Synthesis of Dietetic Structured Lipids from Spent Coffee Grounds Crude Oil Catalyzed by Commercial Immobilized Lipases and Immobilized *Rhizopus oryzae* Lipase on Biochar and Hybrid Support

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Abstract: The aim of this study was the valorization of coffee industry residues, namely spent coffee grounds (SCG) as a source of oil, and silverskin (CS) as a source of both oil and biomass, under the concept of the circular economy. Therefore, crude oil from SCG was used to produce low-calorie structured lipids (SL) for food and pharmaceutical industries, and CS to produce biochar by pyrolysis for biotechnological uses. SL were obtained by acidolysis with caprylic or capric acid, or interesterification with ethyl caprylate or ethyl caprate, in solvent-free media, catalyzed by immobilized *sn*-1,3 regioselective lipases. Silverskin biochar (BIO) was directly used as enzyme carrier or to produce hybrid organic-silica (HB) supports for enzyme immobilization. *Rhizopus oryzae* lipase (ROL) immobilized on Amberlite (AMB), silica (SIL), BIO or HB, and the commercial immobilized *Thermomyces lanuginosus* (Lipozyme TL IM) and *Rhizomucor miehei* (Lipozyme RM IM) lipases were tested. Lipozyme RM IM showed better results in SL production than Lipozyme TLIM or ROL on BIO, SIL or HB. About 90% triacylglycerol conversion was attained after 7 h acidolysis or interesterification. Lipozyme RM IM was more stable in interesterification (80% and 65% activity with ethyl caprylate or ethyl caprate) than in acidolysis (first-order decay) after 10 reuses.

Keywords: coffee silverskin; immobilization; lipase; spent coffee grounds oil; structured lipids

1. Introduction

Worldwide, coffee is a popular beverage and one of the most relevant commodities providing economic benefits for the producing countries. World coffee production is projected to reach 167.4 million bags (60 kg/bag) in 2019/2020. The price/quotation of the coffee bag in July 2020 was approximately US$ 100 in Brazil, which is the largest exporter of coffee in the world and occupies the second position among the consuming countries of this beverage [1]. Consequently, different by-products are produced during coffee processing (e.g., husks and mucilages), coffee roasting (e.g., coffee silverskin) and coffee drink preparation (spent coffee grounds) [2–5]. The current policies

seek to prevent and reduce waste generation, with the proposal of practicing sustainable consumption habits and reusing waste. Growing environmental awareness has triggered an interest in the valorization of agro-food and organic wastes which are generated in large amounts daily, such as spent coffee grounds (SCG) and coffee silverskin (CS), to produce added-value products [2,4–6]. This concept has been used by many countries in the development of integrated biorefineries. CS can be converted by pyrolysis process into biochar [7] and used as enzyme immobilization support [8–10]. The immobilization of enzymes on solid supports may increase both the stability and catalytic properties of enzymes in oil biotransformation reactions. SCG and CS are important sources of edible oils, containing around 10–20 and 2.4–3.4 wt% oil, respectively. These oils are rich in long-chain fatty acids (FA), predominantly linoleic acid (C18:2, 24.4–44.1%) and palmitic acid (C16:0, 22–33.6%) [3,11–13]. The use of SCG oils in the production of cosmetics [14] and biodiesel [15–19] has been reported. Moreover, the utilization of SCG or CS oils to produce structured lipids could be a source of revenue for the coffee sector, together with environmental benefits. However, according to our knowledge, the only work using crude SCG oil for the production of structured lipids was carried out by our group [20].

Structured lipids (SL) are lipids (or fats and oils) that have been modified from their natural biosynthetic form into novel lipids with improved technological, nutritional and/or functional properties [21–24]. The term "modified" means any modification in the structure of the triacylglycerol (TAG), either in the composition or in the position of fatty acids (FA) in TAG. Long-chain fatty acids (L) are metabolized slowly and tend to be deposited in human adipose tissue. In contrast, medium-chain fatty acids (M) are directly absorbed in the stomach and/or intestine providing energy quickly via oxidation. Therefore, they show a lower tendency to be accumulated in the adipose tissue. Low-calorie TAGs are examples of structured lipids with optimized benefits compared to the natural oils and fats, with a caloric value between 5–7 kcal/g against 9 kcal/g of natural fats [25]. These TAGs are usually of the type MLM, since they contain a long-chain fatty acid (L) at the position *sn*-2 and medium-chain fatty acids (M), at the positions *sn*-1,3 [21,22].

Low-calorie SL can be produced enzymatically either (i) by acidolysis of a TAG or oil, containing long-chain fatty acids, with a medium-chain fatty acid [26], or (ii) by interesterification of a TAG or oil with methyl or ethyl esters (fatty acid methyl esters, FAME, or fatty acid ethyl esters, FAEE) of medium-chain fatty acids [27,28] or medium-chain TAG [29]. Reactions catalyzed by enzymes are carried out under mild temperatures, reducing energy consumption, leading to a decrease in the loss of temperature-sensitive molecules (substrates and/or products), and resulting in more environment-friendly and safer processes. In SL synthesis, immobilized lipases have been used, helping to overcome some of the economic constraints for industrial process implementation associated with the high price of enzymes, since they can be reused in batch bioreactors or used in continuous processes.

Currently, the demand for novel supports or improvements in the existing or traditional materials used for enzyme immobilization have been explored to promote high catalytic activity and operational stability. The use of environmentally friendly supports has been reported in the literature for enzyme immobilization, such as chitosan [30], sugarcane bagasse [31] and biochar [8–10,32,33]. Hybrid supports, synthesized by the sol–gel technique, are promising materials since they exhibit both high performance and high functionality for enzyme immobilization [34]. The hybrid inorganic-organic material, obtained from silica and biochar, was successfully used for lipase immobilization and application in the biotransformation of oils [35].

The aim of this study was the valorization of coffee industry residues, namely SCG as a source of oil, and CS as a source of both oil and biomass, under the concept of the circular economy. Therefore, in the present study, the crude oil extracted from SCG was used as a raw material for the synthesis of low-calorie SL, either by acidolysis with caprylic (C8:0) or capric (C10:0) acids or by interesterification with their ethyl esters, in the absence of organic solvent, catalyzed by immobilized lipases. The *Rhizopus oryzae* lipase (ROL) was immobilized on Amberlite IRA 96 (used as a reference), silica, biochar prepared with silverskin, or hybrid support prepared using this biochar and silica, and tested as novel biocatalysts.

The results obtained with the immobilized-ROL preparations were compared with the performance of the commercial immobilized preparations of *Thermomyces lanuginosus* lipase (Lipozyme TL IM) and *Rhizomucor miehei* lipase (Lipozyme RM IM) under similar reaction conditions.

2. Materials and Methods

2.1. Materials

Coffee silverskin (CS) and spent coffee grounds (SCG) were provided by the industry and coffee shops located in Sergipe (Brazil), respectively. Hexane for oil extraction was obtained from Synth (Brazil). *Rhizopus oryzae* lipase (ROL), the silane precursor tetraethoxysilane (TEOS) and Amberlite IRA 96 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). *Thermomyces lanuginosus* lipase (Lipozyme TL IM) and *Rhizomucor miehei* lipase (Lipozyme RM IM) were kindly donated by Novozymes A/S, Denmark. The commercial extra virgin olive oil was purchased in a local market and the Arabic gum was from Synth, Brazil. Caprylic acid (C8:0) (>98% purity), capric acid (C10:0) (>98% purity), ethyl caprylate (C8 Ethyl) (>98% purity), ethyl caprate (C10 Ethyl) (>98% purity) and N-Methyl-N trimethylsilyltrifluoroacetamide (MSTFA; >90%) were from TCI Europe N.V., Belgium Glutaraldehyde (25%, *v/v* aqueous solution) was purchased from Merck, Germany. The standard of mononanadecanoin (99%) was acquired from Larodan Fine Chemicals AB, Sweden, and pyridine with purity *p.a.*, was obtained from Panreac Química S.L.U., Barcelona, Spain. The other reagents used were analytical grade.

2.2. Determination of the Proximate Composition of Coffee Silverskin and Spent Coffee Grounds

The proximate composition of samples of coffee silverskin and spent coffee grounds was determined by quantifying the moisture, ash, lipid and protein contents. The percentage of carbohydrates was determined by a difference of 100. The samples were analyzed in triplicate according to the AOAC method [36].

2.3. Oil Extraction from Spent Coffee Grounds

Before lipid extraction, the water content of SCG and CS samples was removed by drying at approximately 60 ± 2 °C until the humidity was below 10%. For oil extraction, 20 g of dried SCG or CS were extracted with 150 mL hexane under reflux for 8 h in a Soxhlet apparatus. After, the solvent was evaporated at 60 °C using a rotary evaporator and the crude oil recovered [36].

2.4. Quality Parameters and Fatty Acid Composition of Spent Coffee Grounds and Coffee Silverskin Crude Oils

The acidity and UV specific absorbances (K_{232} and K_{270}) assays were carried out following the analytical methods described by the Commission Regulation of the European Union for olive and olive pomace oils (EEC) No 2568/91 [37]. The acidity consists of the content (%) of free fatty acids (FFA), expressed as linoleic acid (MW = 280.4) which is the major fatty acid of SCG and CS oils. The values of specific extinctions at wavelengths of 232 nm and 270 nm, K_{232} and K_{270}, can provide information on the quality of a fat, since they are related to initial and final oxidation products, respectively. The values of K_{232} and K_{270} were assayed for solutions of 1% oil in iso-octane (*w/v*) in a UNICAM UV/V is spectrophotometer, using quartz cells.

The fatty acid composition of SCG and CS crude oils was assayed as fatty acid methyl esters (FAME) in a gas chromatograph (GC) Shimadzu GC-2010 Ultra (Shimadzu, Tokyo, Japan) coupled with a mass spectrometry detector with quadrupole mass analysis (GC/qMS-QP). A SUPELCOWAXTM 10 (Polyethylene glycol) capillary column (30 m × 0.25 mm ID × 0.25 μm film) was used. The injection (1.0 μL) was performed in the split mode (1:5) and helium was used as the carrier gas. The column temperature was programmed as follows: 90 °C for 1 min, a temperature increase at 3 °C·min^{-1} to 270 °C and then held 10 min at this final temperature. Detector and injector temperatures were 270 and 260 °C, respectively. The Shimadzu software GC Solution (Shimadzu, Tokyo, Japan) with an operational system

in SCAN mode, allowing the comparison of the resulting spectra with those of the equipment's spectrum library, was used. In addition, a standard solution containing seven compounds (methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl arachidate, methyl behenate and ethyl linoleate as internal standard) was used for fatty acid methyl ester identification. The quantitative analysis of the esters was performed with the selection of ions that could be monitored by the SIM mode (Single Ion Monitoring—monitoring of selected ions) and quantified separately.

2.5. Preparation of Supports

The coffee silverskin was dried at approximately 60 °C until the moisture content was below 10%. After drying, particles between 9 and 32 mesh were recovered by sieving and used for subsequent oil extraction and biochar production by pyrolysis, after oil extraction (cf. 2.3). The pyrolysis process was performed using a bench-scale equipment consisting of a stainless steel fixed-bed reactor with a vertical furnace. The reactor was fed with 50 g of de-oiled silverskin and then purged with nitrogen gas before starting the process. Then, the silverskin was heated from 25 °C to the 550 °C at a rate of approximately 10 °C min^{-1} and maintained at 550 °C for 15 min. After cooling the reactor, the solid product (biochar) was recovered.

The hybrid support was produced using the sol–gel process, according to Martins et al. [34] with some modifications: 30 mL of tetraethoxysilane (TEOS) were diluted in 36 mL of absolute ethanol (99%) under nitrogen. The ethanolic solution was stirred for about 5 min at room temperature. Then, 0.22 mL of hydrochloric acid (36%, *v/v*) solution in ultra-pure water (pre-hydrolyzing solution) were slowly added to this mixture and stirred (200 rpm) for 90 min at 35 °C. For the preparation of the hybrid support, 3.0 g of biochar was added. Next, the hydrolysis solution (1 mL of ammonium hydroxide dissolved in 6 mL of ethanol) was added. The sample was kept under static conditions for 24 h to finish the chemical condensation. After this period, the gel was removed and washed with hexane and kept in a desiccator for 72 h.

2.6. ROL Immobilization

The immobilization procedures for ROL on Amberlite IRA96 (AMB), silica (SIL), biochar (BIO) and hybrid support (HB) were carried out according to the methodology described by Costa et al. [25] with modifications. For the immobilization by physical adsorption (PA), 1 g of support and the lipase solution (0.25 g of ROL dissolved in 10 mL of sodium phosphate buffer 0.2 M, pH 7.5) were mixed together at 28 °C, under gentle magnetic stirring for 6 h. The immobilized ROL was washed with 20 mL of sodium phosphate buffer and filtered under reduced pressure (three times). A similar methodology was used for the immobilization by covalent reticulation (CR), but after the previous procedure, the particles were soaked in glutaraldehyde aqueous solution (2.5%, *v/v*) (25 mL/1 g support) for 2 h, under gentle magnetic stirring. The immobilized biocatalyst was then washed three times with 20 mL of sodium phosphate buffer, dried under vacuum filtration and stored at 4 °C until use.

2.7. Determination of Hydrolytic Activity

The hydrolytic activity of free and immobilized ROL was assayed by titration of the free fatty acids released during the hydrolysis of extra virgin olive oil in Arabic gum stabilized emulsion, according to the method described by Soares et al. [38]. The amount of enzyme needed to hydrolyze olive oil releasing 1.0 μmol of fatty acids min^{-1}, under the assay conditions, corresponds to one unit of lipase activity (U). The hydrolytic activity of free and immobilized preparations was expressed in U·g^{-1}. The relative activity (%) of the enzyme preparations was calculated considering the highest hydrolytic activity found equal to 100%.

2.8. Acidolysis and Interesterification Reactions

Acidolysis (crude oil with caprylic or capric acid) and interesterification (crude oil with ethyl caprylate or ethyl caprate) reactions were performed batchwise in closed thermostated 20 mL cylindrical

batch reactors according to Mota et al. [20], with modifications. A molar ratio TAG:FA or TAG:FAEE of 1:2 was used. The substrate mixture consisted of 6.52 g of SCG oil and 2.05 g of caprylic acid (C8:0) or 2.45 g of capric acid (C10:0) or 2.33 g of caprylic acid ethyl ester (C8 Ethyl) or 2.45 g of capric acid ethyl ester (C10 Ethyl). The reactions were carried at 50 °C, with Lipozyme TL IM and Lipozyme RM IM, or 40 °C, with immobilized ROL on different supports. A biocatalyst load of 5% (*w/w*) of the total TAG (SCG or CS oil) was used in each experiment (0.27 g of immobilized lipase). Samples of 0.05 mL of reaction medium were taken along the reaction time. In screening experiments, samples were withdrawn at 0, 24, and 48 h. In time-course experiments, samples were taken at 0, 3, 5, 7, 24, 30 and 48 h. All the samples were stored at −18 °C for subsequent analysis. Reactions were carried out in triplicate.

2.9. Batch Operational Stability Tests

The operational stability of the Lipozyme RM IM was assayed under the same conditions followed in the acidolysis and interesterification reactions, along ten consecutive 7 h-batch reuses, according to Mota et al. [20]. Several deactivation models were tested using "Solver" add-in from Excel for Windows, by minimizing the residual sum-of-squares between the experimental data points and those estimated by each model.

2.10. Analysis of Reaction Products

The identification and quantification of reaction products, namely initial and new triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), consumed medium-chain fatty acids (C8:0 or C10:0) or their ethyl esters (C8 Ethyl or C10 Ethyl), and released long-chain fatty acids from the oil, were analyzed by gas chromatography (Agilent Technologies 7820A, Santa Clara, CA, USA) equipped with an on-column injector, a flame ionization detector and a DB5-HT capillary column (15 m × 0.32 mm ID × 0.10 μm film). The procedure followed was adapted from that described by the European Standard (EN 14105) for detection of trace amounts of glycerol, MAG, DAG and TAG in purified biodiesel (FAME) [39], as previously described [20]. A calibration curve for trilinolein was used to quantify the formation of new TAG, due to the lack of standards of MLM or MLL. The conversions (TAG, C8:0, C10:0, C8 Ethyl orC10 Ethyl) were calculated by the ratio between the amount of each consumed compound and its initial amount (%). The yield of the new TAG was calculated as mass yield (%, *w/w*), i.e., the ratio between the amount of new TAG produced and the amount of initial TAG (%).

2.11. Statistical Analysis

For each system with new biocatalysts and/or commercial immobilized lipases, eventual significant differences between the performance of the biocatalysts, for the production of new TAG, was investigated by one-way analysis of variance (ANOVA). The software Statistica, version 8, from Statsoft, Tulsa, OK, USA, was used and post-hoc comparisons were carried out using the Tukey test at a *p*-value of 0.05.

3. Results and Discussion

3.1. Proximate Composition of Coffee Silverskin and Spent Coffee Grounds and Characterization of Respective Oils

The proximate composition values obtained for coffee silverskin and spent coffee grounds samples are presented in Table 1. The moisture content of SCG and CS was 72.6% and 8%, respectively. To better compare the data of Table 1, they were converted into dry weight (d.w.) values: CS was richer in carbohydrates while SCG was richer in protein and oil content. In fact, the protein contents of CS and SCG were 17.1 and 42.3% (d.w.), respectively; the carbohydrate content in CS was 65.7% and was only 5.8% in SCG, while fat contents of CS and SCG were 4.2 and 50.4%, respectively.

Table 1. The proximate composition values obtained for coffee silverskin (CS) and spent coffee grounds (SCG).

Component (%)	CS (This Study)	SCG (This Study)	CS [10]	SCG [40]
Protein	15.8 ± 0.9	11.6 ± 1.2	19 ± 0.3	14.2 ± 0.7
Fat	3.9 ± 0.2	13.8 ± 1.1	2.4 ± 0.1	12.5 ± 1.6
Carbohydrates	60.4 ± 4	1.6 ± 0.7	62.2 ± 0.4	-
Ash	7.9 ± 3.2	0.4 ± 0.2	8.3 ± 0.04	2.0 ± 0.7
Moisture	8 ± 0.9	72.6 ± 0.4	4.8 ± 0.1	65.0 ± 3.0

The variations between the compositions found for SCG and CS in this study and those reported in the literature can be due to different varieties of coffee beans used, different geographic origins of coffee plants with different climate and soil conditions, different age of the coffee plants, different fruit processing and roasting technologies [41–43]. According to the literature, the lipid content of CS and SCG depends on the brewing and extraction methods with values ranging from 1.6% to 3.4% and 10 to 15%, respectively, being triacylglycerols the main components of the oils [2,11,13,42,44].

The parameters related to the quality and fatty acid composition of the crude SCG and CS oils are presented in Tables 2 and 3, respectively. The acidity of CS oil (6.75%) was higher than that of SCG oil (4.6%). The extent of oil degradation is related to the free fatty acid (FFA) percentage of oil, which is an important quality parameter. The low values of K_{232} and K_{270} in both crude SCG and CS oils indicate a low extent of oxidation, mainly in SCG oil. The K_{232} is associated with the presence of conjugated hydroperoxides resulting from the first stage of oxidation, while K_{270} is related to the presence of secondary oxidation products, namely short-chain fatty acids, aldehydes and ketones [45]. These compounds were possibly formed from unsaturated fatty acids such as linoleic acid (C18:2) with high concentrations in SCG and CS oils in the present study.

Table 2. Acidity and UV absorption at 232 and 270 nm (K_{232} and K_{270}) of spent coffee grounds (SCG) and coffee silverskin (CS) oils (average value of three repetitions ± standard deviation).

Parameter	SCG Oil	CS Oil
Free fatty acids (%, *w/w*)	4.6 ± 0.3	6.75 ± 0.2
K_{232}	0.185 ± 0.003	0.257 ± 0.004
K_{270}	0.229 ± 0.005	0.329 ± 0.003

Table 3. Fatty acid composition of spent coffee grounds (SCG) and coffee silverskin (CS) oils.

Fatty Acid Group	Fatty Acid	SCG Oil (%)	CS Oil (%)
Saturated fatty acids (SFA)	Palmitic (C16:0)	32.8	34.4
	Stearic (C18:0)	10.9	9.3
	Arachidic (C20:0)	5.6	7.6
	Behenic (C22:0)	2.8	8.9
Monounsaturated fatty acids (MUFA)	Oleic (C18:1)	11.1	8.3
Polyunsaturated fatty acids (PUFA)	Linoleic (C18:2)	36.8	31.5
Σ SFA		52.1	60.2
Σ MUFA		11.1	8.3
Σ PUFA		36.8	31.5

The fatty acid profiles of SCG and CS oils are very similar since they both present the same fatty acids, with palmitic (C16:0) and linoleic (C18:2) being the major fatty acids. In the spent coffee grounds oil, linoleic acid was found in greater concentration (36.8%), followed by palmitic acid (32.8%) and oleic acid (C18:1; 11.1%). The stearic acid (C18:0) represented 10.9% while arachidonic (C20:0) and behenic (C22:0) fatty acids were detected in lower concentrations. The fatty acid profile of coffee

silverskin found in this study shows that the concentration of palmitic acid (34.4%) was higher than that of linoleic acid (31.5%), followed by the content of stearic acid with 9.3%. The amounts of C22:0, C18:1 and C20:0 acids were between 7.6 and 8.9%. In both SCG and CS oils, the total concentration of saturated fatty acids (52.1 and 60.2% of SFA) was higher than polyunsaturated fatty acids (36.8 and 31.5% of PUFA) and monounsaturated fatty acids (11.1 and 8.3% of MUFA), respectively (Table 3).

Similar results, concerning the fatty acid profile of the oils under study, were reported in the literature. Costa et al. [11] and Toschi et al. [13] characterized silverskin oil, also obtained by hexane extraction, where the C18:2 was the major fatty acid (24.4 and 29.2%), followed by C16:0 (22 and 27.5%), C22:0 (15.4 and 11.5%) and C20:0 (13.5 and 10.8%), respectively. In those studies, saturated fatty acids were found in higher concentrations (62.7%), followed by polyunsaturated fatty acids (29.3%) and monounsaturated fatty acids (7%). Therefore, these profiles were similar to the fatty acid profiles observed in the present study. Araújo et al. [46], Tarigan et al. [3] and Passadis et al. [17] obtained SCG oil samples with a higher concentration of linoleic acid (44.1–45.43%) and palmitic acid (31.31–33.43%), respectively. The total saturated fatty acid content was 43%, total monounsaturated fatty acid content was 11.1% and the total polyunsaturated fatty acid content was 45.4% [3,17,46].

Thus, from the characterization of the SCG and CS biomasses and respective oils, the following possible applications were selected to be tested: (i) the silverskin of the extracted coffee was used for biochar production since its oil content is low and (ii) crude spent coffee grounds oil was used as raw material for the synthesis of low-calorie structured lipids. The literature already reports biochar as a promising material to be used as enzyme immobilization support [8–10,33]. However, no hybrid support of silverskin biochar and silica was tested yet. This hybrid support was produced by the sol–gel method for lipase immobilization and applied to the synthesis of structured lipids.

3.2. Determination of Hydrolytic Activity

Table 4 presents the relative hydrolytic activities of the biocatalysts, namely ROL immobilized on Amberlite IRA96 (AMB), silica (SIL), biochar prepared with silverskin (BIO), or hybrid support prepared with silverskin biochar and silica (HB). It is important to notice that Amberlite resin IRA96 (anion exchange resin with a weak macroreticular base) was used as a reference. This is a support reported in the literature for the immobilization of ROL which was used as biocatalysts for the production of structured lipids [26]. The highest hydrolytic activity of 239 U·g^{-1} (std = 15.8) was obtained for ROL immobilized in HB by physical adsorption which corresponds to a relative activity of 100%. This hydrolytic activity is 2.3 times the activity of ROL immobilized on the same support by covalent reticulation (105 U·g^{-1}; std = 23.6). In fact, ROL-HB obtained by covalent reticulation presented the lowest hydrolytic activity among the biocatalysts tested (44% relative activity). The activities of ROL, immobilized by physical adsorption on biochar (156 U·g^{-1}; std = 11.7) or silica (153 U·g^{-1}; std = 11.9), were similar to those obtained by their counterparts prepared by covalent reticulation (63–65% relative activities). The lowest activity obtained by physical adsorption was when ROL was immobilized on the reference support Amberlite (135 U·g^{-1}; std = 29.3; 57% relative activity). When the immobilized preparations were obtained by covalent reticulation, the activity of ROL on Amberlite (160 U·g^{-1}; std = 27.7) was slightly higher than those of ROL on SIL (155 U·g^{-1}; std = 28.3) and ROL on BIO (151 U·g^{-1}; std = 31.2).

It is important to note that in the present study, the hydrolytic activities of the ROL preparations on the different supports (AMB, SIL, BIO and HB), by physical adsorption and covalent reticulation, were determined only to verify the efficiency of the immobilization protocols. However, the hydrolytic activity of an immobilized ROL is not always correlated with its catalytic activity in other reactions, such as acidolysis and interesterification [20,26,47]. Several authors reported the immobilization of ROL on several types of support (Accurel MP 1000, Lewatit VP OC 1600, Eupergit C, Amberlite IRA 96, magnetic nanoparticles) with hydrolytic activity ranging from 35.5 to 4150 U·g^{-1}. These biocatalysts were used in the synthesis of functional structured lipids [20,26,47]. Therefore, all the biocatalysts were

used for screening in the acidolysis reaction with coffee grounds oil with caprylic acid (C8:0) or capric acid (C10:0).

Table 4. Determination of relative hydrolytic activity of *Rhizopus oryzae* lipase (ROL) immobilized on different supports (Amberlite IRA96—AMB; silica—SIL; biochar—BIO; and hybrid support—HB) by physical adsorption (PA) or covalent reticulation (CR).

	Biocatalysts	Relative Hydrolytic Activity (%)
PA	ROL-AMB	57
	ROL-SIL	64
	ROL-BIO	65
	ROL-HB	100
CR	ROL-AMB	67
	ROL-SIL	65
	ROL-BIO	63
	ROL-HB	44

3.3. Screening of Biocatalysts in Acidolysis and Interesterification Reactions

The screening of new biocatalysts was carried out for the production of low-calorie TAG after 24 and 48 h acidolysis of crude SGC oil with caprylic (C8:0) or capric acid (C10:0), in solvent-free media.

Figure 1 shows that all the new biocatalysts and commercial immobilized lipases presented catalytic activity in acidolysis or interesterification reactions. Figure 1a indicates that ROL immobilized on the hybrid support by physical adsorption showed a slightly better yield of new TAG than ROL immobilized on Amberlite IRA 96 or biochar: 13.7 and 15.4% with C8:0 and 12.8 and 15.8% with C10:0, after 24 and 48 h acidolysis, respectively. When ROL immobilized on silica by physical adsorption was used, the reaction was rather slow and only after 48 h acidolysis, the yield in new TAG containing C8:0 or C10:0 was 8.2% and 10.6%, respectively. A preference of ROL for C10:0 over C8:0 was only observed after 48 h acidolysis for ROL immobilized by physical adsorption on Amberlite or silica supports.

Figure 1b shows that ROL immobilized on silica by covalent reticulation showed the highest yields of new TAG with C8:0 (16.4%) and with C10:0 (21.9%), after 24 h acidolysis. The decrease in the new TAG yield was observed for this biocatalyst after 48 h-reaction, probably due to the reversibility of the acidolysis reaction. In fact, acidolysis consists of a two-step reversible reaction: first, the hydrolysis of the ester bonds in TAG occurs which is followed by the esterification of the new fatty acids in TAG, until equilibrium is attained [48].

The biocatalysts prepared by physical adsorption on hybrid support showed a slightly better hydrolytic activity and higher yield in the new TAG. However, considering covalent crosslinking, the ROL immobilized in AMB showed greater hydrolytic activity, while the lipase immobilized in silica provided greater yield in new TAG by acidolysis. This proves that the hydrolytic activities of lipase preparations used in this study are not directly related to their catalytic activity in acidolysis, as previously observed for other biocatalysts applied in the production of structured lipids [26,47].

The performance of the commercial immobilized lipases, Lipozyme RM IM and Lipozyme TL IM, in the acidolysis of crude SCG oil with C8:0 or C10:0, and also in the interesterification with the respective ethyl esters (C8 Ethyl and C10 Ethyl) is shown in Figure 1c. For all the systems tested, the yields in new TAG were always much higher using these commercial biocatalysts than the values attained with immobilized ROL preparations prepared by us (Figure 1a,b). In the acidolysis with C8:0 or C10:0, the commercial preparations showed yields of new TAG greater than 50%, with the exception of the reaction with C10: 0 catalyzed by Lipozyme TL IM (39.4% after 24 h and 48.8% yield after 48 h). A preference of Lipozyme RM IM towards C10:0 and of Lipozyme TL IM towards C8:0 was observed in acidolysis.
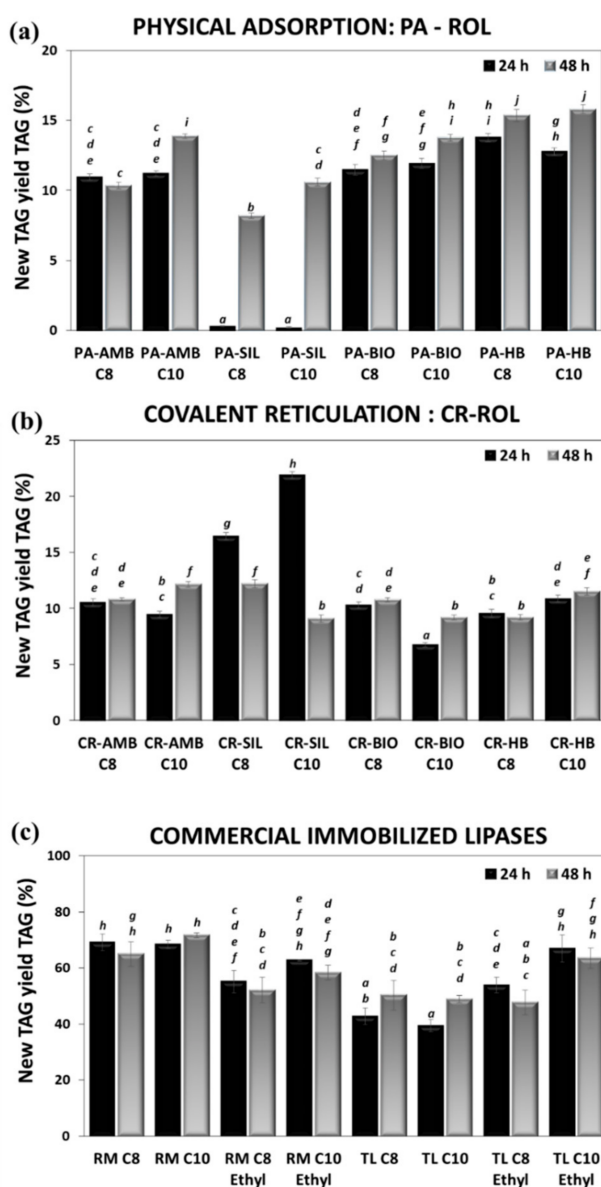
Figure 1. New TAG yield obtained (**a**) by acidolysis reactions of crude spent coffee grounds oil with caprylic acid (C8) or capric acid (C10), at 40 °C, catalyzed by *Rhizopus oryzae* lipase (ROL) immobilized by physical adsorption (PA) or (**b**) immobilized by covalent reticulation (CR) on Amberlite IRA96 (AMB), silica (SIL), biochar (BIO) or hybrid support (HB), or (**c**) New TAG yield obtained by acidolysis or interesterification with C8 Ethyl or C10 Ethyl catalyzed by the commercial enzymes Lipozyme RM IM (RM) or Lipozyme TL IM (TL), after 24 (■) or 48 h (▨) reaction at 50 °C, in solvent-free media. For each biocatalyst and reaction system, different superscript indexes indicate significant differences based on the Tukey test ($p \leq 0.05$).

Due to these results obtained with the commercial immobilized enzymes, the interesterification reaction of the coffee grounds oil with C8 Ethyl or C10 Ethyl was only carried out with these biocatalysts (Figure 1c). In the interesterification reaction, yields of new TAG were greater than 50%, either with Lipozyme RM IM or Lipozyme TL IM. The preference for capric acid ethyl ester over caprylic ethyl ester was observed for both Lipozyme RM IM and Lipozyme TL IM. However, the yields in new TAG were higher by acidolysis than by interesterification, when Lipozyme RM IM was used. Conversely, a preference of Lipozyme TL IM towards interesterification over acidolysis was observed. Similar behavior of Lipozyme TL IM was observed when crude olive pomace oil was

used to produce SL rich in caprylic acid [20]. Lipozyme TL IM showed to be an efficient catalyst mainly in the interesterification reactions. However, this biocatalyst presents lower physical and mechanical stability than Lipozyme RM IM. The mechanical stability of the support is also an important and crucial parameter for many applications of immobilized lipases. Biocatalyst particles with low mechanical stability will disintegrate during the bioprocesses, affecting negatively biocatalyst catalytic activity, its operational and thermal stability [49]. Therefore, the kinetic studies were performed only with Lipozyme RM IM to optimize the reaction-time and avoid reversible reactions, since lipases are hydrolytic enzymes that reversibly catalyze the cleavage of the ester bonds of the triacylglycerols [48].

3.4. Acidolysis and Interesterification Kinetics

The acidolysis kinetics of crude SGC oil with caprylic (C8:0) or capric acid (C10:0) and of interesterification with ethyl caprylate (C8 Ethyl) or ethyl caprate (C10 Ethyl), catalyzed by Lipozyme RM IM, was evaluated along 48 h reaction time. The time-courses were followed by the production of new TAG, TAG consumption, medium-chain fatty acid (C8 or C10) or medium-chain fatty acid ethyl ester (C8 Ethyl or C10 Ethyl) consumptions, monoacylglycerol (MAG) and diacylglycerol (DAG) contents in the reaction medium (Figure 2). As already mentioned, the enzymatic acidolysis reaction is considered as a two-step reversible reaction: hydrolysis followed by esterification. Through these steps, TAGs are hydrolyzed, the long-chain fatty acids of the original TAG are released, and the medium-chain fatty acids present in the reaction medium are incorporated into acylglycerols yielding new TAGs, until the equilibrium is attained. Thus, in acidolysis as well as in interesterification (ester–ester exchange), DAG and MAG are intermediate compounds. During the reactions catalyzed by the *sn*-1,3 regioselective lipase (Lipozyme RM IM), only the long-chain FA at positions *sn*-1,3 of TAGs of SCG oil are released to the reaction medium and C8:0 (or C10:0) molecules are esterified to form new DAG or new TAG. The obtained structured lipids can be new TAG of the types MLL or MLM, depending on if only one or both original long-chain fatty acids (L) at positions *sn*-1,3 were replaced by the C8 or C10 (medium-chain fatty acids, M).
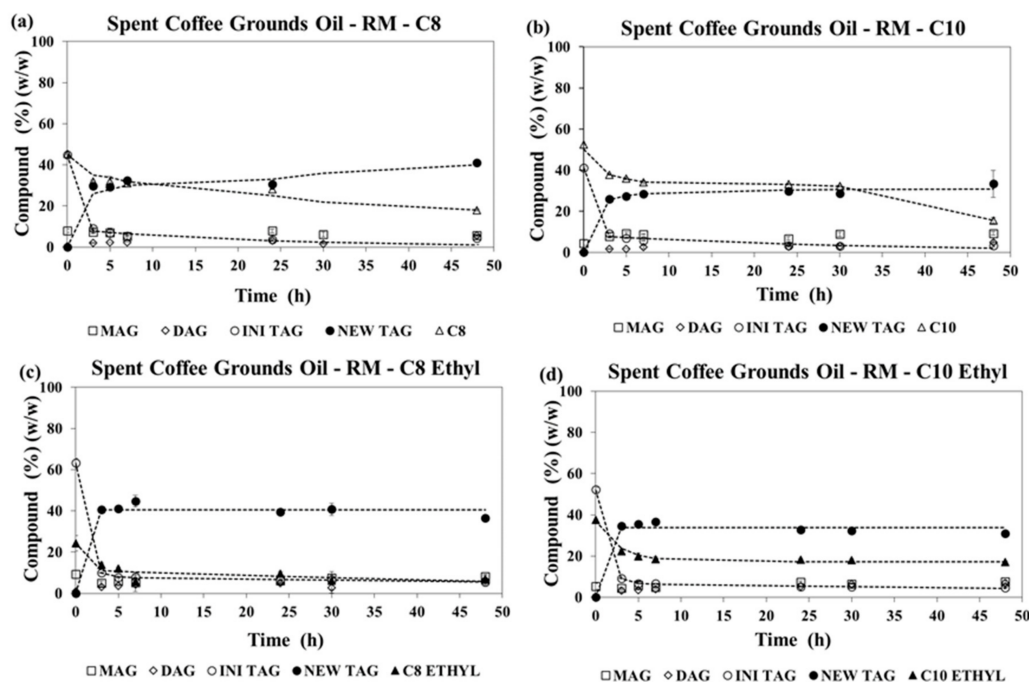

Figure 2. Time-course of acidolysis or interesterification of crude spent coffee grounds oil with (**a**) caprylic acid (C8:0); (**b**) capric acid (C10:0); (**c**) caprylic acid ethyl ester (C8 Ethyl); or (**d**) capric acid ethyl ester (C10 Ethyl), catalyzed by Lipozyme RM IM, at 50 °C in solvent-free media.

Figure 2 shows that both acidolysis and interesterification attained a quasi-equilibrium, in terms of new TAG formation, after around 7 h reaction time. The intermediate acylglycerols, MAG and DAG, did not very much increase along the 48 h reaction.

To better analyze and compare the different time-courses, TAG conversion and yield of new TAG obtained by acidolysis or interesterification of crude spent coffee grounds oil with caprylic acid or capric acid or their ethyl esters (C8 Ethyl or C10 Ethyl), catalyzed by Lipozyme RM IM, were calculated. The results are presented in Figure 3. Reactions were rather fast, reaching TAG conversion values of about 90% in less than 7 h reaction, independently from the reaction or medium-chain fatty acid or respective ethyl ester used. Considering the production of new TAG, Lipozyme RM IM showed its preference for the acidolysis with C8 over the interesterification with C8 Ethyl (75% and 61%, after 7 h reaction, respectively). Concerning the production of new TAG containing C10, no preference between acidolysis or interesterification was observed, during the first 7-h reaction (new TAG yields of 70%). However, after attaining the maximum new TAG yield, a slight decrease in yield was observed along the time-course, mainly in the interesterification reactions. Again, this may be due to the reversibility of these two-step reactions.
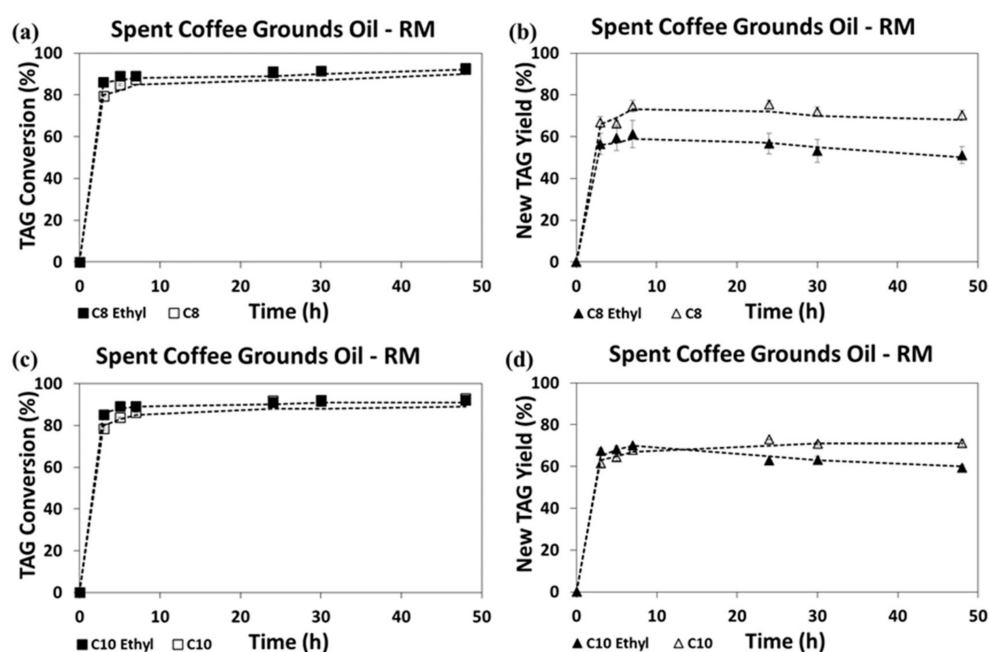

Figure 3. TAG conversion and yield of new TAG obtained by acidolysis or interesterification of crude spent coffee grounds oil with (**a**) caprylic acid (C8:0); (**b**) capric acid (C10:0); (**c**) caprylic acid ethyl ester (C8 Ethyl); or (**d**) capric acid ethyl ester (C10 Ethyl), catalyzed by Lipozyme RM IM, at 50 °C in solvent-free media.

Mota et al. [20] immobilized ROL on magnetite nanoparticles (MNP-ROL) and used the novel biocatalyst in the production of structured lipids from crude spent coffee grounds and olive-pomace oils, by acidolysis with capric acid, or by interesterification with ethyl caprate. The performance of MNP-ROL was compared with that of Lipozyme TL IM. For both oils, Lipozyme TL IM showed a preference for interesterification over acidolysis: after 7 h-interesterification of SCG oil with C10 Ethyl, a new TAG yield of 65% was obtained while only 48% was achieved after 48 h acidolysis. MNP-ROL catalyzed reactions were faster and acidolysis was preferred over interesterification with yields of 50% new TAG after 3 h reaction with the SCG oil.

Willett and Akoh [50] used menhaden oil for the production of structured lipids, by acidolysis with capric acid or by interesterification with capric acid ethyl ester, catalyzed by immobilized recombinant lipase from *Candida antarctica* (Lipozyme 435) and Lipozyme RM IM. They also observed higher

incorporation levels of C10:0 by interesterification with capric acid ethyl ester as the acyl donor than by acidolysis with capric acid. Nunes et al. [51] showed that Novozym 435, Lipozyme RM IM and Lipozyme TL IM were efficient catalysts to incorporate caprylic and capric acids in virgin olive oil, either in a system with hexane or in the absence of a solvent. The highest values of molar incorporation in TAG were observed in solvent-free media for all the lipases used, which is the preferred option for the food industry. All the biocatalysts tested showed a preference for C10 (27.1–30.4 mol% incorporation in TAG) over C8 (20–25 mol% incorporation) after 24 h acidolysis.

3.5. Batch Operational Stability Tests

The reuse of biocatalyst with high stability is a way to make enzymatic processes economically feasible. Operational stability tests were carried out for Lipozyme RM IM in 10 consecutive batch reuses, either in acidolysis or interesterification, since similar results in the time-course reactions with crude SCG oil were obtained. The duration of each batch was 7-h because it was the time needed to attain the maximum new TAG yield. Acidolysis and interesterification activities were evaluated in terms of new TAG yield. The residual activity of Lipozyme RM IM, at the end of each 7 h-batch, is presented in Figure 4. For both systems, in terms of new TAG yield, the observed behavior can be described by first-order deactivation kinetics or sigmoid model described by logistic differential equations.
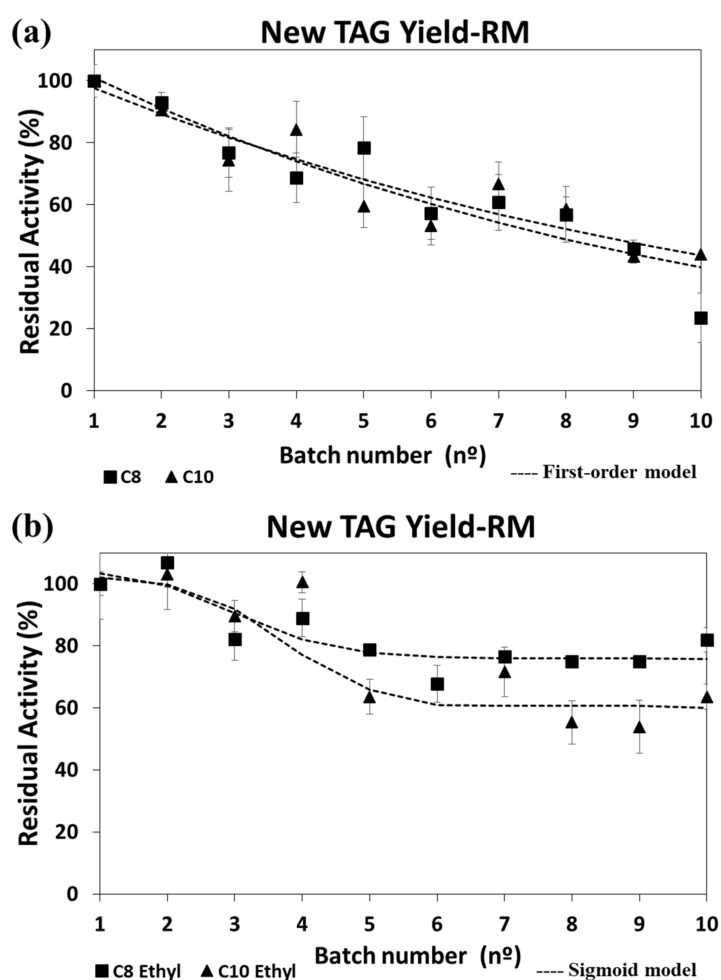

Figure 4. Residual activity of Lipozyme RM IM, assayed by new TAG Yield, along ten 7 h-consecutive batches of (**a**) acidolysis of crude spent coffee grounds oil with caprylic acid (C8) or capric acid (C10) described by first-order deactivation kinetics, or (**b**) interesterification reactions with caprylic acid ethyl ester (C8 Ethyl) or capric acid ethyl ester (C10 Ethyl) described by logistic differential equations (Sigmoid model).

Thus, the first-order model is given by the following general equation:

$$A_t = A_0 e^{-k_d \times t} \tag{1}$$

where A_t is the biocatalyst residual activity (%) at time t (or batch n), A_0 is the initial activity and k_d is the deactivation rate constant.

The sigmoid model is given by the following logistic differential equation:

$$A_t = B + \frac{A - B}{1 + e^{-(c + d \times t)}} \tag{2}$$

where A is the value of the upper plateau (initial activity), B is the value of the lower plateau (final activity), c and d are constants. In batch reuses, t is replaced by the batch number, n.

When the deactivation is described by the first-order model (Equation (1)), the half-life time ($t_{1/2}$) of the biocatalyst, referring to operation time required for half the biocatalyst activity to be lost, is given by Equation (3):

$$t_{1/2} = \frac{Ln2}{k_d} \tag{3}$$

The deactivation models fitted to the experimental data, the determination coefficients of these models, as well as half-life times of each biocatalyst are presented in Table 5. A good fit of the first-order or sigmoid model to the experimental data points (batch number, residual activity) was observed.

Table 5. Deactivation models fitted to Lipozyme RM IM in the systems with caprylic (C8:0) or capric (C10:0) acids, and caprylic acid (C8 Ethyl) or capric acid (C10 Ethyl) ethyl esters, respective determination coefficients (R^2) and half-life times ($t_{1/2}$) of the biocatalysts.

System	Model	Model Equation	R^2	Half-Life Time (h)
		New TAG Yield		
C8:0	First-order	$A_t = 112.13 e^{-0.10 \times n}$	0.934	47
C10:0	First-order	$A_t = 106.87 e^{-0.09 \times n}$	0.929	54
C8:0 Ethyl	Logistic model	$A_t = 75.82 + \frac{29.43}{1 + e^{-(4.04 - 1.34 \times n)}}$	0.877	Not attained
C10:0 Ethyl	Logistic model	$A_t = 60.63 + \frac{42.04}{1 + e^{-(5.58 - 1.50 \times n)}}$	0.884	Not attained

Figure 4 shows that the deactivation of Lipozyme RM IM in acidolysis followed a similar first-order model, either in presence of caprylic or capric acid, with half-life times of 6.7 and 7.7 batches, corresponding to 47 and 54 h, respectively (Table 5). In interesterification reactions, the deactivation profile of the biocatalyst was well described by a sigmoid model. When caprylic acid ethyl ester was used, a decrease in the initial activity to around 80% of the initial value was observed after the third batch, but this residual activity was maintained along the subsequent reuses of Lipozyme RM IM. In the interesterification of SCG oil with capric acid ethyl ester, Lipozyme RM IM presented a decrease in its initial activity along the first five batches to c.a. 65%. Again, this residual activity was maintained thereafter. The sigmoid model might suggest a series-type inactivation of the biocatalyst, since this profile is observed on thermodynamically stable intermediates on the folding/unfolding pathway of proteins [52]. The residual activity observed in the lower plateau may be due to the presence of a stable and active intermediate form of the enzyme. In these situations, it was not possible to calculate the half-life of Lipozyme RM IM because the lower plateaus occurred for residual activity values higher than 50% of the initial activity (Figure 4).

Lipozyme RM IM presented better operational stability in interesterification than in acidolysis.

Mota et al. [20] observed that *R. oryzae* lipase immobilized on magnetic nanoparticles showed higher operational stability in the interesterification of crude SCG oil with C10 Ethyl than in the acidolysis with capric acid, in solvent-free media. In both reactions, this biocatalyst followed a series-type Sadana deactivation model represented by a parabolic profile that opens downwards [53].

No deactivation of Lipozyme RM IM was observed by Nunes et al. [51] after 10 consecutive 23-h uses in the acidolysis of virgin olive oil with capric acid, in solvent-free media. However, in the acidolysis of virgin olive oil with caprylic acid, Lipozyme RM IM followed a first order deactivation kinetics and exhibited a half-life of 299 h [51]. The operational stability of Lipozyme RM IM in this work carried out by Nunes et al. [51] was much higher than that noticed in the present study. It is important to notice that in Nunes' work, virgin olive oil with an acidity lower than 0.8% was used, whereas in the present work, crude oil from SCG with 4.6% FFA was used. The presence of high amounts of free fatty acids in the reaction medium can interfere with the stability of some lipases, as previously observed [20,54]. Moreover, in the studies of Nunes et al. [51], the operational stability was evaluated along 23-h consecutive batches, while in the present work, operational stability tests were performed in 7 h batches. It is possible that during 23 h batches a slight deactivation might occur without being detected due to the extent of each batch which compensates for the slower reaction rates.

Zhang et al. [29] produced structured low-calorie TAG by interesterification of soybean oil with TAG rich in caprylic and capric acyl groups, in solvent-free media, catalyzed by Lipozyme TL IM. This biocatalyst was used in a pilot-scale packed-bed batch reactor (to avoid particle disintegration) and reused for 25 successive batch reactions (16 min/batch). No significant reduction in catalytic efficiency was observed during a total of 6.7 h operation.

4. Conclusions

The results obtained in this study are of utmost importance showing that the wastes from coffee extraction industries can be used to produce added-value products, decreasing environmental impacts, and improving the revenues of the coffee-related industries. The crude SCG oil can be used to produce low-calorie structured lipids either by acidolysis or interesterification, catalyzed by *sn*-1,3 regioselective lipases. Silverskin can be used to produce biochar for direct use as an enzyme carrier or to produce hybrid organic-silica supports. Best results were obtained with Lipozyme RM IM: 61–75% new TAG after 7 h reaction and high operational stability during interesterification or acidolysis.

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