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Continuous enzymatic interesterification of lard and soybean oil blend: Effects of different flow rates on physical properties and acyl migration

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ABSTRACT

Continuous enzymatic interesterification is an alternative to chemical interesterification for lipid modification technology which is economically viable for large scale use. A blend of 70% lard and 30% soybean oil was submitted to continuous enzymatic interesterification in a glass tubular bioreactor at flow rate ranging from 0.5 to 4.5 mL/min. The original mixture and the reaction products obtained were examined to determine melting and crystallization behavior by DSC, and analyzed for regiospecific fatty acid distribution. Continuous enzymatic interesterification changed the mixture, forming a new triacylglycerol composition, verified by DSC curves and variation in enthalpy of melting values. The regiospecific distribution of fatty acids was changed by flow variations in the reactor. In the continuous enzymatic interesterification reaction the flow rate of 4.5 mL/min, was more advantageous than slower flow rates, reducing acyl migration and increasing process productivity.

1. Introduction

Fatty acids present in human milk have a highly specific positional distribution in triacylglycerols (TAGs), and this configuration has been implicated as the main factor determining the efficiency of human milk absorption [1]. Pancreatic and gastric lipases selectively hydrolyze fatty acids at *sn*-1,3 positions, producing free fatty acids and 2-monoacylglycerols. 2-Palmitoylglycerol is more efficiently absorbed than palmitic acid in free form, the latter producing insoluble soaps with calcium and magnesium [2].

The structure of lipids in human milk can be reproduced artificially by means of the enzymatic interesterification reaction using *sn*-1,3 specific lipase as a catalyst [3–7]. Chemical interesterification produces fats which have a random distribution of fatty acids in TAGs molecules, a process known as randomization [8]. However, when the aim is to produce lipids with highly specific compositions for functional and medical applications, enzymatic interesterification methods are better suited [9].

Structured lipids (SLs) are TAGs that have been modified to change the fatty acid composition and/or their positional distribution in glycerol backbone by chemically and/or enzymatically catalyzed reactions and/or genetic engineering. More specifically, SLs are modified TAGs with improved nutritional or functional properties [10].

Enzymatic interesterification offer the advantage of greater control over the positional distribution of fatty acids in the final product, due to lipases' fatty acid selectivity and regiospecificity [11]. In addition, the technique allows for the modification of fats without the use of chemical reagents, while the use of specific lipases for the *sn*-1,3 positions enables synthesis of specific TAGs [5].

Lipase-catalyzed reactions involve a combination of hydrolysis and esterification/interesterification [12]. Since the reaction takes place on the ester bond, no *trans* isomers are formed [13]. The water must be removed from the reaction in order to minimize hydrolysis and increase esterification, thereby raising the conversion rate. In the presence of excessive water, hydrolysis is predominant, resulting in an accumulation of glycerol, free fatty acids, monoacylglycerols and diacylglycerols. However, water is essential to maintain the enzymatic reaction because it sustains the enzyme integrity. In order to refine the first step of the reaction it is necessary to achieve a balance between hydrolysis and esterification [10].

In the effort to produce reactions that are more technologically uniform and economically viable, the use of enzymes in immobilized form has substantially increased stability and provide a broader range of applications, important for developing bioconversion processes [14]. Immobilization is also associated with

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greater productivity, automation of processes and continuous operations, precise control over reactions, ease of separation of products obtained, stabilization of enzymatic activity, as well as recovery and reuse of enzymes [15,16].

Continuous reactors are used conjunction with immobilized enzymes for large scale in production of SLs lipids. The systems enable industrial scale production, minimize costs and facilitate control of the process, besides producing few byproducts and offering ease of operation. In continuous reactors, since the substrate comes into contact with a large amount of enzyme, the reaction time is shorter compared to non-continuous reactors, resulting in lower acyl migration [12,17]. Different types of oils and fats are used as substrates for producing structured lipid substitutes for human milk fat: tripalmitin, lard, bovine milk fat, soybean oil, canola oil, borage oil, and sunflower seed oil. Of these alternatives, lard has the most favorable characteristics for use as a raw material in the production of these SLs. In contrast to human body fat and that of other mammals, lard has a fatty acid distribution with palmitic acid predominantly esterified at the *sn*-2 position, the same types of structure found in human milk fat [15,18,19]. Soybean oil has the advantage of having a high essential fatty acid content.

In a recent study, Silva et al. [20] employed different lard:soybean oil blends to produce a structured lipid substitute for human milk fat by continuous enzymatic interesterification, but observed some degree of acyl migration.

The aim of the present study was to assess the influence of flow in the reactor on the physicochemical properties of the SLs produced from a blend of 70% lard and 30% soybean oil. Therefore, this study shall determine the optimal conditions of this process while minimizing acyl migration in the end product.

2. Experimental

2.1. Materials

Lard and soybean oil were obtained from local commerce (São Paulo, Brazil). Commercial immobilized lipase from *Thermomyces lanuginosus* (Lipozyme TL IM) was kindly supplied by Novozymes Latin America Ltd. (Araucária, Brazil). The enzyme activity of the lipase was 250 IUN/g. All other reagents and solvents were analytical or chromatographic grade.

2.2. Methods

2.2.1. Reactant blend preparation

Lard and soybean oil were blended at 70:30 (w/w) proportion. The blend was prepared after complete melting of the fats at 70–75 °C for 30 min under magnetic stirring. The blends thus obtained were stored at −18 °C until analysis.

2.2.2. Performance of interesterification reactions

The enzymatic interesterification was carried out in a continuous tubular glass bioreactor (height 34 cm, internal diameter 2 cm) equipped with an external jacket to maintain constant temperature and fixed bed to support the enzyme (70 g), and with a peristaltic pump (VC 360II Ismatec, Switzerland). Soybean oil was initially introduced into the reactor at a flow rate of 1 mL/min to remove air and water from the enzyme until the free fatty acids (CA-5A, AOCS, 2009)[21] of the interesterified oil presented a stable value (0.58 g oleic acid/100 g fat). The residence time was experimentally determined and was defined as the time needed to fill the empty spaces of the reactor packed with immobilized enzyme, using a flow rate of 1 mL/min. The jacketed column was heated by water bath (RE 112, Lauda, Germany) to maintain the bed enzyme at 60 °C (according to the manufacturer's recommendations) [20].

2.2.3. Flow rate variation

After conditioning of the enzyme, the blend (70:30) was pumped into the reactor and was submitted to the flow rate variation. This blend was chosen for having the fatty acid composition closer to that of human milk fat. The mixture was introduced into the reactor in the following flow rates: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 mL/min, sequentially, controlled by a peristaltic pump. Each different interesterified sample was collected (50 mL) after discarding the first 100 mL to avoid cross-contamination with the previous sample, and stored at −18 °C until analysis. The residence times of the blend in the reactor ranged from 120 min (0.5 mL/min) to 13 min (4.5 mL/min).

2.2.4. Melting and crystallization behaviors

Calorimetric analysis of the crystallization and melting behaviors of the samples was performed on a DSC 4000 Perkin-Elmer Thermal Analyzer fitted with an SP Intracooler Cooling System. Melted samples (~5 mg) were placed in the 50 μL aluminium recipients of the calorimeter (BO14-3017 recipient and BO14-3003 cap, Perkin-Elmer) which were then hermetically sealed. An empty recipient (as per above) served as a reference. The instrument was calibrated with indium.

The following temperature program was used according to method Cj 1–94 of the American Oil Chemists' Society [21,22]: for crystallization curves, samples were kept at 80 °C for 10 min to eliminate all crystal nuclei, cooled to −60 °C at a rate of 10 °C/min and kept at this temperature for 30 min; for melting curves, samples were heated from −60 °C to 80 °C, at a rate of 5 °C/min. All data were treated using the Pyris software program. Thermograms were analyzed to determine onset and endset of crystallization and melting, maximum temperature of main peaks (°C), and enthalpies of crystallization and melting (J/g). All samples were analyzed in triplicate.

2.2.5. ^{13}C NMR analyses

A proton-decoupled ^{13}C NMR was used to analyze the positional distribution of fatty acids on the triacylglycerol backbone [23–25]. Lipid samples (250 mg) were dissolved in 0.5 mL of deuterated chloroform ($CDCL_3$) in 5 mm NMR tubes, and NMR spectra were recorded on a Bruker Advance DPX spectrometer operating at 300 MHz. The ^{13}C spectra of the lipid samples were acquired with a spectral width of 2332.090 Hz, pulse of 10.2 μs, and a relaxation delay of 30 s. Determination of ^{13}C was performed at a frequency of 75.8 MHz with a 5 mm multinuclear probe operating at 30 °C, using the method described by Vlahov [23,26]. The results showed the compositions of saturated fatty acids, oleic acid and linoleic + linolenic acids in *sn*-2 and *sn*-1,3 positions. The determination of positional compositions of fatty acids in triacylglycerols was carried out by using the frequency ranges 172.4–173.1 ppm where *sn*-2 and *sn*-1,3 positions can be seen, respectively.

3. Results and discussion

The flow of substrate in the enzymatic reactor is a key parameter for continuous processes. Slow flow rate can lead to acyl migration and a behavior similar to that of non-continuous processes [27], whereas high flow rate can cause incomplete interesterification. Shin et al. [15] assessed the effect of flow (between 3 and 10 mL/min) on continuous interesterification of blends of milk fat, palm stearin and linseed oil. Their results showed that lower flow, resulting in longer contact time with enzymatic bed, produced a higher degree of interesterification. However, acyl migration was not assessed in the cited study.

Because a simple physical mixture results in retention of the original absorption rates of the individual TAGs, the different structural compositions of TAG between structured lipids and simple

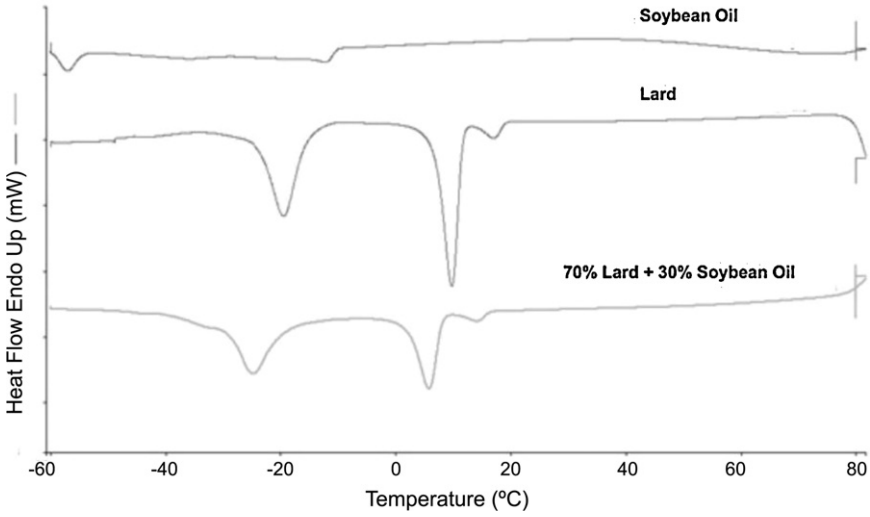

Fig. 1. Crystallization curves obtained by DSC for lard, soybean oil and 70% lard:30% soybean oil blend, prior to continuous enzymatic interesterification reaction.

physical mixture may lead to different hydrolysis and absorption rates, resulting in different metabolic fates. So, the interesterification of lard and soybean oil gives such benefit to the product. Besides, our study showed improvements in this area because low-cost raw materials were used (lard and soybean oil) and were obtained quantities of saturated fatty acids at *sn*-2 position higher than that of the commercial product Betapol [20].

3.1. Melting and crystallization behaviors

Cooling and heating phenomena were assessed by DSC for the mixture, before and after the continuous enzymatic interesterification reaction, at different flow rates in the reactor.

The profile of the crystallization curves (Fig. 1) of lard, soybean oil and a blend of both, revealed characteristic distinct peaks for each sample.

During crystallization, a reduction in the sum of enthalpies occurred upon addition of 30% soybean oil to lard (Table 1). This is due to the fact that the energy release (exothermal phenomenon) is proportional to the amount of lard contained in the mixture [28]. Also, a tendency toward lower temperatures of crystallization peaks was evident for the 70:30 blend compared to lard alone, because the unsaturated fatty acid level in soybean oil is higher than in lard [29]. No significant difference was found among crystallization enthalpies of the 70:30 blend or the SLs obtained, at different mixture flow rates in the reactor (Table 1).

Table 1
Sum of enthalpies of crystallization and melting of lard, soybean oil and 70% lard:30 soybean oil blend, before and after continuous enzymatic interesterification, at different mixture flow rates in reactor.

	Crystallization (ΔH) J/g	Melting (ΔH) J/g
Lard	−29.39 ± 5.4	28.90 ± 1.4
Soybean oil	−8.88 ± 1.1	17.33 ± 2.2
70% lard + 30% soybean oil	−23.34 ± 5.5	20.80 ± 3.0
Different flow rates in the reactor (mL/min)		
0.5	−22.58 ± 3.1	13.24 ± 0.8
1.0	−20.43 ± 4.2	11.86 ± 1.6
1.5	−21.75 ± 1.6	12.15 ± 0.3
2.0	−22.71 ± 1.9	11.96 ± 0.3
2.5	−24.56 ± 3.6	12.82 ± 0.5
3.0	−25.07 ± 4.3	14.91 ± 1.8
3.5	−22.24 ± 2.7	13.58 ± 0.9
4.0	−20.82 ± 3.2	10.51 ± 0.7
4.5	−24.59 ± 4.2	11.78 ± 2.0

Comparison of samples of the 70:30 blend, before and after continuous enzymatic interesterification (Figs. 1 and 2), revealed a change in the profiles of crystallization thermograms. This indicates that interesterification occurred in all samples at different flow rates, changing their crystallization behaviors.

The interesterified samples of the mixture submitted to different flow rate in the reactor had very similar crystallization thermogram profiles (Fig. 2). Similarly, values of the sum of enthalpy of crystallization (Table 1) also differed only slightly. This result suggests that variation in flow did not lead to any significant changes in the crystallization properties of the SLs.

The results for melting were akin to those for crystallization. The melting curve profiles showed characteristic peaks for lard, soybean oil and the 70:30 blend (Fig. 3), which differed to one another owing from the different compositions of these samples [20].

The melting thermograms of the 70:30 blend submitted to the different flow rates are shown in Fig. 4. The results obtained show a considerable change in the melting pattern of the SLs compared to the pattern for the mixture before the reaction.

During the melting process, the system absorbs energy (endothermic phenomenon) in a much more complex manner, since this energy reflects the melting of the different crystalline forms (α, β and β-prime) present in the sample [30].

The values for the sum of enthalpies of melting were lower following interesterification (Table 1). This shows that the interesterification reaction took place at all flow rate, forming new triacylglycerols [20] which in turn affected melting behavior.

The melting profiles of the SLs obtained for different flow rates (Fig. 4) show that the curves are similar across all samples, suggesting that the variation in the flow of the mixture in the reactor had no marked impact on melting behavior of the SLs.

3.2. Analysis of regiospecific distribution of fatty acids

The stereospecific distribution has implications on both nutritional and technological qualities of oils and fats. The triacylglycerol structure of human milk fat most conducive for absorption by newborns is that formed by unsaturated fatty acids esterified at the *sn*-1 position, saturated fatty acids at the *sn*-2 position, and medium-chain fatty acids at the *sn*-3 position [31].

Using the NMR data for fatty acids in the *sn*-2 and *sn*-1,3 positions it is possible to calculate the fatty acid composition of the mixture. The fatty acid composition calculated by NMR compared

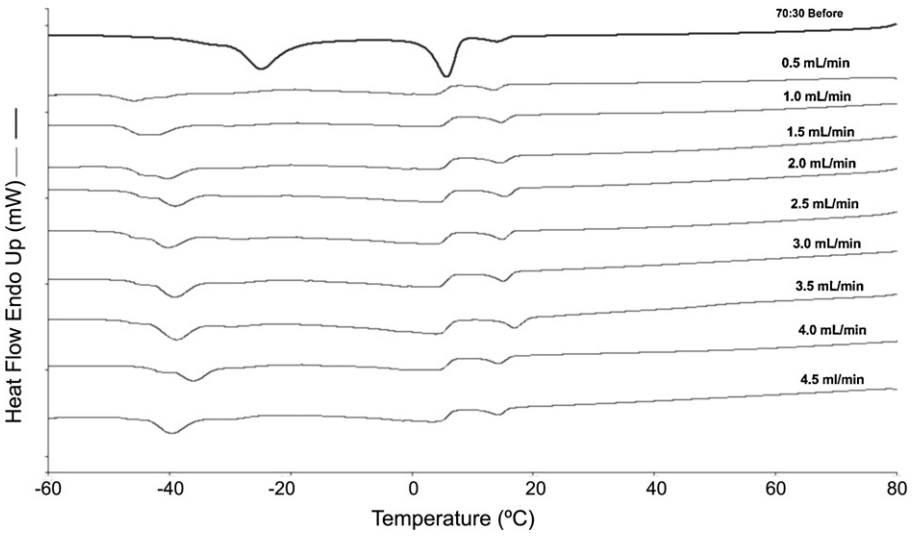

Fig. 2. Crystallization curves obtained by DSC for 70% lard:30% soybean oil blend, after continuous enzymatic interesterification at different mixture flow rates in reactor.

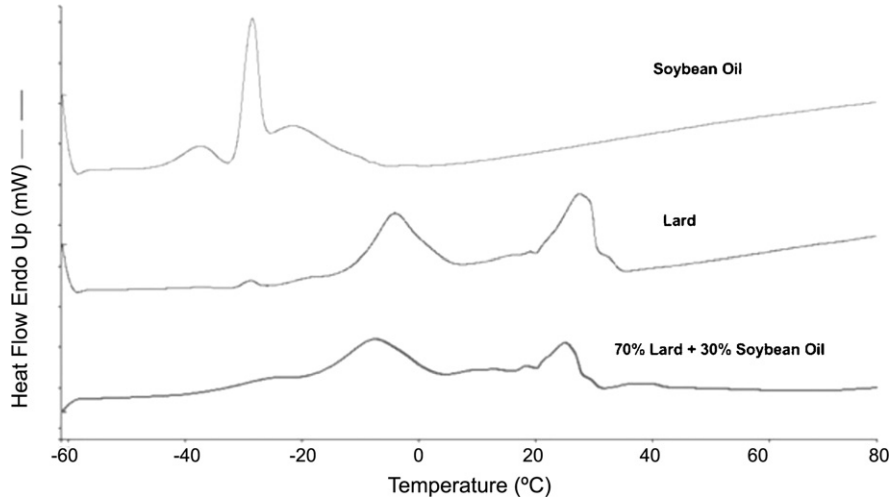

Fig. 3. Melting curves obtained by DSC for lard, soybean oil and 70% lard:30% soybean oil blend, prior to continuous enzymatic interesterification reaction.

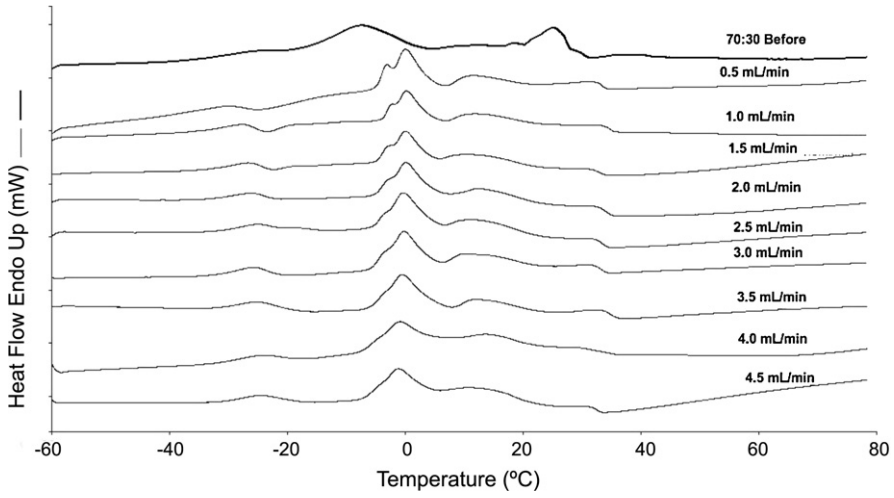

Fig. 4. Melting curves obtained by DSC for 70% lard:30% soybean oil blend, after continuous enzymatic interesterification at different mixture flow rates in reactor.

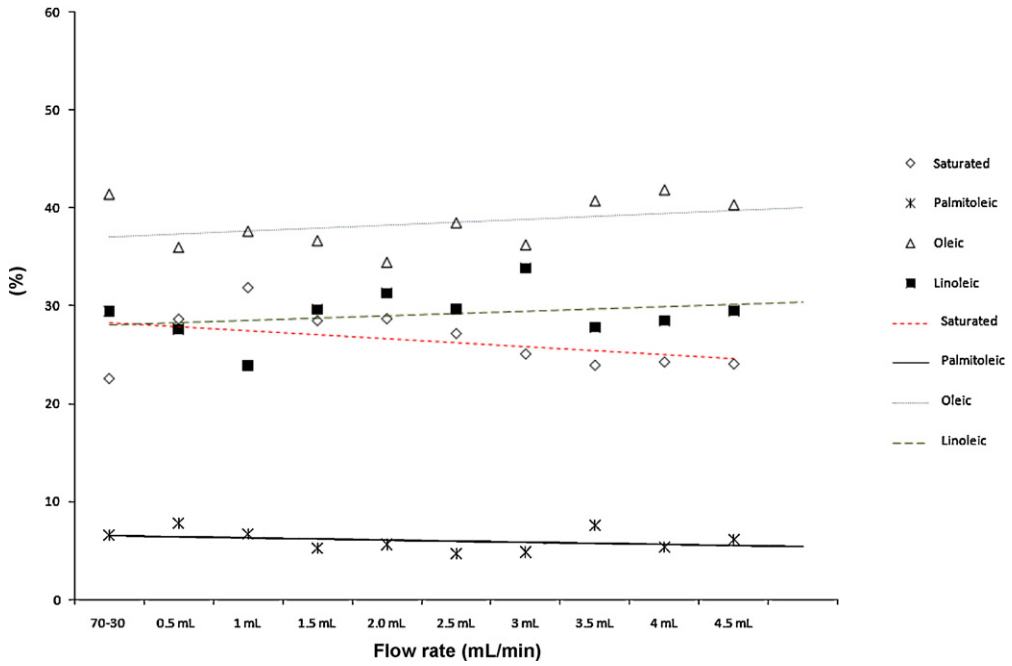

Fig. 5. Regiospecific distribution of fatty acids at *sn*-1,3 positions of 70% lard:30% soybean oil blend, before and after continuous enzymatic interesterification at different mixture flow rates in reactor.

with that obtained by gas liquid chromatography (GLC) showed were similar results.

The fatty acid profile at the *sn*-1,3 positions of the 70:30 blend, before and after continuous enzymatic interesterification, at different mixture flow rates in the reactor are given in Fig. 5, while profiles at the *sn*-2 position are shown in Fig. 6.

According to literature [32–35], acyl migration is a thermodynamic process and is very difficult to stop fully in actual reactions. Because acyl migration is an undesirable but unavoidable side reaction in the enzymatic production of specific structured lipids, the minimization of acyl migration is a key to improving the quality of targeted structured lipids. Therefore, acyl migration as well as acyl incorporation should be considered together in the structured lipids production from the standpoints of both yield and quality of structured lipids. There have been no possible methods to fully stop the acyl migration, but minimization of acyl migration can be expected under optimized conditions. Acyl migration generally involves migration from *sn*-1,3 to *sn*-2 positions, but also occurs with migration of acyls from the *sn*-2 into the *sn*-1,3 positions. There are many factors that could possibly influence acyl migration, such as reaction temperature, time, acyl chain length, water activity, acid, base, reactor type, behavior of lipase and others.

The enzyme Lipozyme TL IM is *sn*-1,3 specific and therefore fatty acids initially located at the *sn*-2 position should largely remain in this position, despite some degree of acyl migration to *sn*-1,3 positions [36]. The lipase used in this study is *sn*-1,3 specific, but the

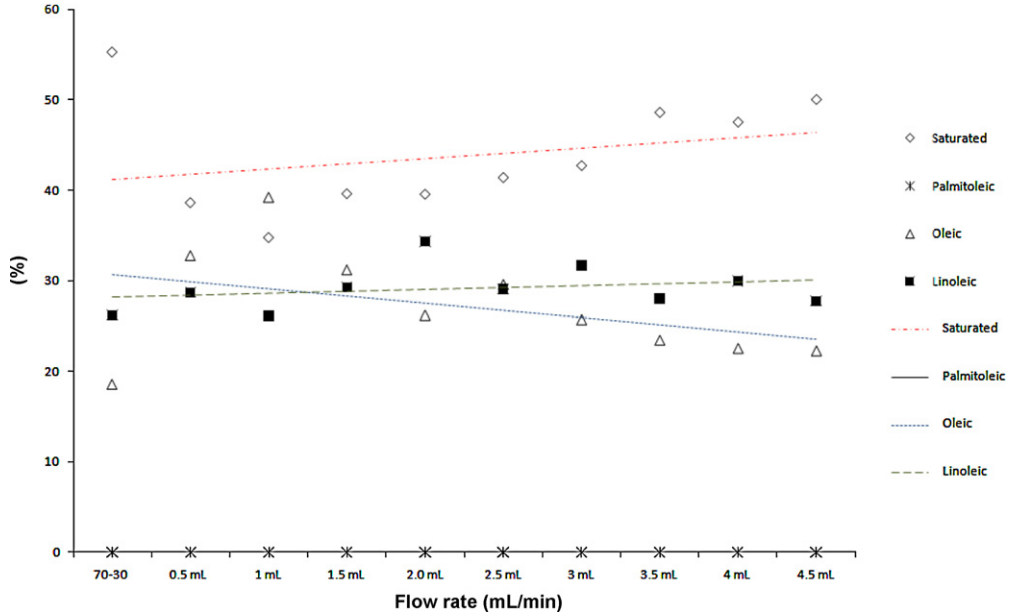

Fig. 6. Regiospecific distribution of fatty acids at *sn*-2 position of the 70% lard:30% soybean oil blend, before and after continuous enzymatic interesterification, at different mixture flow rates in reactor.

conditions employed in the reactor allowed the acyl migration and thus interesterification was not completely specific to the *sn*-1,3 positions. As can be seen in Fig. 5, at *sn*-1,3 positions, the percentage of saturated fatty acids increased in lower flow rates, indicating that those fatty acids migrated from *sn*-2 position to the *sn*-1,3 positions. With increasing of the flow rate, acyl migration reduced, and for flow rates like 4.5 mL/min the levels of saturated fatty acids are close to the original blend. Analysis of physical properties such as melting and crystallization behavior shows that, despite the original blend and structured lipids (at 4.5 mL/min) have very similar regiospecific distribution, the physical properties are different, indicating that the interesterification was efficient.

Analysis of the regiospecific distribution of fatty acids at the *sn*-2 position (Fig. 6) shows that, at lower flow rate, the level of saturated fatty acids is lower than in the pre-reaction mixture. In the samples obtained at greater flow rate, this level rises to attain levels closer to those seen in the non-interesterified mixture. These results indicate that, when the reaction is slower as result of lower flow rates, there is a greater tendency for acyl migration, i.e. saturated fatty acids migrate from *sn*-2 to *sn*-1,3 positions. This is an undesirable phenomenon given that the structure of triacylglycerols of human milk fat ideal for absorption, have saturated fatty acids predominantly at the *sn*-2 position. On the other hand, at greater flow rate, this migration occurs to a lesser degree, maintaining a high proportion of saturate fatty acids at the *sn*-2, position, albeit at lower amounts than those of the initial 70:30 blend.Costales-Rodrígez et al. [17] deemed a flow of 300 g lipidic substrate/h in continuous reactors acceptable for industrial applications. In the present study, the highest flow was 4.5 mL/min, corresponding to approximately 246 g substrate/h, assuming a density of the 70:30 mixture of 0.9 g/mL [13]. Therefore, the flow of 4.5 mL/min can be considered adequate for industrial uses.

In addition, quality parameters such as tocopherol level, final acidity, partial acylglycerols and color, are better after continuous than non-continuous interesterification, due to the shorter contact time under the former process. This confirms the advantage of the use of continuous operation in enzymatic interesterification [13,17].

Therefore, higher mixture flow rate in the continuous enzymatic interesterification reactor, such as 4.5 L/min, are more advantageous than slower flow rate for two reasons: a lesser degree of acyl migration and greater productivity of the process. In this study, the minimum residence time of the mixture within the reactor was around 13 min, consistent with the 15 min residence time described by Osório et al. [12].

4. Conclusion

Continuous enzymatic interesterification changed the 70% lard:30% soybean oil mixture, forming a new triacylglycerol composition. This was evidenced by the changes in the physical properties of the samples seen in the melting and crystallization curves obtained by DSC, and also by the variation in the enthalpy of melting values. Varying mixture flow in the reactor had no marked impact on the melting and crystallization behavior of the samples. However, changes in the regiospecific distribution of fatty acids were seen at the *sn*-1,3 and *sn*-2 positions at different flow rates in the reactor, changes not reflected in the physical properties analyzed. At lower flow rate, the saturated fatty acid level was lower at the *sn*-2 position and higher at the *sn*-1,3 positions. This acyl migration is undesirable since the structure of triacylglycerols of human milk for optimal absorption has saturated fatty acids at the *sn*-2 position. Therefore, it can be concluded that lower residence time at higher flow rate in the continuous enzymatic interesterification reactor, such as 4.5 mL/min, are more advantageous than lower flow rate for two main reasons: a lesser degree of acyl migration and greater productivity of the process.

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