J Am Oil Chem Soc (2013) 90:391–400
DOI 10.1007/s11746-012-2182-z

ORIGINAL PAPER

Optimization of Reaction Conditions in the Enzymatic Interesterification of Soybean Oil and Fully Hydrogenated Soybean Oil to Produce Plastic Fats

Consuelo Pacheco · Camila Palla · Guillermo H. Crapiste · María E. Carrín

Received: 9 May 2012 / Revised: 19 September 2012 / Accepted: 9 November 2012 / Published online: 2 December 2012
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Abstract Semisolid fats obtained from oils and fats through enzymatic interesterification have interesting applications. The effect of certain reaction parameters (enzyme concentration, moisture content, reaction time, substrate ratio, temperature, and agitation level) over the enzymatic interesterification of fully hydrogenated soybean oil (FHSO) and refined soybean oil (SO) using two immobilized enzyme types (Lipozyme RM IM and Lipozyme TL IM), was studied with a fractional factorial design (FFD). The reaction products were analyzed with respect to melting point (mp), by-products content and triacylglycerols (TAG) composition. It was found that substrate ratio, reaction time, and their interaction presented the most significant contributions to mp, varying this from 43.4 to 61.5 °C. The highest contributions to by-product content were presented by time and its interaction with the amount of molecular sieves, mainly for Lipozyme TL IM. Through the models obtained, theoretical conditions to achieve minimal by-product generation and mp were found, being 5.0 % (w/$w_{subst.}$) of any of both lipases, 24 h, 70:30 (oil:-fat, % w/w), 65 °C, 230 rpm, and absence of molecular sieves. Regression models for TAG groups as a function of significant factors and interactions were constructed, offering useful information to establish the reaction conditions for obtaining a product with a target mp or chemical composition.

C. Pacheco · C. Palla · G. H. Crapiste · M. E. Carrín (✉)
PLAPIQUI (Universidad Nacional del Sur, CONICET),
Camino La Carrindanga Km 7,
8000 Bahía Blanca, Argentina
e-mail: mcarrin@plapiqui.edu.ar

Keywords Lipase-catalyzed interesterification · Melting point · TAG · DAG · MAG · Experimental design · Structured lipids · Soybean oil

Introduction

Plastic fats, which are used by the food industry as shortenings or as the base stock in the elaboration of margarines, need to possess specific physical attributes [1]. Most native oils have limited applications in their unmodified forms [2] and low oxidative stability; hence several technologies have been developed to modify them. Partial hydrogenation has been applied for many years, but the resulting increase in the product melting point (mp) is not only attributable to the formation of saturated fatty acids (FA), but also to the generation of *trans* FA [3]. This initially desirable side reaction soon became unwanted because several studies have shown that *trans* FA may have an adverse effect on human health. High contents of *trans* FA obtained in partially hydrogenated products and the destruction of essential FA generated by this process have imposed a challenge for the food industry and new technologies have been developed. Blending different oils and fats appeared to be a tempting option because its simplicity, but phase separation during storage and formation of relatively coarse crystals [1, 4] are some of the reasons why this process has not resulted in the end of the search. Interesterification (IE) of a fat and edible oils or a mixture of oils seems to be free of all these drawbacks and appears to be the chosen future technology for plastic fats production. It consists in the rearrangement of FA among and within triacylglycerols (TAG) obtaining a mixture of TAG different from the initial one and hence with modified physical characteristics [1] and crystallization behavior [5].

The FA profile of the initial mixture remains unchanged thus it could be considered as a more natural process than hydrogenation. Moreover, enzymatic interesterification (EIE) has some advantages over chemical IE, such as enhanced selectivity (fewer by-products are generated), mild reaction conditions (pressure, temperature) leading to reduced costs and energy consumption [6] and decreased degradation of minor compounds present in the oil or fat as tocopherols [4]. On the other hand, if a *sn*-1,3-specific lipase is used, most FA in the *sn*-2 position (which are more easily absorbed) are maintained. These FA are unsaturated ones in most oils, so it is possible to obtain a more natural fat [2] using EIE instead of chemical IE. Immobilized lipases provide another advantage to the process, which is related to the facility of product recovery and the possibility of reusing them lowering process and investment capital costs [4]. The selection of a solvent-free system incorporates another cost reducing factor and involves an ecofriendly alternative.

In the present work, soybean oil (SO) was selected as the raw material considering its nutritional qualities, abundance, considerable economic value and wide functionality. Some IE studies between SO and fully hydrogenated SO (FHSO) have been carried out including chemical [7–9] and enzymatic catalysis [10]. In this last work, a model for the solid fat content of the interesterified blends was obtained, but no other physicochemical property was studied, nor was TAG composition determined. Unlike most of the published works in which only substrates ratio remain variable or more reaction conditions are considered but varied one-at-a-time, in this work the influence of a considerable number of reaction parameters over the EIE of SO and FHSO in a solvent-free system was determined through experimental design in order to have a mathematical tool to establish experimental conditions to get specific goals (regarding physical and/or chemical properties). The factors (independent variables) considered for two different immobilized enzymes were: (i) enzyme concentration, (ii) moisture content, (iii) time, (iv) substrates ratio, (v) temperature, and (vi) agitation level, while the responses (dependent variables) measured were: mp, by-product generation and TAG content (S_3, US_2, U_2S, and U_3, being S: saturated FA and U: unsaturated FA). The first objective of the present study was to analyze in detail the influence of the reaction parameters considered (and their interactions) over the mp and by-products generation in interesterified products of SO and FHSO binary blends. The second goal was to construct regression models for all responses in order to obtain a concrete tool for predicting them when producing semisolid products in the experimental domain considered. Finally, the evaluation of the performance of the generated mathematical models was carried out establishing the reaction conditions to obtain a product with minimum by-products generation (to maximize TAG yield) and low mp (to assure that the enzymatic reaction has proceeded).

Experimental

Materials

Refined SO (approximate composition of predominant FA as FAME: 53.0 % C18:2 (*cis, cis*-9,12-octadecadienoic acid), 20.0 % C18:1 (*cis*-9-octadecenoic acid), 11.0 % C16:0 (hexadecanoic acid), 6.0 % C18:3 (*cis*, *cis*, *cis*-9,12,15-octadecatrienoic acid), according to AOCS Official Method Ce 2-62 and Ce 1e-91 [11]; peroxide value (PV): 1.16 meq/kg, according to AOCS Official Method Cd 8-53 [11]; calculated iodine value IV: 129.1, according to AOCS Official Method Cd 1c-85 [11], moisture content: 0.05 %(w/w), measured with a Karl Fischer titrator Mettler DL18, according to the AOCS Official Method Ca 2e-84 [11]) was provided by Molinos Río de la Plata SA (Buenos Aires, Argentina) and FHSO (approximate composition of predominant FA as FAME: 83.8 % C18:0 (octadecanoic acid), 12.3 % C16:0 (hexadecanoic acid), 1.4 % C18:1 (*cis*-9-octadecenoic acid); PV: 0.20 meq/kg; IV: 2.14, moisture content: 0.15 % (w/w)) was kindly provided by Calsa S.A. (Buenos Aires, Argentina). Immobilized lipases from *Rhizomucor miehei* [EC number 3.1.1.3, Lipozyme RM IM, immobilized on ion-exchange resin, *sn*-1,3 specific, with an original water content of 5.1 % (w/w), determined by the Karl Fisher Titration Method] and *Thermomyces lanuginosa* [EC number 3.1.1.3, Lypozyme TL IM, immobilized on granulated silica gel, *sn*-1,3 specific, with an original water content of 3.6 % (w/w)] was a generous gift of Novo Nordisk (Bagsvaerd, Denmark) and they were used as received. Molecular sieves (3 Å) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were obtained from Fluka (Buchs, Switzerland). All standards (1,2,3-trioctadecanoyl-glycerol, 1,2,3-trihexadecenoyl-glycerol, 1,2,3-trioctadecadienoyl-glycerol, 1,2,3-trioctadecenoyl-glycerol, 1,2,3-trihexadecanoyl-glycerol, 1,2,3-tridecanoyl-glycerol, 1,2-distearoyl-3-palmitoyl-*rac*-glycerol, 1,3-dipalmitoyl-2-oleoylglycerol, 1,3-dioleoyl-2-palmitoyl-glycerol, 1,2-dilinoleyl-3-palmitoyl-*rac*-glycerol, 1,2-dioleoyl-3-stearoyl-*rac*-glycerol, 1-palmitoyl-2-oleoyl-3-linoleoyl-*rac*-glycerol, 1,2-distearoyl-3-oleoyl-*rac*-glycerol, 1,3-dipalmitoyl-*rac*-glycerol, 1-monopalmitoyl-*rac*-glycerol, octadecenoic acid, tetradecane) were of purity greater than 98 % and were obtained from Sigma Chemical Co. (St. Louis, USA). Pyridine was from J. T. Baker (Phillipsburg, USA). All other reagents, gases and solvents were of analytical or chromatographic grade.

Methods

Experimental Design

Fractional factorial designs (FFD) are widely used in experiments involving several factors where it is necessary to study the joint effect of the factors on a response. They allow the effects of a factor to be estimated at several levels of the other factors, yielding conclusions that are valid over a range of experimental conditions [12]. Moreover, they are far less time- and experimental work-consuming than the classical one-factor-at-a-time experiments. When there are only two levels for each factor, it is assumed that the response is approximately linear over the chosen range of the factor levels although the addition of interaction terms to the main effects or first-order model contributes to representing some curvature in the response function obtaining a twisted plane [12].

In this study, experiments with each enzyme type (RM IM and TL IM) were performed with a 2^{6-2}_{IV} FFD including the central point. Reactions were run randomly and carried out in duplicate to clearly discriminate between random and systematic experimental errors, except at the central point where four replicates were made. Chromatographic and DSC analyses were also replicated twice. Factors and levels of the FFD are described in Table 1. The corresponding levels for each factor were determined after a thorough literature search and taking into account certain considerations about the system under study. For the enzyme concentration range selection the values considered were the most frequently used in the literature, as well as the two enzymes used [4, 13]. For the factor time, 1 h was chosen as a time to evaluate the initial stage of the reaction, while 24 h was considered as a moment in which the reaction would reach the stationary state [14]. Regarding the substrates ratio, two extreme ratios were considered for screening a wide range of this variable. Temperature levels were determined based on optimal temperatures for the enzymes used and setting the lower factor level in a higher value than the melting point of the FHSO for reducing mass transfer limitations. Agitation and system moisture levels were fixed according to values found in the literature [4, 15]. Moisture levels were determined according to the molecular sieves' mass added to the reaction system and not according to the water content because moisture is contributed by both substrates and enzymes, and they are added in different quantities in the different experiments. Therefore, it would have been impossible to establish fixed levels of water content.

Reaction Protocol

Blends constituted by SO and FHSO (total substrate weight 1 g) were placed in a screw-capped test tube, preheated in a water bath with a temperature controller (65–75 °C) and magnetic agitation (230–520 rpm) and, after 5 min of homogenization, biocatalyst (5–10 % w/w of substrates) and molecular sieves (0–10 % w/w of substrates) were added to start the reaction. Molecular sieves had been previously dried at 100 °C for 24 h. Reactions were stopped by removing enzymes by filtering with Whatman Grade 1 filter paper. Reaction products were stored below 0 °C.

Analytical Methods

Melting Point Determinations

Samples were analyzed by DSC with a Perkin-Elmer Pyris 1 calorimeter (Waltham, USA) adapting the AOCS Method Cc1-25 [11] to improve the reading of the corresponding temperature. The sample's mp was considered as the temperature at which the thermogram reached a straight and horizontal line when the sample was subjected to a heating rate of 5 °C/min from 10 to 65 °C. The Official Method [11] recommends a heating rate of 0.5 °C/min when visual inspection is performed. In this work, different heating rates were tested during the DSC experimentation with the same sample (and thermal history), assuring that the same result was obtained when a heating rate of 5 °C/min was used. In order to obtain a defined end of peak for mp determination, free fatty acids (FFA) were removed from the products according to the deacidification method described by Carrín et al. [13]. An empty aluminum pan was used as a blank. Samples of 5–8 mg were placed in aluminum pans and they were placed in an oven at 70 °C for an hour to

Table 1 Factors and levels for the experimental design

	Low level (−1)	Central point (0)	High level (+1)
Enzyme concentration (% $w/w_{subst.}$)	5	7.5	10
System moisture (% $w_{molecular\ sieves}/w_{subst.}$)	10	5	0
Time (h)	1	12.5	24
SO:FHSO (% w/w)	30:70	50:50	70:30
Temperature (°C)	65	70	75
Agitation level (rpm)	230	375	520

ensure that no air bubbles had been trapped. Afterwards, they were held at refrigerator temperature for at least 16 h before analysis in order to guarantee the same thermal history for all samples.

TAG Analysis

TAG quantification was performed by GC by means of a 4890D series gas chromatograph (Agilent, Hewlett-Packard) equipped with a FID (adapted from IRMM Method EUR 20831 EN) [16]. The injector was used in split mode (split ratio of 1:70) and held at 360 °C. A metallic capillary column (MXT-65TG, 30 m × 0.25 mm × 0.1 μm film thickness; Restek, Bellefonte, USA) was used. The FID temperature was constant at 380 °C. The column oven temperature was increased first from 200 to 350 °C at a rate of 15 °C/min and then to 360 °C at a rate of 0.2 °C/min. Hydrogen was used as the carrier gas at a linear velocity of 33.6 cm/s. An internal standard method was used to quantify TAG using tripalmitin as standard. Relative response factors of all available standard TAG were correlated with their relative residence time [17] in order to quantify TAG whose standards were not available. Data acquisition and peak integration were performed using HP 3398A GC Chemstation Software (Hewlett-Packard, 1998). The analysis was carried out grouping TAG in four different categories according to the number of saturated (S) and unsaturated (U) FA in the molecule [7–9]. Consequently, content of TAG in products is reported as S_3, US_2, U_2S, and U_3 (% w/w on total TAG).

By-Products Analysis

FFA, MAG and DAG were prepared and analyzed by GC according to Planck & Lorbeer [18], using the AOCS Official Method Cd 11b-91 [11] with modifications. A 4890D series gas chromatograph (Agilent, Hewlett-Packard) was used with a metallic capillary column (MXT-65TG, 15 m × 0.25 mm × 0.10 μm film thickness; Restek, Bellefonte, USA). Hydrogen at 45 cm/s was used as the carrier gas. A split ratio of 1:30 was used during injection. Oven temperature was programmed as follow: 40 °C for 4 min, then increased up to 365 °C at 25 °C/min, and held at this temperature for 15 min. Injector and FID temperatures were 320 and 370 °C, respectively. The internal standard method was used to quantify each group of by-products (FFA, MAG, and DAG) with a calibration curve for each one, being tetradecane the internal standard for FFA and glyceryl tridecanoate for MAG and DAG. For the case of FFA, the standard used was oleic acid, while monopalmitin and dipalmitin were the standards considered for MAG and DAG, respectively. The presence of glycerol was evaluated with this technique. Data acquisition and peak integration were carried out using HP 3398A GC Chemstation Software (Hewlett-Packard, 1998).

Statistical Analysis

Data obtained from the experimental design was analyzed using the software Design-Expert® (Stat-Ease Inc., 2006). Five responses were studied: mp (°C), by-products generation (% w/w of total product) and S_3, US_2, and U_3 contents (% w/w of total TAG). By-products generation was calculated as the difference between the sum of FFA, MAG, and DAG contents in the reaction product and the ones in the control systems (blends at similar conditions without enzyme). The main and interaction effects of the six factors under study with regard to each response were determined for each enzyme type (Lipozyme RM IM and Lipozyme TL IM). Their significance was evaluated by analysis of variance (ANOVA, $P \leq 0.05$) and multiple linear regression models were obtained as follows:

$$\hat{y} = \beta_0 + \sum_{i=1}^{6} \beta_i x_i + \sum_{i=1}^{6} \sum_{j \neq i, j=i}^{6} \beta_{ij} x_i x_j \quad (1)$$

where $\hat{y}$ is the response being studied, β_0 the intercept, β_i main effect coefficient for the ith factor, β_{ij} interaction model coefficient for the interaction between factor ith and jth, and x_i and x_j are each of the factors considered.

They were validated by their statistical coefficients [R^2, R^2_{adj} and lack of fit (LOF) value] and then used to predict models' responses at one point of the design region. Prediction intervals ($\alpha = 0.1$) for these observations were constructed according to Eq. (2).

$$\hat{y}(\mathbf{x}_0) - t_{\alpha/2,n-p}\sqrt{\hat{\sigma}^2(1 + \mathbf{x}_0'(\mathbf{X}'\mathbf{X})^{-1}\mathbf{x}_0} \leq y_0 \leq \hat{y}(\mathbf{x}_0) + t_{\alpha/2,n-p}\sqrt{\hat{\sigma}^2(1 + \mathbf{x}_0'(\mathbf{X}'\mathbf{X})^{-1}\mathbf{x}_0} \quad (2)$$

where $\mathbf{x}_0$ is vector of the levels of the regressor variables at the point of interest; $\mathbf{X}$ matrix of the levels of the independent variables; n total observations; p number of terms present in the model; $\hat{y}(\mathbf{x}_0)$ estimated mean response at $\mathbf{x}_0$; $t_{\alpha/2,n\text{-}p}$ upper $\alpha/2$ percentage point of the t distribution with n-p degrees of freedom; $\hat{\sigma}^2$ estimate of the error variance. The calculation of the prediction intervals was performed with the software Maple 11.0 (Waterloo Maple Inc., 2007).

Results and Discussion

The characterization of the raw materials (SO and FHSO) presented in the "Experimental" Section was completed with the data reported in Table 2. As expected, major TAG of SO and FHSO were of the triunsaturated (U_3) and trisaturated (S_3) groups, respectively. Melting points of

Table 2 Characterization of soybean oil (SO) and fully hydrogenated SO (FHSO)

Composition (w/w%)	SO	FHSO
FFA	1.01 ± 0.08	0.88 ± 0.14
MAG	0.11 ± 0.01	0.15 ± 0.02
DAG	1.13 ± 0.04	0.62 ± 0.08
TAG[a]		
PPP	ND[b]	0.25 ± 0.02
PPSt	ND	4.08 ± 0.23
PPO	0.66 ± 0.03	0.22 ± 0.01
PPL	2.85 ± 0.07	0.13 ± 0.04
PPLn	0.13 ± 0.07	0.28 ± 0.01
PStSt	0.07 ± 0.04	29.20 ± 0.91
POSt	0.75 ± 0.04	0.51 ± 0.29
POO	2.58 ± 0.10	0.32 ± 0.01
PLSt	2.18 ± 0.03	ND
POL	8.56 ± 0.13	0.50 ± 0.03
PLL	14.47 ± 0.71	ND
PLLn	0.53 ± 0.07	ND
StStSt	1.79 ± 0.27	59.91 ± 1.23
StStO	0.28 ± 0.09	0.29 ± 0.09
StOO	0.94 ± 0.16	0.17 ± 0.04
StStL	2.29 ± 0.15	ND
StOL	3.90 ± 0.20	ND
OOL	8.28 ± 0.63	ND
StLL	4.65 ± 0.49	ND
OLL	18.26 ± 0.27	ND
LLL	20.39 ± 0.57	ND
LLLn	3.96 ± 0.72	ND
Melting point (°C)	–	70.0 ± 0.1

[a] *P* palmitic acid, *St* stearic acid, *O* oleic acid, *L* linoleic acid, *Ln* linolenic acid

[b] *ND* non-detected value

binary blends were: 67.3, 64.5 and 61.1 for ratios 30:70, 50:50 and 70:30 (w/w, SO:FHSO), respectively. Glycerol was not present in any of the interesterified samples showing that in none of them total hydrolysis was achieved. The average FFA content for all reaction products was of 5.4 %, being the lowest level approximately 2 %, corresponding to reactions performed with molecular sieves and 5 % of Lipozyme RM IM for 1 h, in either of the two levels of the remaining factors. The highest levels of FFA were ca. 11.0 % and they were obtained with Lipozyme TL IM, in the presence of molecular sieves and when the reaction time was established as 24 h, independently of the levels of the rest of the factors. With regard to MAG, almost imperceptible quantities were found in all samples, being 0.80 % the average value determined. DAG content in all samples analyzed ranged from 3.2 to 12.8 % for Lipozyme RM IM and from 5.3 to 17.1 % for Lipozyme TL IM, with average proportion of 8.8 and 9.7 %, respectively. As expected, the maximum content was obtained for the samples in which the FFA content was also at its maximum level. The by-products content of all controls (samples with no enzyme) was similar (approximately 0.95 % FFA, 0.15 % MAG, and 1.3 % DAG). Tristearin 1,2,3-trioctadecanoyl glycerol composition was studied in particular due to the property of this TAG to impart a waxy mouthfeel. In the 30:70 (SO:FHSO) control samples its composition was ca. 42.5 %, while after EIE it ranged from 7.3 to 16.5 % depending on the reaction conditions. For the other substrate ratio, the starting tristearin composition was of 19.2 % and it reached values ranging from 1.2 up to 8.0 %. These results showed that the FA rearrangement EIE produces among the TAG mixture can have a marked effect on the content of this undesirable TAG.

Reaction Parameters Study

Main Effects and Interactions Analysis

Experiences of the FFD and data obtained for the experimental design responses are summarized in Table 3. MAG composition data are not shown there because they were too low, as stated above. With regard to TAG groups, U_2S was not included because it can be obtained from the data of other TAG groups. Moreover, it was the only TAG group that presented little variation along all the experiments.

The results of the ANOVA for the response mp showed that factors *D* (substrates ratio) and *C* (time), and the interaction between them (CD) contributed 77 and 81 % of the total mean squares of factors and interactions, for Lipozyme RM IM and Lipozyme TL IM, respectively. Enzyme concentration (*A*) and its interaction with time (*C*) presented a contribution of around 10 % for both biocatalysts, while the contribution of the rest of the significant factors and interactions did not exceed 2.7 %, demonstrating limited practical significance.

Forssell et al. [19] found for the EIE of tallow and rapeseed oil with *Mucor miehei* lipase that time, enzyme dosage, and temperature decreased the mp of the products. These results are in agreement with ours. System moisture was the only factor that was not considered significant by itself for both lipases, but as it was present in significant interactions, it would be wrong to completely disregard its effect. It is known that IE lowers the mp of the corresponding blends not only as a consequence of TAG profile modification but also as a result of partial glycerides appearance during the process [20, 21]. So our result that the factor time had a negative effect with regard to the mp is in agreement with that. It seems to be obvious that *D* had

Table 3 Experimental design responses

No	Factors[a]						mp[b] (°C)		By-products (% w/w)[b]				TAG (% w/w)[b,c]					
	A	*B*	*C*	*D*	*E*	*F*			FFA	DAG	FFA	DAG	S_3	US_2	U_3	S_3	US_2	U_3
							RM IM	TL IM	RM IM		TL IM		RM IM			TL IM		
1	−1	−1	−1	−1	−1	−1	61.1 ± 0.1	60.7 ± 0.2	1.8 ± 0.3	3.2 ± 0.5	3.1 ± 0.3	5.3 ± 1.6	25.8 ± 1.4	36.8 ± 2.6	9.3 ± 0.6	21.3 ± 1.9	40.6 ± 2.5	11.5 ± 1.1
2	−1	−1	−1	−1	1	1	58.3 ± 0.1	58.0 ± 1.2	5.0 ± 1.0	8.9 ± 2.1	5.5 ± 0.5	10.0 ± 2.2	25.1 ± 0.1	37.1 ± 0.6	12.1 ± 0.2	23.1 ± 1.3	39.8 ± 1.1	9.0 ± 0.4
3	−1	−1	−1	1	−1	1	56.4 ± 0.1	55.9 ± 1.0	2.8 ± 0.4	5.2 ± 0.4	2.7 ± 0.3	6.3 ± 0.8	12.0 ± 0.3	18.4 ± 0.5	40.0 ± 0.5	9.1 ± 1.1	21.0 ± 2.6	38.5 ± 1.5
4	−1	−1	−1	1	1	−1	56.5 ± 0.0	53.8 ± 0.4	2.6 ± 0.6	4.3 ± 1.0	3.7 ± 0.1	6.0 ± 1.0	11.4 ± 1.3	17.0 ± 1.9	41.5 ± 1.6	9.3 ± 1.5	18.9 ± 0.4	41.6 ± 0.3
5	−1	−1	1	−1	−1	1	54.3 ± 0.5	51.8 ± 0.4	6.1 ± 0.2	10.3 ± 0.1	11.3 ± 1.7	14.3 ± 1.4	15.0 ± 1.7	42.7 ± 3.0	17.1 ± 0.7	23.1 ± 1.5	45.1 ± 1.5	6.9 ± 0.4
6	−1	−1	1	−1	1	−1	57.3 ± 0.4	55.6 ± 1.1	3.6 ± 0.4	5.9 ± 0.9	6.6 ± 0.4	11.8 ± 1.6	14.2 ± 1.4	42.5 ± 1.2	25.7 ± 0.1	18.1 ± 1.3	43.3 ± 2.1	19.9 ± 1.7
7	−1	−1	1	1	−1	−1	44.1 ± 0.3	43.4 ± 0.9	6.0 ± 0.2	10.5 ± 0.2	6.8 ± 0.6	11.9 ± 1.0	2.9 ± 0.5	20.3 ± 0.1	45.2 ± 0.2	4.3 ± 0.6	22.6 ± 1.3	42.3 ± 1.6
8	−1	−1	1	1	1	1	45.2 ± 0.2	47.5 ± 0.6	6.4 ± 0.5	10.7 ± 1.2	9.1 ± 0.9	12.3 ± 0.7	3.1 ± 0.4	20.6 ± 2.3	54.1 ± 4.3	4.3 ± 0.7	21.8 ± 1.4	46.4 ± 3.0
9	−1	1	−1	−1	−1	1	61.5 ± 0.0	59.8 ± 1.2	3.6 ± 0.1	6.2 ± 0.2	5.0 ± 0.1	9.2 ± 0.2	22.7 ± 1.5	37.9 ± 0.4	12.6 ± 0.4	21.4 ± 1.7	42.1 ± 2.4	4.8 ± 1.5
10	−1	1	−1	−1	1	−1	60.8 ± 0.1	58.8 ± 0.4	4.3 ± 0.1	7.3 ± 1.0	4.5 ± 0.7	9.3 ± 0.9	24.8 ± 0.7	39.8 ± 2.8	8.8 ± 0.9	19.2 ± 1.6	42.5 ± 1.2	8.7 ± 0.0
11	−1	1	−1	1	−1	−1	58.9 ± 0.0	58.5 ± 0.0	3.2 ± 0.5	5.2 ± 1.1	3.7 ± 0.3	6.9 ± 0.1	10.9 ± 1.2	16.6 ± 1.9	43.4 ± 1.8	8.1 ± 1.0	20.2 ± 1.5	41.3 ± 0.8
12	−1	1	−1	1	1	1	56.1 ± 0.1	56.2 ± 1.4	4.4 ± 0.6	8.0 ± 1.3	5.2 ± 0.3	9.5 ± 1.1	10.1 ± 0.8	16.6 ± 4.5	44.6 ± 6.3	8.4 ± 1.0	18.8 ± 2.6	43.8 ± 2.1
13	−1	1	1	−1	−1	−1	56.8 ± 0.4	55.1 ± 1.0	4.5 ± 1.3	8.2 ± 2.7	5.4 ± 0.4	10.0 ± 1.8	15.8 ± 1.4	46.2 ± 2.5	7.2 ± 0.5	17.5 ± 1.3	46.8 ± 1.7	6.3 ± 0.6
14	−1	1	1	−1	1	1	57.1 ± 0.2	56.8 ± 0.4	4.6 ± 0.2	8.6 ± 0.6	7.3 ± 0.7	11.8 ± 1.7	15.0 ± 1.7	45.8 ± 3.2	19.6 ± 2.2	19.8 ± 1.1	48.1 ± 0.7	4.4 ± 0.9
15	−1	1	1	1	−1	1	44.7 ± 0.3	43.7 ± 0.4	6.2 ± 0.8	9.2 ± 0.4	5.2 ± 0.5	9.8 ± 1.7	3.1 ± 0.4	20.3 ± 2.3	49.2 ± 4.6	4.5 ± 0.2	22.0 ± 0.5	41.7 ± 0.8
16	−1	1	1	1	1	−1	47.2 ± 0.5	47.8 ± 0.9	5.5 ± 0.2	9.1 ± 0.6	5.4 ± 1.5	8.8 ± 1.1	4.8 ± 1.9	20.6 ± 1.4	57.0 ± 2.8	4.5 ± 0.9	21.3 ± 1.7	47.7 ± 1.7
17	0	0	0	0	0	0	52.3 ± 0.2	54.1 ± 0.3	7.5 ± 0.1	12.8 ± 1.0	6.3 ± 1.2	11.5 ± 1.3	9.7 ± 0.2	30.8 ± 0.7	30.6 ± 1.3	11.4 ± 0.2	35.1 ± 0.4	24.1 ± 1.3
18	1	−1	−1	−1	−1	1	58.8 ± 0.4	58.3 ± 1.3	4.7 ± 0.1	8.4 ± 0.2	5.3 ± 0.6	9.7 ± 1.2	17.9 ± 2.7	41.9 ± 0.7	9.2 ± 0.7	16.1 ± 1.7	42.6 ± 1.5	11.3 ± 1.3
19	1	−1	−1	−1	1	−1	53.2 ± 0.1	56.1 ± 0.4	5.0 ± 0.5	8.3 ± 0.1	5.8 ± 0.0	9.1 ± 0.3	16.0 ± 1.8	41.6 ± 2.9	12.6 ± 3.9	14.7 ± 2.0	41.8 ± 0.2	11.6 ± 0.8
20	1	−1	−1	1	−1	−1	53.9 ± 0.1	53.3 ± 0.4	4.0 ± 0.9	6.1 ± 1.0	4.0 ± 1.4	7.1 ± 0.4	8.2 ± 0.9	19.5 ± 2.2	38.6 ± 5.5	6.6 ± 0.3	19.3 ± 0.1	44.3 ± 0.8
21	1	−1	−1	1	1	1	49.5 ± 0.7	51.5 ± 1.0	4.7 ± 1.0	8.2 ± 2.1	5.6 ± 0.1	9.3 ± 1.2	4.7 ± 1.1	19.8 ± 2.0	43.2 ± 3.1	5.2 ± 0.7	20.4 ± 2.2	42.2 ± 3.9
22	1	−1	1	−1	−1	−1	56.3 ± 0.7	56.3 ± 1.1	5.4 ± 0.1	9.1 ± 0.1	8.0 ± 1.2	14.2 ± 1.3	16.9 ± 3.2	43.9 ± 4.0	8.6 ± 1.2	18.7 ± 2.9	44.5 ± 2.8	12.2 ± 1.7
23	1	−1	1	−1	1	1	55.1 ± 0.1	53.5 ± 0.7	6.5 ± 0.6	10.6 ± 0.9	11.2 ± 1.1	16.0 ± 1.9	13.9 ± 1.6	45.8 ± 3.2	13.7 ± 0.9	18.5 ± 1.0	46.9 ± 0.2	11.9 ± 0.7
24	1	−1	1	1	−1	1	45.7 ± 0.2	47.7 ± 0.5	6.9 ± 0.8	12.3 ± 1.2	11.7 ± 0.5	17.1 ± 0.8	3.4 ± 0.4	21.4 ± 2.4	39.5 ± 1.5	4.4 ± 0.2	23.0 ± 1.3	37.5 ± 0.2
25	1	−1	1	1	1	−1	44.3 ± 0.6	45.6 ± 0.9	6.6 ± 0.9	11.0 ± 1.5	8.4 ± 0.8	14.4 ± 1.3	3.6 ± 0.1	21.4 ± 0.3	41.7 ± 0.7	3.7 ± 0.5	21.0 ± 0.8	45.8 ± 0.1
26	1	1	−1	−1	−1	−1	56.3 ± 0.1	54.8 ± 0.4	5.1 ± 0.4	8.6 ± 0.1	6.7 ± 0.5	10.8 ± 1.6	18.0 ± 2.0	44.5 ± 3.1	7.3 ± 0.3	15.0 ± 0.4	45.4 ± 0.2	7.3 ± 0.7
27	1	1	−1	−1	1	1	50.8 ± 0.4	52.3 ± 1.0	6.7 ± 1.3	11.8 ± 2.5	6.6 ± 0.3	12.0 ± 1.2	17.3 ± 2.1	45.4 ± 0.7	7.6 ± 0.7	14.3 ± 1.7	45.1 ± 1.0	3.9 ± 1.4
28	1	1	−1	1	−1	1	52.4 ± 0.1	53.7 ± 1.0	6.0 ± 0.9	10.2 ± 1.5	8.1 ± 0.8	9.1 ± 1.4	7.4 ± 0.1	17.8 ± 1.2	45.4 ± 1.0	6.2 ± 0.9	19.9 ± 2.5	43.2 ± 1.0
29	1	1	−1	1	1	−1	48.1 ± 0.1	50.5 ± 0.0	6.0 ± 0.5	10.5 ± 1.2	5.9 ± 1.4	10.5 ± 0.9	6.8 ± 0.8	19.1 ± 2.2	43.2 ± 0.5	5.4 ± 0.2	20.7 ± 0.4	41.8 ± 0.4
30	1	1	1	−1	−1	1	56.0 ± 0.2	57.2 ± 0.3	5.4 ± 1.0	9.2 ± 0.8	8.6 ± 0.5	11.4 ± 0.9	15.6 ± 0.9	47.6 ± 1.7	4.4 ± 0.6	19.7 ± 2.0	46.9 ± 0.9	5.2 ± 0.7
31	1	1	1	−1	1	−1	54.8 ± 0.4	54.4 ± 1.1	5.1 ± 0.1	8.9 ± 0.5	6.4 ± 0.6	11.5 ± 1.3	13.1 ± 0.2	49.6 ± 0.7	6.9 ± 1.3	17.2 ± 1.1	48.4 ± 2.4	6.3 ± 1.1
32	1	1	1	1	−1	−1	45.3 ± 0.4	45.6 ± 0.9	5.4 ± 0.2	8.8 ± 0.8	5.9 ± 1.5	11.1 ± 0.8	4.2 ± 0.2	22.0 ± 1.1	41.3 ± 2.2	4.6 ± 0.6	20.1 ± 0.9	43.9 ± 0.1
33	1	1	1	1	1	1	44.2 ± 0.2	44.5 ± 0.8	5.8 ± 0.7	10.5 ± 1.4	7.9 ± 1.2	10.5 ± 0.6	2.9 ± 0.3	21.1 ± 2.4	47.1 ± 4.8	3.7 ± 0.5	18.9 ± 0.6	44.3 ± 1.5

[a] See Table 1 for more details

[b] Mean value ± standard deviation

[c] Total TAG basis

a negative effect on mp, i.e. a higher proportion of oil in the blend led to a lower mp of the corresponding interesterified product. When the content of oil in the reaction mixture was higher ($D = 1$), the decrease in the mp of the resulting product with time was more marked. When a higher proportion of fat is present in the blend, mass transfer/diffusional limitations may appear and the esterification step may be retarded [15], increasing the concentration of by-products in the medium. If this were the case, the interaction effect should have been opposite. The values of secondary products generation under both conditions (low and high levels of the substrate ratio) also demonstrated that this phenomenon did not occur as their average values were similar for equal reaction times (for example, 9.2 and 9.9 % when $C = -1$, and 10.7 and 13.5 % when $C = 1$, when Lipozyme RM IM was at 5 % in the reaction media). However, the comparison of the TAG composition changes for samples in the low and high levels of the substrates ratio when time increases from 1 to 24 h could explain the described effect. For Lipozyme RM IM at 10 %, in the oil enriched mixtures the decrease in S_3 was of 50 % while the increase in US_2 was of 16 % when time shifted from its low to its high level. On the other hand, for systems with higher fat content ($D = -1$) the corresponding percentages resulted in 14 and 8 % (change percentages of U_3 and U_2S being similar for both conditions at about 6 and 5 %, respectively). From these results it can be inferred that S_3 is the main TAG group responsible for the decrease in mp.

Regarding factor *A* (enzyme concentration), it presented a negative correlation with the response under study, i.e. a higher catalyst concentration led to a lower mp of the obtained products. As stated above, chemical changes in composition due to the interesterification reaction decrease the product's mp, as well as these changes increase with enzyme concentration. Concerning interaction AC, for both lipases it was observed that with a 24-h reaction time ($C = 1$) the fats produced had a similar mp over the analyzed range of the amount of enzyme, while for a 1-h reaction time ($C = -1$) the lower the enzyme concentration, the higher the mp obtained. If at 24 h the reaction reached its thermodynamic equilibrium, product compositions also depended upon enzyme type and the water quantity it introduces into the system. However, this result would indicate that an increase in lipase concentration would not lead to significant changes in the global composition of the products, thus obtaining a comparable mp under both conditions and for both enzymes. In contrast, at 1 h of reaction time, systems with twice the enzyme content (and, therefore, twice the number of active sites) will produce bigger changes in samples composition, producing a greater decrease in their mp.

With regard to the second response (by-products generation), only 8 and 7 main effects and interactions were considered significant for Lipozyme RM IM and Lipozyme TL IM, respectively (Table 4, coefficients with p value < 0.05), being *A*, *C*, *D*, and BC the most significant ones for the former (86.7 % of mean squares of factors and interactions) and *A*, *C*, *F*, and BC for the latter (81.9 % of mean squares of factors and interactions). For the main effects, their high levels increased the generation of by-products. Substrate ratio (*D*) only gave a significant effect when Lipozyme RM IM was used. This means that each lipase may have different preferences for the substrate it acts on. Enzyme load had a positive effect on this response, probably due partially to the extra water incorporated by the enzyme [4, 18, 22]. When time was set to a low level, both enzymes acted similarly with respect to this response, while reaction products obtained at 24 h with Lipozyme TL IM contained much more secondary products than those obtained with Lipozyme RM IM. It can be said that higher yields of purified products could be obtained using the latter. Lipozyme TL IM is immobilized over silica gel particles. The acyl migration effect silica gel has over FA during biodiesel production has been demonstrated elsewhere [23] and through the analysis of 1(3),2-DAG and 1,3-DAG we have also observed the same effect in our systems (data not shown). The phenomenon of acyl migration implies the spontaneous displacement of a FA from the *sn*-2 position to one of the outer positions in the same glyceride to reach a more stable configuration. Considering the *sn*-1,3-specificity of the enzymes, the resulting 1,3-DAG have no opportunity of being directly reesterified to TAG, thus contributing to by-products generation.

In the case of BC interaction, for 1 h of reaction time, moisture reduced systems ($B = -1$) gave lower by-products probably due to the lack of water molecules necessary to initiate the hydrolysis step [15]. Conversely, after 24 h of reaction time, secondary products generation in those systems was higher than in the original moisture level ones ($B = +1$), principally when Lipozyme TL IM was participating in the reaction. It is beyond discussion that in lipase-catalyzed reactions a reduction to some extent in the moisture system limits the hydrolysis step, generating lower levels of secondary products. Thus, the result obtained was opposite to the expected one. It can be thought that some kind of interaction between the molecular sieves and the different substrates and products, or even with lipases or supports of biocatalysts occurs, since results differed from one enzyme to the other. Definitively this result deserves a further study to elucidate the possible mechanisms involved. Torres et al. [15] found that the presence of molecular sieves favored the EIE between corn oil and tristearin, reacting at 45 °C. Our results were not in accordance with those. Probably, the different conditions used in both reaction systems contributed to a change in the behavior of each reaction system.

Table 4 Regression coefficients (RC) of mp and by-products generation models and their significance *P* values

Source	Mp (°C)				Byproducts generation (% w/w)			
	Lipozyme RM IM		Lipozyme TL IM		Lipozyme RM IM		Lipozyme TL IM	
	RC	*P* value	RC	*P* value	RC	*P* value	RC	*P* value
Intercept	53.158	<0.001	53.068	<0.001	12.548	<0.001	14.260	<0.001
A Enzyme concentration	−1.642	<0.001	−0.871	<0.001	1.613	<0.001	1.805	<0.001
B System moisture	−0. 003	0.941	0.028	0.785	0.251	0.344	−0.462	0.138
C Time	−2.670	<0.001	−2.685	<0.001	0.905	0.001	2.834	<0.001
D Oil:fat	−3.652	<0.001	−3.132	<0.001	0.659	0.016	–	
E Temperature	−0.784	<0.001	−0.406	<0.001	0.406	0.128	0.388	0.211
F Agitation level	−0.314	0.001	−0.050	0.626	0.308	0.247	2.237	<0.001
AB	−0.562	<0.001	−0.606	<0.001	–		–	
AC	1.330	<0.001	1.073	<0.001	−0.463	0.084	–	
AE	−0.756	<0.001	−0.752	<0.001	–		–	
AF	0.323	<0.001	0.192	0.063	–		–	
BC	0.234	<0.001	–		−1.641	<0.001	−2.001	<0.001
BF	–		–		−0.553	0.040	−0.914	0.005
CD	−1.786	<0.001	−1.547	<0.001	–		–	
CE	0.903	<0.001	0.710	<0.001	−0.613	0.024	−0.760	0.017
CF	–		–		–		1.645	<0.001
DE	0.137	0.0020	–		–	–	–	
DF	–		–		−0.502	0.062	–	
DG	–		–		–	–	–	
R^2	0.997		0.979		0.723		0.869	
R^2_{adj}	0.995		0.972		0.628		0.804	
Curvature		0.120		0.150		0.130		0.109
LOF		0.190		0.315		0.925		0.489

LOF lack of fit

Depending on the selected main effects and interactions for each response, linear regression models were set up in order to successfully fit the experimental data. Some main effects which turned out to be non-significant were added to regression models in order to maintain hierarchy [12]. The coefficients for all models according to Eq. (1) with their corresponding *P* values are presented in Tables 4, 5, and 6. The statistical parameters [R^2 R^2_{adj}, curvature and lack of fit (LOF) value] for each model are also presented, demonstrating that models obtained to relate variables with responses can be taken to analyze the system behavior into the studied variable range (LOF and curvature *p* values were higher than 0.05 in all cases). Coefficients of TAG models show that few interactions had any effect over any of considered TAG groups.

All determination coefficients show a good correlation between the observed values and the considered factors and their interactions.

Analyzing the range of the mp values obtained, numeric optimizations were carried out using the software Design-Expert®, establishing, as objective functions, the minimization of both melting point and by-product generation. Minimization of by-product generation is directly related to the intention of obtaining maximum product (TAG) yield. Minimization of the melting point function corresponds to the objective of assuring the enzymatic reaction has proceeded. Such optimization yielded the following optimal factor levels, independently of which lipase was considered: 5 % (% w/w_{subst}) of biocatalyst, 70:30 (oil:fat, % w/w), 65 °C, agitation at 230 rpm and 24 h of reaction time. Analyzing these conditions, the low level of enzyme concentration and temperature, and the absence of molecular sieves confer an advantage from an economical point of view giving this result an even more attractive significance. Prediction intervals according to Eq. (2) were similar for both lipases, resulting: 43.4–46.0 °C for mp,

Table 5 Regression coefficients of TAG models and their significance *P* values for interesterified fats obtained using Lipozyme RM IM

Source	Ln ($\%S_3$)	*P* value	% US_2	*P* value	% U_3	*P* value
Intersection[a]	2.28	$<10^{-4}$	31.29	$<10^{-4}$	28.05	$<10^{-4}$
A	−0.09	$<10^{-4}$	1.35	$<10^{-4}$	−2.42	$<10^{-4}$
B	–		0.62	0.016	−0.20	0.679
C	−0.32	$<10^{-4}$	1.94	$<10^{-4}$	1.840	$<10^{-3}$
D	−0.58	$<10^{-4}$	−11.76	$<10^{-4}$	16.64	$<10^{-4}$
E	−0.03	0.017	–	–	1.91	$<10^{-3}$
Interactions[a]						
AC	0.11	$<10^{-4}$	–	–	−2.08	$<10^{-4}$
AE	−0.04	0.0232	–	–	–	0.0016
BD	–	–	−0.90	$<10^{-3}$	1.91	$<10^{-3}$
CD	−0.16	$<10^{-4}$	–	–	–	–
CE	–	–	–	–	1.43	0.004
R^2	0.980		0.974		0.958	
R^2_{aj}	0.977		0.971		0.952	
Curvature		0.996		0.110		0.250
LOF		0.144		0.822		0.927

[a] *A* enzyme concentration, *B* system moisture, *C* time, *D* oil:fat mass ratio, *E* temperature, *F* agitation level

Table 6 Regression coefficients of TAG models and their significance *P* values for interesterified fats obtained using Lipozyme TL IM

Source	Ln ($\%S_3$)	*P* value	$\%US_2$	*P* value	$\%U_3$	*P* value
Intersection[a]	2.30	$<10^{-4}$	32.49	$<10^{-4}$	25.86	$<10^{-4}$
A	−0.11	$<10^{-4}$	–	–	–	–
B	–		0.45	0.062	−1.19	0.007
C	−0.11	$<10^{-4}$	1.31	$<10^{-4}$	–	–
D	−0.60	$<10^{-4}$	−11.88	$<10^{-4}$	17.04	$<10^{-4}$
F	–	–	–	–	−1.17	0.008
Interactions[a]						
AC	0.08	$<10^{-4}$	–	–	–	–
BD	–	–	−0.83	$<10^{-3}$	1.77	$<10^{-4}$
CD	−0.15	$<10^{-4}$	−0.58	0.018	–	–
R^2	0.959		0.977		0.963	
R^2_{aj}	0.956		0.975		0.961	
Curvature		0.070		0.09		0.311
LOF		0.896		0.845		0.315

[a] *A* enzyme concentration, *B* system moisture, *C* time, *D* oil:fat mass ratio, *E* temperature, *F* agitation level

3.6–18.1 % for by-products generation, 2.3–5.3 % for S_3, 16.2–25.6 % for US_2, and 40.3–51.9 % for U_3. The obtained experimental values at those conditions fell inside their corresponding intervals (mp: 44.7 ± 0.6 °C, by-products generation: 15.3 ± 2.4 %, S_3: 4.0 ± 0.2 %, US_2: 22.3 ± 1.8 %, and U_3: 41.3 ± 2.5 %, for Lipozyme RM IM, and mp: 43.9 ± 0.9 °C, by-products generation: 14.8 ± 1.3 %, S_3: 4.6 ± 0.8 %, US_2: 22.0 ± 1.2 %, and U_3: 41.0 ± 1.1 %, for Lipozyme TL IM), validating not only the optimized results, but also the models obtained. Moreover, regarding the content of tristearin, its value was low enough (ca. 1.7 %) to avoid the undesirable waxy mouthfeel it imparts. Petrauskaite et al. [7] reported a similar mp (47 °C) for chemically interesterified blends of similar substrates and in the same proportion.

Conclusions

Binary blends of SO and FHSO were effectively interesterified using immobilized lipases (Lipozyme RM IM and Lipozyme TL IM) at different levels of processing variables. Products with different TAG composition and by-products content were obtained, modifying their mp. It was demonstrated that experimental designs varying one variable at a time are incomplete ones since interactions among factors can be highly significant. The most important variables for obtaining semi-solid shortenings with minimum by-product generation were: enzyme concentration, time, and substrates ratio. Both lipases presented different behavior when molecular sieves were used.

Regression models for all responses (by-products generation, mp, S_3, US_2, and U_3) were constructed and validated afterwards, obtaining useful tools for predicting semisolid products physical and compositional properties. Optimal variables levels could be established (independently of which lipase was considered: 5 % (% w/w_{subst}) of biocatalyst, 70:30 (oil:fat, % w/w), 65 °C, agitation at 230 rpm and 24 h of reaction time, without molecular sieves in the reaction medium) for reaching not only the desired physical property, but also the highest yield of TAG. Subsequent confirmation assays validated the results obtained.

Acknowledgments This work was financially assisted by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), and the Universidad Nacional del Sur (UNS) of Argentina.

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