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Interesterification of milkfat and soybean oil blends catalyzed by immobilized *Rhizopus oryzae* lipase

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ABSTRACT

Milkfat (MF)/soybean oil (SBO) blends ranging from 50% to 100% of milkfat (w/w) were enzymatically interesterified with a sn-1,3 specific lipase from *Rhizopus oryzae* immobilized on polysiloxane–polyvinyl alcohol matrix, in a solvent free medium. Interesterification progress was monitored by following the changes in the relative proportions of 50-carbon triacylglycerols (TAGs) to 44-carbon TAGs (50/44 ratio) in the reaction. The starting materials and products were also analyzed in terms of consistency measured in a texturometer. All reactions gave interesterified (IE) products with lower consistency than non-interesterified (NIE) MF:SBO blends and interesterification degree varied from 0.54 to 2.60 in 48 h reaction. The highest interesterification degree was achieved for 65:35 MF:SBO blends, which gave 76% reduction in the consistency. These results showed the potential of the immobilized lipase to change the TAGs profile of the MF:SBO blend allowing to obtain cold-spreadable milkfat.

1. Introduction

Milkfat is the third main lipid source for human nutrition having better flavor and mouth feel than any other edible fat. It has also good nutritional qualities as a source of essential fatty acids and fat-soluble vitamins [1,2]. Furthermore, it is a very complex mixture of thousands of different triacylglycerols with a high percentage of saturated fatty acids [3,4]. During recent years a shift in consumer preference is seen toward products containing less and healthier fat [1]. This change has given rise to new challenges for dairy industries to develop products that combine desirable nutritional and organoleptical characteristics with low production cost [1,5].

An alternative to achieve this target is to replace milkfat by partial hydrogenated vegetable oils, in the form of margarines and spreads. However, vegetal oil hydrogenation process generates also high amount of trans fatty acids as by-product. The ingestion of these fatty acids has been found to increase both the low density lipoprotein cholesterol (LDL-cholesterol), and decrease the high density lipoprotein cholesterol (HDL-cholesterol). This has motivated the food industry to reformulate products that could take advantage of the beneficial effects of milkfat as conjugated linoleic acids [6].

An interesting approach is to blend milkfat with vegetable oils either in the presence (interesterification reaction) or without catalyst (physical mixture). The former yielded a product with better spreadability properties and can be catalyzed by chemical or enzymatic routes [7]. Enzymatic interesterification is advantageous in comparison to chemical interesterification because enzymes (lipases) allows carrying out processes under mild reaction conditions (temperature and pressure) that results better preservation of the milkfat flavor [3,8].

In this work, the soybean oil (SBO) was chosen to be interesterified with milkfat (MF) due to its nutritional qualities, considerable economic value and high functionality [9]. The effect blends at different mass fractions between soybean oil and milkfat was evaluated in interesterification reactions using a commercial food grade lipase from *Rhizopus oryzae* immobilized on polysiloxane–polyvinyl alcohol hybrid matrix. This enzyme was previously selected showing to be the most suitable biocatalyst, among seven other lipases, to mediate this reaction [10]. The consistency was also evaluated, considering that the interesterification reaction modifies the texture of the interesterified blends [9], turning it more spreadable under cool temperature.

2. Materials and methods

2.1. Materials

A commercial food grade lipase from *R. oryzae* (L036P, Biocatalysts, Cardiff, England) in a crude form was used without further purification. Tetraethoxysilane (TEOS) and polyvinyl alcohol (PVA, MW 88,000) were acquired from Aldrich Chemical Co. (Milwaukee, WI, USA). Hydrochloric acid (minimum 36%), ethanol (minimum

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99%) and polyethylene glycol (PEG 1500 g/mol) were supplied by Synth (São Paulo, Brazil). Milkfat was obtained from commercial butter (Aviação, purchased in a local market), totally melted at 50–65 °C, in a microwave oven, followed by centrifugation to separate the aqueous phase. Commercial soybean oil (Liza Type 1, purchased in a local market) having the following composition in fatty acids: (w/w): 0.05% lauric, 0.01% myristic, 11.5% palmitic, 4.2% steriac, 21.4% oleic, 53.45% linoleic and 7.5% linolenic with average molecular weight 869.31 g/mol. Solvents were of standard laboratory grade (Synth, SP, Brazil). All the other reagents were of analytical degree.

2.2. Support synthesis and immobilization procedure

A polysiloxane–polyvinyl alcohol hybrid support (POS–PVA) was prepared by the hydrolysis and polycondensation of tetraethoxysilane according to methodology previously described [11] rending particles having the following properties: average pore diameter (22.91 Å); surface area BET (461.00 m^2/g) and porous volume (0.275 cm^3/g) [12]. The activation of POS–PVA particles was carried out with sodium metaperiodate (0.5 M) under agitation during 90 min, at 25 °C, in a dark place. Afterwards, the carrier was filtered under vacuum and washed in abundance with distilled water and phosphate buffer pH 8.0 (0.1 M) and dried at 60 °C for 24 h. Activated particles were soaked into hexane under stirring (100 rpm) for 1 h at 25 °C. Then, excess of hexane was removed and lipase was added at a ratio of 1:4 g of enzyme/gram of support. PEG-1500 (5 mg/g) was added together with the enzyme solution at a fixed amount (100 μL/g of support). Lipase-support system was maintained in contact for 16 h at 4 °C under static conditions. The immobilized lipase derivative was filtered (nylon membrane 62HD from Scheiz Seidengazefabrik AG, Thal Schweiz, Switzerland) under high pressure vacuum and thoroughly rinsed with dried hexane to attain immobilized derivatives with low water contents (<10%). To perform the assays, four immobilized derivatives were prepared having average hydrolytic activities of 4860 ± 340 U/g, using olive oil as substrate [13].

2.3. Interesterification of milkfat with soybean oil

The interesterification reactions were performed in duplicate under inert nitrogen atmosphere, in cylindrical glass reactors (80 mL), containing 40 g of binary blends of MF/SBO 50:50, 65:35, 80:20 and 100:0. Blends were incubated with immobilized derivative on POS–PVA at fixed loading of 970 U/g of reaction medium. Reactions were carried out for 48 h, at 45 °C, under magnetic agitation. The progress of the reaction was monitored by determining the free fatty acid contents, triacylglycerols (TAGs) compositions and consistency for both non-interesterified (NIE) blends and interesterified (IE) products.

2.4. Triacylglycerols (TAGs) profile in interesterification reactions

TAGs were analyzed by gas chromatograph using a Varian CG 3800 model (Varian, Inc., Corporate Headquarters, Palo Alto, CA, USA) equipped with flame-ionization detector and 3% OV1 Silpt-WBM 100/120 mesh in Silco Var packed column (Restek, Frankel Commerce of Analytic Instruments Ltda, SP, Brazil). Nitrogen was used as the carrier gas with a flow rate of 40 mL/min. The detector and injector temperatures were 350 and 370 °C, respectively. The column temperature was first set to 80 °C for 1 min and then programmed at 50 °C min^{-1} to 210 °C, which was kept constant for 1 min. Finally, the column temperature was programmed at 6 °C min^{-1} to 340 °C and kept constant for 2 min. The injection volume was 1 μL. Before injection, samples were diluted in 2 mL of heptane and tetradecane was added as internal standard. The chromatograms were processed using a Varian data integrator version 4.51 computational program. For the determination of TAGs calibration curve, milkfat standard from the Community Bureau of Reference Materials [14] was used. The groups of TAGs were identified by carbon number (CN), that is, the sum of the carbons of the fatty acids residues from one TAG. Based on these results, interesterification degree was calculated according to Eq. (1):

$$ID = \left| \left(\frac{[C_{50}]}{[C_{44}]} \right)_t - \left(\frac{[C_{50}]}{[C_{44}]} \right)_0 \right| \quad (1)$$

where C_{50} and C_{44} are the peaks with carbon number equal to 50 and 44, corresponding, respectively, to a highly variable and the most stable group of peaks during the reaction; the index "*t*" and (0) represent the concentrations of TAG at given reaction time and at initial reaction, respectively.

2.5. Free fatty acids content

The AOCS [15] official method Cd 3d-63 was used for determination of total free fatty acids (FFAs). FFAs were expressed as percent oleic acid (w/w). Results allowed calculating the hydrolysis degree according to Eq. (2):

$$H(\%) = \frac{AG_f}{AG_t} \times 100 \quad (2)$$

where AG_f = total concentration of fatty acids formed in the reaction (mM); AG_t = total concentration of fatty acids that would be liberated, considering the total hydrolysis of triacylglycerols in the reaction medium (mM).

2.6. Consistency

The consistency of the raw materials and interesterified products were determined using a texture analyzer (Model QTS-25 Brookfield, Middleboro, MA, USA) controlled by the Texture Pro software v. 2.1. Samples were heated in microwave oven (55–62 °C) for complete melting of the crystals, and conditioned in cubic silicone moulds (edge of 25 mm) for 48 h at 10 °C. The probe TA15 was used, corresponding to an acrylic cone with angle of 45°. Tests were carried out under the following conditions: total of cycles 1, distance = 10 mm, speed = 120 mm/min, time = 5 s; determination of the force in compression (gf), in duplicate. Measurements were used to calculate the "yield value", defined according to Eq. (3) [16]:

$$C = K \cdot \frac{W}{p^{1.6}} \quad (3)$$

where *C* = "yield value" (gf/cm^2); *K* = a constant depending on cone angle (4700 for 45°); *W* = maximum compression force (gf), for 5 s; *p* = penetration depth (in 0.1 mm).

3. Results and discussion

3.1. Compositional modifications

Fig. 1 shows that TAG composition for MF:SBO NIE blends displayed different profiles in relation to pure milkfat. Such profiles were a result of the soybean oil TAG composition, which has triglycerides with C_{52} and C_{54} carbon species predominant in its composition. Therefore, all blends showed lower C_{24}–C_{50} triglyceride levels and higher C_{52} and C_{54} triglyceride levels.

The observation of Fig. 2 allows comparing the triglyceride NIE blend profiles with the corresponding IE products at 48 h reaction. Independent of the blend composition TAG carbon specie C_{54} decreased. With an exception 50:50 blend (Fig. 2), TAG carbon species 34–42 decreased while carbon species 46–52 increased. For 50:50 blends an increase in C_{40}–C_{52} triglyceride levels occurred.

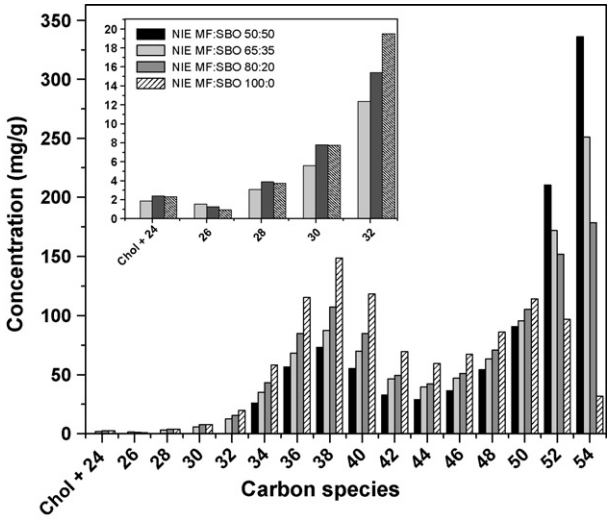

Fig. 1. Triglycerides concentration profile for different non-interesterified (NIE) milkfat (MF) and soybean oil (SBO) blends. The groups of TAGs were identified by carbon number in the fatty acids residues.

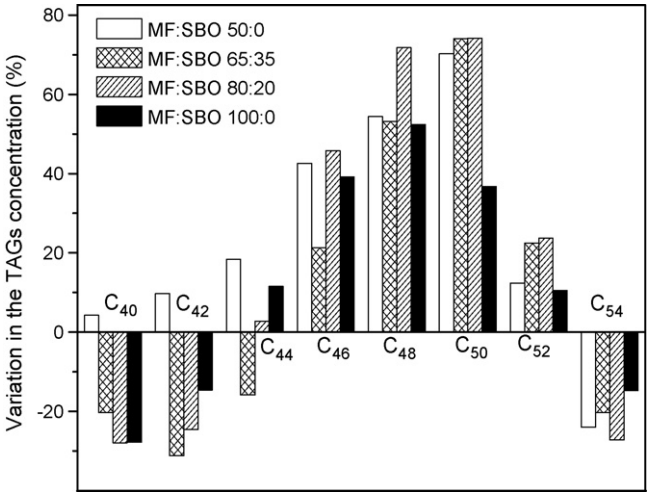

Fig. 3. Variation in TAGs concentration after 48 h of enzymatic interesterification in relation to the initial blends composition using different milkfat (MF) and soybean oil (SBO) proportions.

For all experiments, the highest variation on the concentration occurred for TAG C_{50}, while TAG C_{44} did not change noticeably (Fig. 3). Thus, the ratio of the relative proportions of 50-carbon species to 44-carbon species (50/44) was used to measure the evolution of TAG species by calculating the interesterification degree according to Eq. (1). These trends in carbon species modifications were also reported by Aguedo et al. [4], using different raw materials and lipase source.

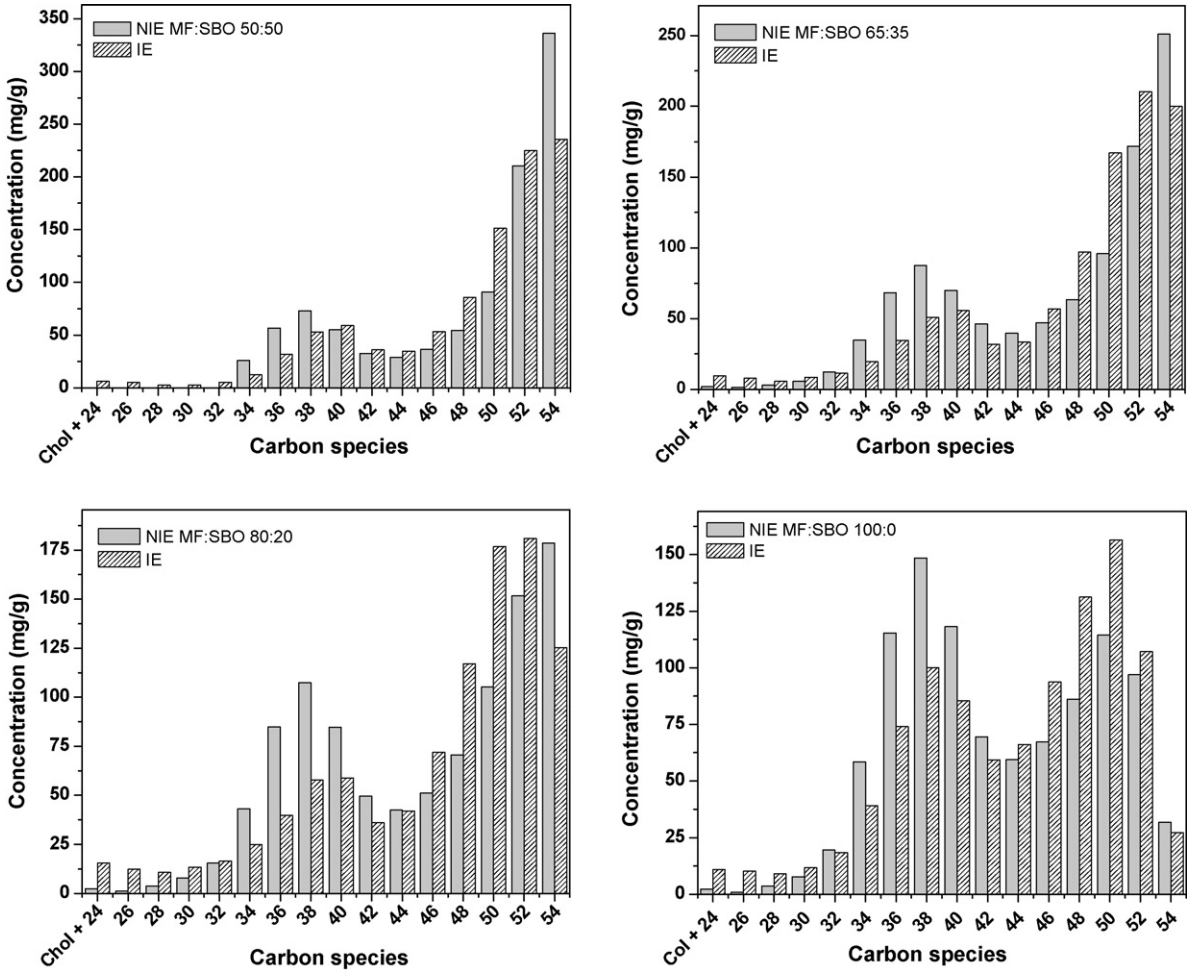

Fig. 2. Triglycerides concentration profile obtained in the interesterification reactions for MF:SBO blends 50:50, 65:35, 80:20, 100:0, catalyzed by lipase from *Rhyzopus oryzae*. The groups of TAGs were identified by carbon number in the fatty acids residues.

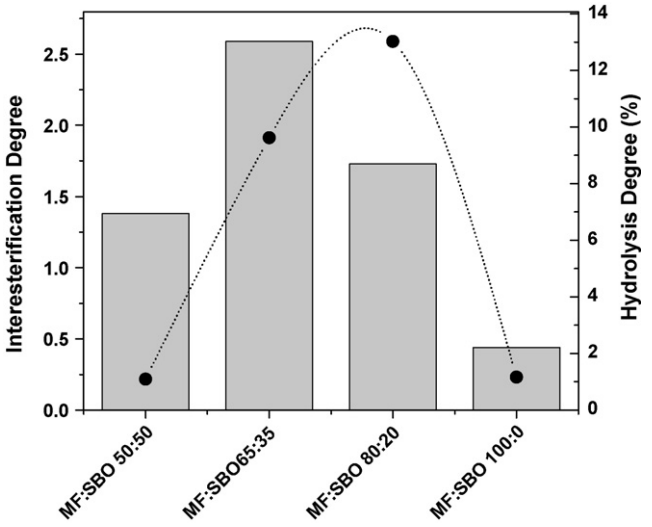

Fig. 4. Interesterification degree (gray bars) and hydrolysis degree (dotted black line) at 48 h, for MF:SBO blends 50:50, 65:35, 80:20, 100:0.

Fig. 4 displays values attained for interesterification and hydrolysis degrees for each reaction performed. The highest interesterification degree (2.60) was obtained using 65:35 MF:SBO blend. As milkfat proportion increased in the blend, a fall in the interesterification degree was observed, probably as a result of the reaction limitation imposed by the loss of TAGs containing unsaturated 18-carbon fatty acids in soybean oil, which represent the majority of its fatty acid make-up. The enzyme used in this work, lipase from *R. oryzae*, distinguishes between FA with different length. Under optimum conditions (either in the presence of organic solvent or solvent-free systems) this lipase prefers C_{18} fatty acids and C_2–C_4 alcohols [17]. Therefore, blends containing high soybean oil amounts may favor the reaction as found in this work for 65:35 MF:SBO blend, which appears to be the best combination of fatty acids for the interesterification reaction mediate by lipase from *R. oryzae*. Note that blend containing similar proportions of milkfat and soybean oil (50:50), slowdown the reaction rate and lower interesterification degree was obtained.

From the quantity of the free fatty acids present in medium was possible to calculate the hydrolysis degree (%) according to Eq. (3) (Fig. 4). Blends 65:35 and 80:20 showed the highest hydrolysis degrees: approximately 10% and 13%, respectively. Regarding the IE products from blend 50:50 and 100:0 MF:SBO, lower hydrolysis degrees were verified (<2%).

The presence of hydrolysis products in the bulk phase is a complex function of the water concentration in the liquid phase, the amount of adsorbed water on the immobilized derivative, and the concentration of starting materials and IE products, which affects the hydrophilicity of the reaction medium. Some insight into the reason for reversal reaction may be gained by considering the enzyme mechanism. The interesterification reaction is a special case of fatty acids transference that involves, at molecular level, sequential reactions of hydrolysis and synthesis [18]. The first stage involves hydrolysis of triacylglycerols to produce diacylglycerols, monoacylglycerols, and free fatty acids. Accumulation of hydrolysis products will continue during interesterification until equilibrium is established [18]. When water is not in high quantity, reesterification will occur and the interesterification will be favored.

3.2. Consistency of interesterified products

Food texture is recognized as a sensory quality that manifests itself in different ways. Some of the sensory attributes identified as describing the texture of solid foods are consistency and hardness. The consistency is an important functional aspect for plastic fats, which are mixtures of solid fat crystals and liquid oil. The ratio between the two phases and the crystalline character of the solid phase determine sample consistency and firmness [19]. Moreover, consistency is a critical factor in determining the functionality and consumer acceptance of table spreads [20].

Table 1
Consistency values for different non-interesterified (NIE) and interesterified product (IE) obtained from milkfat (MF) and soybean oil (SBO) blends.

Proportion MF:SBO	Consistency value (gf/cm^2)	
50:50	NIE	138
	IE	48
65:35	NIE	1159
	IE	276
80:20	NIE	3246
	IE	1953
100:0	NIE	6074
	IE	4260

Table 1 shows the consistency of both NIE blends and IE products, represented as yield value (gf/cm^2) at 10 °C. The results showed that for all NIE blends consistency decreased in relation to pure milkfat. Similar results were described in the literature using milkfat and different vegetable oils, such as canola oil, linseed and rapeseed oils [4,21].

For NIE blends, the increase of soybean oil content in the blend strongly influences its consistency, due to the crystalline network dilution and the formation of a weaker structure [22]. The lowest addition of soybean oil (80:20 NIE blend) resulted in a great fall in the NIE blend consistency (47%) in relation to the original milkfat (6074 gf/cm^2). The highest addition (50:50 NIE blend) produced the highest effect in the consistency, with a final value of the 138 gf/cm^2 (98% of decrease in comparison to the milkfat). However, it is important to note that this value was outside the yield value range (200–800 gf/cm^2) considered by Haighton [23] as appropriate to a fat with satisfactory plasticity and spreadability properties.

After interesterification, all IE products displayed lower consistency in comparison to the corresponding NIE blends. The greatest alterations in yield values of the IE products was observed in blends 65:35 and 80:20 (MF:SBO). The original NIE blends resulted in yield values of 1159 and 3246 gf/cm^2; after interesterification, these values decreased, respectively, to 276 and 1953 gf/cm^2, which corresponded to a reduction of 76% and 40%.

These results show that the enzymatic interesterification reaction can modulate the consistency of the milkfat by incorporating unsaturated soybean oil fatty acids into its TAGs. Moreover, besides the consistency reduction, the literature relates that interesterification can also influence the sensorial properties of fats, hence directing their application in food products [24].

From all assays performed, only the IE product from blend 65:35 resulted in yield value inside the range from 200 to 800 gf/cm^2 with satisfactory plasticity and spreadability properties for use at refrigeration temperatures, according to Haighton [23] criteria. Thus, among the mass proportions studied in this work, the blend 65:35 MF:SBO was the most suitable, since this resulted in the highest interesterification degree (2.60) and reduction consistency (76%).

4. Conclusions

This study demonstrated that enzymatic interesterification modified the triacylglycerol composition, and consequently the consistency properties of the MF:SBO blends. Results showed the potential of the lipase from *R. oryzae* for the interesterification

of milkfat with soybean oil. The highest interesterification degree (2.60) was achieved with the medium of 65% of milkfat, which gave 76% reduction in the consistency value. Therefore, the feasibility of using this lipase to reduce the milkfat consistency and turning it more spreadable under cool temperature has been demonstrated.

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