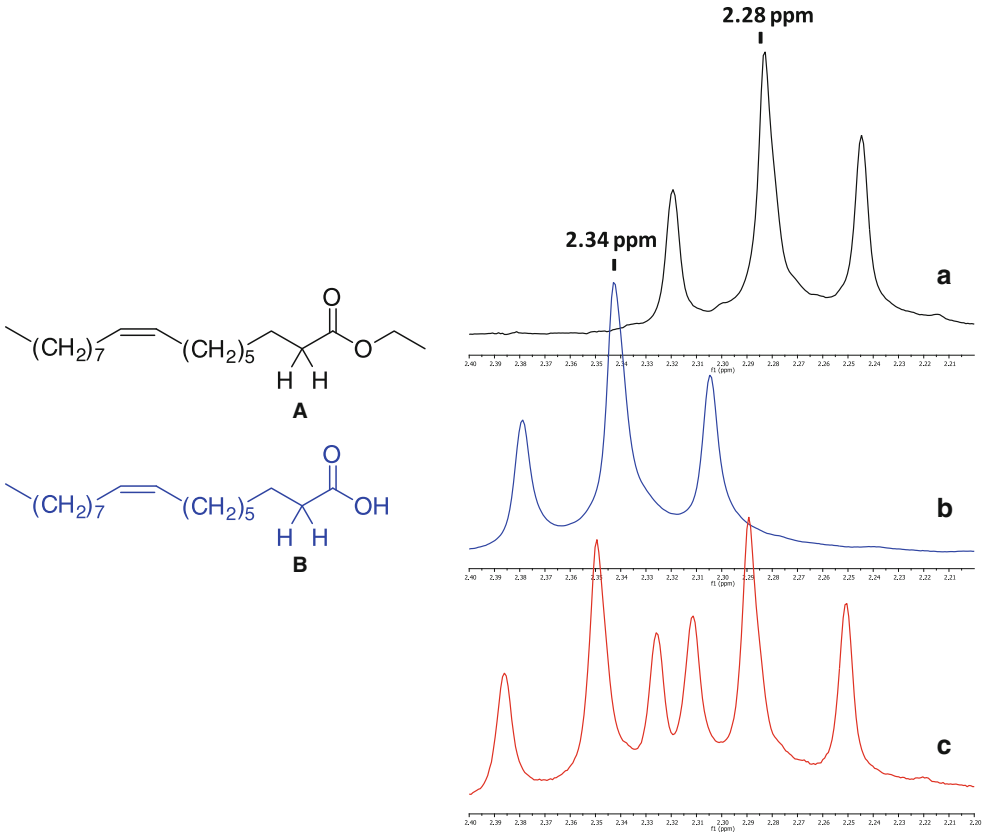

Fig. 2 Example of a signal overlap on the ^{1}H NMR spectra. **a** α-CH_2 protons of ethyl oleate; **b** α-CH_2 protons of oleic acid; and **c** mixture of ethyl oleate and oleic acid (50:50). Expansions were between 2.20–2.40 ppm

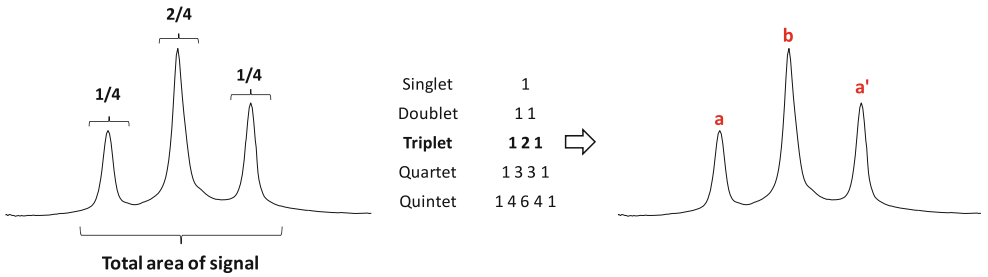

Fig. 3 Triplet for the deployment of resonance signals according to the n+1 rule

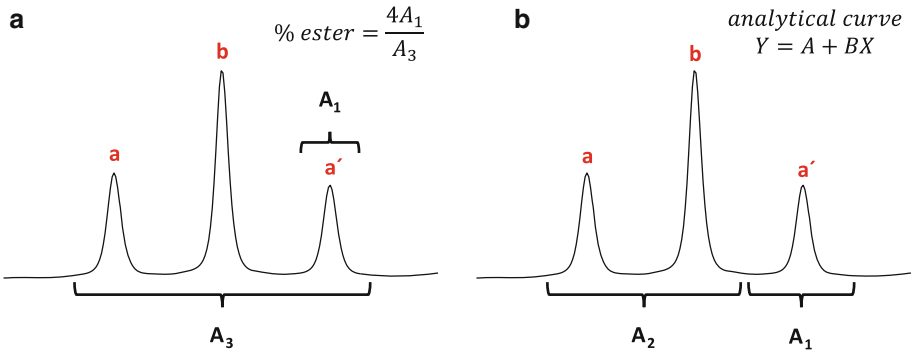

Fig. 4 Methods of quantification of biodiesel by ^{1}H NMR employed in the esterification reaction of oleic acid. **a** Equation for direct quantification; **b** use of analytical curve ($A_1 = a'$, $A_2 = a + b$, $A_3 = a + b + a'$)

time, hydration level and enzyme turnover. All experiments were run in Erlenmeyer flasks on an orbital shaker (130 rpm) at 32 °C. After the reactions, the immobilized enzyme was filtered in a qualitative filter paper and the products were washed with distilled water (1 × 10 mL), concentrated by low pressure, dried with $MgSO_4$ and analyzed by GC and ^{1}H NMR.

2.3.1 Amount of Biocatalysts

To evaluate the amount of catalyst in the reaction, oleic acid (1 eq., 17.7 mmol, 0.5 g), the respective alcohol (methanol, ethanol, *n*-propanol and *n*-butanol, 1.5 mL) and the enzyme in several proportions (0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 % w/w in relation to the oleic acid) were added in Erlenmeyer flasks. After 24 h, the reactions were extracted with *n*-hexane, evaporated and analyzed by GC-FID and ^{1}H NMR.

2.3.2 Reaction Time

The reactions were conducted in Erlenmeyer flasks on orbital shaker (130 rpm) at 32 °C at different reaction times (1, 4, 8, 15 and 24 h). The amount of enzyme in these experiments was 5.0 %. After the reactions, the products were extracted with *n*-hexane, evaporated and analyzed by GC-FID and ^{1}H NMR.

2.3.3 Hydration Level

The presence of water in the reaction medium was evaluated. In this experiment, several mixtures of water and alcohols were prepared: 0.5, 1.0, 1.5, 2.0 and 4.0 % in relation to the volume of alcohols. The reactions were conducted in Erlenmeyer flasks on a shaker (130 rpm), at 32 °C for 24 h employing 5.0 % of enzyme. After the reactions, the products were extracted and analyzed by GC-FID and ^{1}H NMR.

2.3.4 Turnover of the Enzyme

Successive reactions with the same immobilized enzyme were carried out to evaluate the turnover capacity and stability of the biocatalyst. The enzyme was utilized ten times for each alcohol (methanol, ethanol, *n*-propanol and *n*-butanol) and the time of witch reaction was 24 h. The enzyme was filtered, washed with *n*-hexane and used again. After each reaction, the enzyme was filtered in qualitative

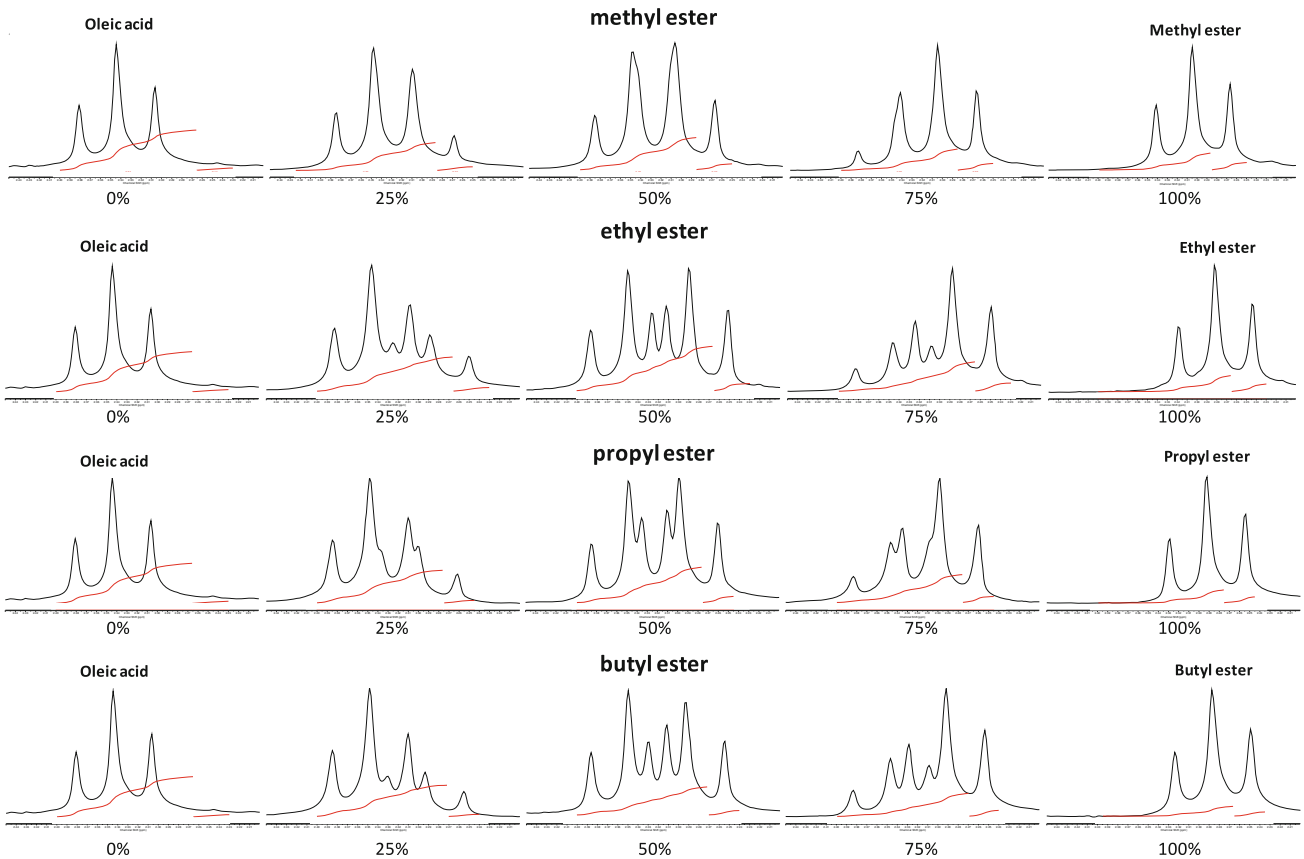

Fig. 5 Overlap for all esters produced and oleic acid in different concentrations (method B)

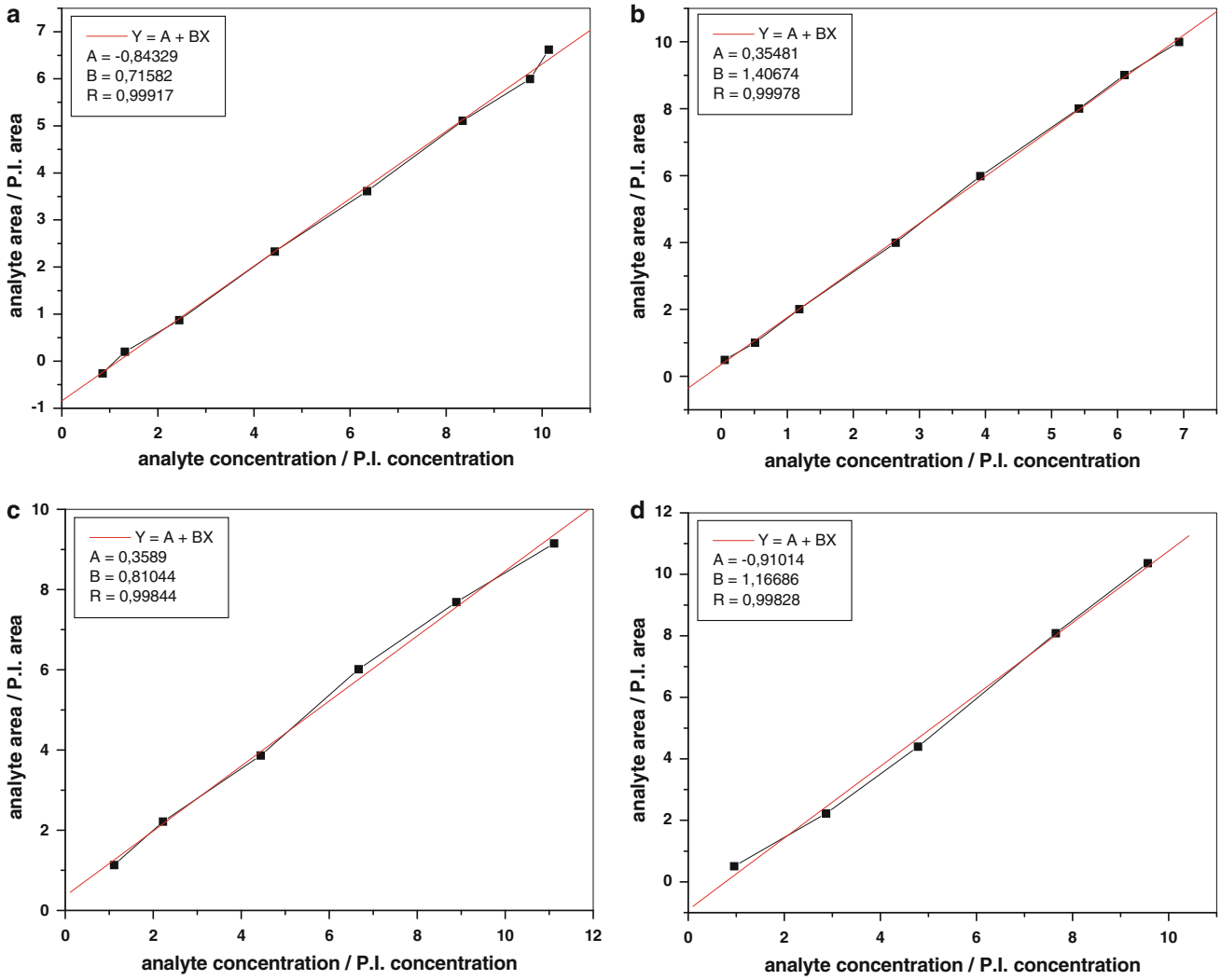

Fig. 6 Analytical curves obtained by GC-FID for the quantification of enzymatic reactions. **a** MeOH; **b** EtOH; **c** *n*-PrOH; **d** *n*-BuOH

filter paper, washed with 20 mL of *n*-hexane P.A., dried at 25 °C for 1 h and reutilized. After the reactions, the products were extracted and analyzed by GC-FID and ^{1}H NMR.

2.4 GC-FID Quantification

Four analytical curves were prepared for the quantification of the reaction catalyzed with lipase CALB. Mixtures of analyte (ester) and internal standard (tricaprin) were prepared and analyzed by GC-FID. For quantification, the areas of the analyte (respective ester) and internal standard were obtained by GC-FID and plotted with the concentration of these compounds. The linear regressions of the curves were used for the quantification of the enzymatic reactions. The GC-FID is the most employed method to quantify these reactions, therefore it was used in this study to compare the results with the ^{1}H NMR quantification.

2.5 ^{1}H NMR Quantification

The quantification by ^{1}H NMR was based on areas of α-CH_2 protons of oleic acid (2.34 ppm) and α-CH_2 of the respective esters produced (methyl: 2.30 ppm; ethyl: 2.28 ppm; *n*-propyl: 2.29 ppm and *n*-butyl: 2.29 ppm). Due to the ester formation, the signal of α-CH_2 protons (triplet) is more shielded when compared with the acid (2.34 ppm). However, this difference is small, resulting in a partial overlap these signals (α-CH_2 of acid with the α-CH_2 of esters) when there exists a mixture of these compounds (Fig. 2).

Through the phenomenon called *spin–spin* deployment, the NMR signals undergo deployment, which can be empirically explained by the $n + 1$ rule. According to this rule, which proton "feel" the equivalents number of protons (n) of the neighbor carbon atom that the proton is connected. Therefore, the resonance signal "unfold" on $n+1$ components. These deployments respect the Pascal triangle. The quantification by NMR of the signals of the

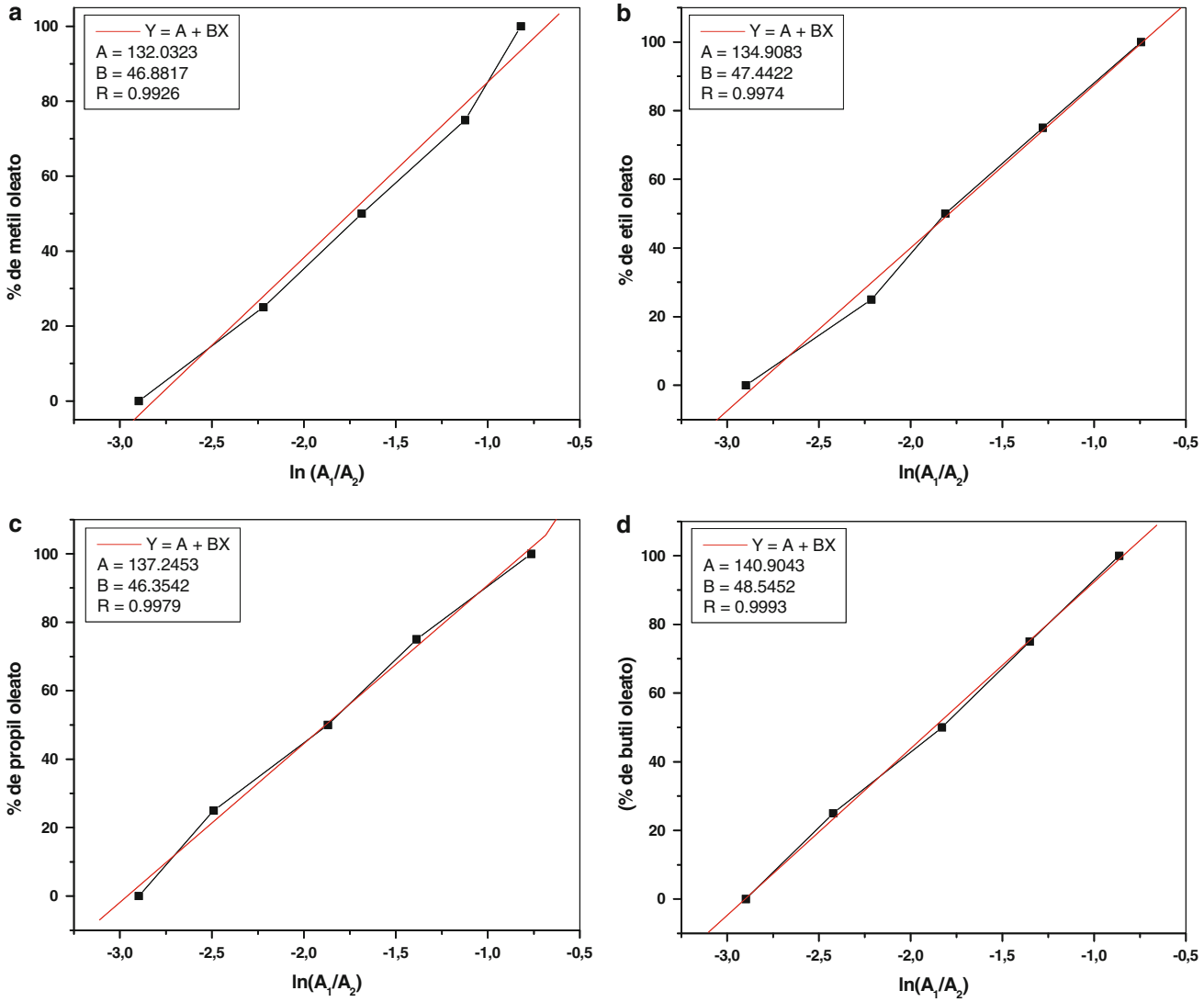

Fig. 7 Analytical curves obtained by ^{1}H NMR analysis (method B) for the quantification of enzymatic reactions. **a** MeOH; **b** EtOH; **c** *n*-PrOH; **d** *n*-BuOH

α-CH_2 atoms of esters was based on the triplet formed, whose total area is the sum of external components of the triplet (smaller intensities, a and a′), which have an area equal to 2/4 (1/4 for each external signal) and of total area of internal component (bigger intensity, b) that have a total area of 2/4 in relation the total area of triplet (Fig. 3).

Based on the deployment of the signals, the ^{1}H NMR quantification was conducted in two forms; method A: through a simple equation it was possible to calculate directly the amount of ester formed through of the external signal on the right side of the ester triplet; method B: an analytical curve obtained by ration of areas of triplet components (Fig. 4).

In method A, the numerator of the equation is multiplied by four as it represents 1/4 of the total area of the signal (a′). This external signal was chosen because it does not suffer any overlap from the signals of oleic acid. In method B, mixtures of oleic acid and respective ester were prepared in different proportions (0, 25, 50, 70 and 100 %) and analyzed by ^{1}H NMR. The relation between the areas (A_1/A_2, a′/a+b) was conducted and it was possible to obtain the analytical curves and use them to calculate the yield of the enzymatic reactions. The overlap of the signals of all esters produced and the oleic acid are showed in Fig. 5.

The ^{1}H NMR quantification employing two methods was conducted only for the first experiment (amount of catalyst), to compare the analytical methods, because in these experiments, the yields varied of 1.0–97 %. To verify these methodologies by NMR, the GC-FID analysis was realized.

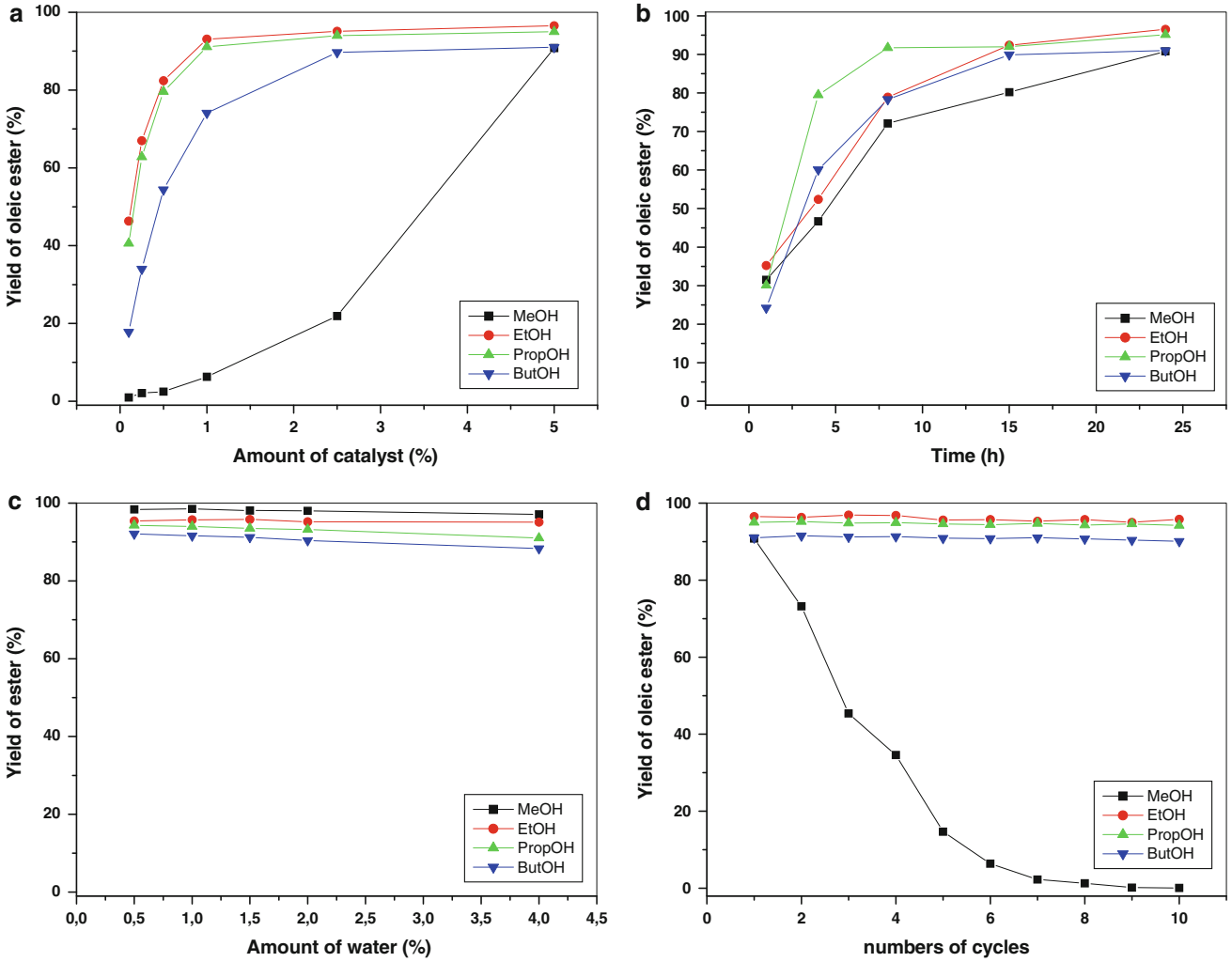

Fig. 8 Results of esterification reactions of oleic acid (quantified by GC-FID): **a** amount of catalyst, **b** reaction time **c** hydration level **d** turnover of the enzyme

3 Results and Discussion

3.1 Analytical Curves

The GC-FID analysis provided four analytical curves by the *Microcal Origin 6.0* program, with the respectives linear regressions, showing a good correlation among the points (Fig. 6). The curves were used for all quantifications of enzymatic reactions and the results were compared with those of the ^{1}H NMR analysis.

Four analytical curves for four alcohols were obtained employing method B of quantification by ^{1}H NMR (Fig. 7) with a good correlation among the points.

3.2 Effect of the Amount of Catalyst

The discussion below was based on GC-FID quantification, as it is the standard quantification method. In the study of the amount of catalyst employing the esterification reaction of oleic acid, it was possible to observe that the larger the amount of biocatalyst used, the higher the yield of reaction and was used until 5.0 % of catalyst (Fig. 8a). When methanol was used in the reaction, the behavior of the curve was very different from that of other alcohols. The results are showed in Table 1.

It is possible to observe that was used ethanol and *n*-propanol on the esterification, 1.0 % of enzyme was already enough to increase of yield (93.1 and 91.1 %, entry 4). A recent study demonstrated that 2.24 % of *C. antarctica* lipase (Novozyme 435®) under similar reaction conditions provided an yield of 95 % with ethanol before 24 h [24]. The *n*-butanol also showed a good yield (91.0 %, entry 6) with 5.0 % of enzyme. A different behavior in relation to that of other alcohols when methanol was used and were necessary 5.0 % of enzyme (90.8 %, entry 6).

3.3 Effect of Time Reaction

The transesterification reaction time also varies greatly from a low of 8 h for immobilized lipase from *Pseudomonas cepacia* with *Jatropha* oil in ethanol to a high of 90 h for the same free enzyme transesterifying soybean oil with methanol [25]. A space-time of between 127 and 51 min using *Candida* sp. immobilized on textile with petroleum ether and 10 % water as co-solvents and constant enzyme activity during 500 h of operation. A conversion of 90 % with a space–time of 2.7 h using Novozyme 435® in a solvent-free system producing fatty acid methyl ester (FAME) from waste oil. The enzymes kept their activity for 100 days when three PBRs in series where used with intermediate glycerol removal and methanol addition [26].

The results showed that the reaction time ranging of 1–24 h, just the *n*-propanol provided a yield of 91.7 % after 8 h of reaction (entry 9), what show that the enzyme has good tolerance to *n*-propanol. This is the time that can be equivalent to acid catalysis [27]. For other alcohols (ethanol and *n*-butanol), 15 h were necessary for the yield to achieve 90 % (92.4 and 89.9 %, entry 10). Just to methanol needed 24 h to obtained a satisfactory yield (90.8 %, entry 11) (Fig. 8b).

3.4 Hydration Level

Another fundamental factor in this type of reaction is the amount of water in the alcohol, i.e., the hydration level. The biocatalyst frequently requires a certain amount of water in the reaction to maintain activity [28]. This is an important factor in transesterification and esterification when enzymes are used. Water is essential to maintain the specify tridimensional structure of the enzyme and the total removal may lead to irreversible changes in the structure [29]. The enzyme activation involves the unmasking and restructuration of its active site through conformational changes of lipase, which require the presence of an oil–water interface. The lipase activity usually depends on the interfacial area available. An increase in the water addition increase the amount of water available to form oil–water droplets, increasing the interfacial area available. The good water contends is the accord between the minimum hydrolysis and the maximum activity of enzyme on the reaction [30].

The results of our study suggest that the presence of water directly affects the yield of reaction with methanol. When anhydrous methanol was used, the yield was 90.8 % with 5.0 % of enzyme (entry 6). However, the 0.5 % increase in water did the meant that the income increase in 7.6 % (98.4 %, entry 12). This may be due to the absence of water, the methanol can be removing the structural water of enzyme and dehydrating the enzyme, this way doing with lose the activity by modification of structure. In the presence of water, this effect is filled, increasing its efficiency. The reaction yield was followed according to the chain length alcohols (methanol until 98.4–92.1 % for *n*-butanol, entry 12), but with the increase of water amount, the yield was decreasing, showing that a little amount of water is needed to offer a good yield (Fig. 8c).

Table 1 Yields of the reactions of oleic acid esterification with various alcohols in the studied parameters

	Parameter	Yield (% of oleic ester)[a]			
	Amount of catalyst (%)	MeOH	EtOH	*n*-PrOH	*n*-BuOH
Entry					
1	0.1	1.0	46.3	40.6	17.8
2	0.25	2.1	67.0	62.8	34.0
3	0.5	2.5	82.4	79.6	54.4
4	1.0	6.3	93.1	91.1	74.1
5	2.5	21.9	95.1	94.0	89.7
6	5.0	90.8	96.5	95.0	91.0
Reaction time (h)					
7	1	31.5	35.2	30.1	24.2
8	4	46.7	52.4	79.5	60.1
9	8	72.1	78.9	91.7	78.3
10	15	80.2	92.4	92.0	89.9
11	24	90.8	96.5	95.1	91.0
Water (%)					
12	0.5	98.4	95.4	94.3	92.1
13	1.0	98.5	95.7	94.0	91.6
14	1.5	98.1	95.8	93.5	91.2
15	2.0	98.0	95.2	93.2	90.4
16	4.0	97.1	95.1	91.0	88.3
Turnover (cycles)					
17	1	90.8	96.5	95.0	91.0
18	2	73.2	96.3	95.2	91.5
19	3	45.4	96.9	94.8	91.2
20	4	34.6	96.8	94.9	91.3
21	5	14.7	95.6	94.6	90.9
22	6	6.4	95.7	94.4	90.8
23	7	2.3	95.3	94.7	91.0
24	8	1.3	95.7	94.3	90.7
25	9	0.2	95.0	94.6	90.4
26	10	0.09	95.8	94.2	90.1

[a] All yields were calculated by GC-FID

3.5 Reuse of the Enzyme

Excellent results were obtained for all alcohols except methanol. For this alcohol, at the end of the 10 reaction

Table 2 Comparison of the quantification methods employed (GC-FID and ^{1}H NMR)

Catalyst (%)	Yields[a]											
	MeOH			EtOH			*n*-PrOH			*n*-BuOH		
	CG	NMR A	NMR B	GC	NMR A	NMR B	GC	NMRA	NMR B	GC	NMR A	NMR B
0.1	1.0	–	–	46.3	38	45	40.6	29	39	17.8	6	16
0.25	2.1	–	–	67.0	61	68	62.8	50	61	34.0	21	32
0.5	2.5	–	–	82.4	75	84	79.6	70	81	54.4	43	53
1.0	6.3	0.5	4	93.1	88	94	91.1	83	92	74.1	62	75
2.5	21.9	9	23	95.1	89	94	94.0	87	96	89.7	82	91
5.0	90.8	84	90	96.5	90	96	95.0	87	96	91.0	85	92

A method by ^{1}H NMR using an simple equation (% ester = $4A_1/A_3$) for direct quantification, *B* method by ^{1}H using analytical curve

[a] The yields were calculated by GC-FID and by methods A and B by ^{1}H NMR

cycles, the income was 90.8 % (cycle 1, entry 17) fell to 0.09 % (cycle 10, entry 26). This huge drop comes is due to the fact that methanol can inhibit the enzyme. The enzyme activity was drastically reduced for methanol, which was used dried (Fig. 8d). For all other alcohols during the 10 reaction cycles, the yields did not change significantly 95.8, 94.2 and 90.1 for ethanol, *n*-propanol and *n*-butanol, respectively), showing that the enzyme can be reused for the preparation of esters for at least 10 cycles. The results are shown in Table 1.

Studies have shown that the degree of deactivation is proportional to the number of carbon atoms present in the alcohol, which, methanol has a greater influence [31, 32] and the use of ethanol in methanol increases the rate of speed of esterification and transesterification reactions [33]. It has been reported that lipase is inactivated by the use of methanol (insoluble) in the form of droplets into oil and fat [34, 35]. In transesterification, the immobilized lipase from *C. antarctica* (Novozyme 435®) was inactivated by using 1.5 equivalents of methanol in relation to the oil. However, this effect can be minimized if methanol is added in steps [36].

The enzymatic reactions presented in this work with the lipase CALB performance can be improved by performing a study of different types of media that are most appropriate for this enzyme in the production of biodiesel.

3.6 Comparison Between GC-FID and ^{1}H NMR

In the comparison of the quantification methods employed, was evaluated for the experiment 1 (amount of catalyst) (Table 2).

The quantification by ^{1}H NMR using method B showed better results in comparison to method A. This could be because, as is done in method A direct integration of the signal to obtain the integral value and used as a low-power device (200 MHz), the integration of the external signal does not correctly represents the actual value. In method B, the ratio of the integrals is placed on a curve, which minimizes this effect, resulting in a better correlation between the points and minor errors. For both methods of quantification by ^{1}H NMR, is not possible when the conversion yields are low (less than 5.0 % when analyzed by GC-FID) as an external signal such as NMR measurement used in the triplet is very small, making it difficult to obtain the integral.

The all spectrums and chromatograms are in supplementary data.

4 Conclusions

The enzymatic esterification of fatty acid for the biodiesel production with aliphatic alcohols showed excellent yields including was possible the reuse the enzyme to ten cycles and keep the yield to reactions. The esterification with methanol showed a little dependence of water on the reaction environment, due to the dehydration of enzyme and its inactivation. The quantification by ^{1}H NMR showed very efficient even when there was a partial overlap of the signals, allowing the quantification of reaction with residual fatty acid quickly and non-destructive.

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