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Optimization of the Enzymatic Interesterification of Milk Fat and Canola Oil Blends Using Immobilized *Rhizopus oryzae* Lipase by Response Surface Methodology

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Summary

Blends of milk fat and canola oil (MF:CNO) were enzymatically interesterified (EIE) by *Rhizopus oryzae* lipase immobilized on polysiloxane-polyvinyl alcohol (SiO_2-PVA) composite, in a solvent-free system. A central composite design (CCD) was used to optimize the reaction, considering the effects of different mass fractions of binary blends of MF:CNO (50:50, 65:35 and 80:20) and temperatures (45, 55 and 65 °C) on the composition and texture properties of the interesterified products, taking the interesterification degree (ID) and consistency (at 10 °C) as response variables. For the ID variable both mass fraction of milk fat in the blend and temperature were found to be significant, while for the consistency only mass fraction of milk fat was significant. Empiric models for ID and consistency were obtained that allowed establishing the best interesterification conditions: blend with 65 % of milk fat and 35 % of canola oil, and temperature of 45 °C. Under these conditions, the ID was 19.77 % and the consistency at 10 °C was 56 290 Pa. The potential of this eco-friendly process demonstrated that a product could be obtained with the desirable milk fat flavour and better spreadability under refrigerated conditions.

Key words: interesterification, *Rhizopus oryzae* lipase, milk fat, canola oil, experimental design

Introduction

Milk fat, with its unique and attractive flavour, is an extensively used dairy product (*1*). In addition, milk fat has beneficial effects on health maintenance and disease prevention, because it contains butyric and conjugated linoleic acids (CLA), which have anti-carcinogenic properties (*2*,*3*).

However, milk fat has attributes that limit its consumption and use (*4*), mainly due to its poor spreadability when refrigerated (*1*). To overcome this problem, various alternatives, such as fractionation, selective blending, and chemical or enzymatic processes to produce specialty milk fat ingredients have been applied (*5*). Among these processes, the enzymatic interesterification (EIE) is a promising technique for the production of structured lipids (*6*).

Enzymatic processes are interesting because enzymes are recyclable, eco-friendly and non-toxic materials (*7*). Lipase-catalyzed interesterification also has advantages compared to the chemical process, such as mild conditions, fewer side products (diacylglycerols, monoacylglycerols, and free fatty acids) and reaction specificity (substrate and positional specificity and stereospecificity) (*8*). When compared to simple fat blending, enzymatic interesterification results in new products with different triacylglycerol (TAG) composition, and consequently modified physical properties (*9*).

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Interesterification of milk fat has been carried out with immobilized lipases, in both solvent and solvent--free systems, in a batch-type reactor or a continuous packed-bed reactor, at temperatures from 37 to 80 °C, with binary blends using vegetable oils (*10*).

In the present work, milk fat and canola oil blends were enzymatically interesterified in solvent-free systems, using the *Rhizopus oryzae* lipase immobilized in polysiloxane-polyvinyl alcohol (SiO_2-PVA), to assess the effect of different proportions of milk fat mass and different temperatures on the composition as well as texture properties of the obtained product. The objective is to develop a process with a potential to obtain a more spreadable product under domestic refrigeration conditions, which maintains the characteristics of milk fat but with a lower content of saturated fats and enriched with essential fatty acids.

The chosen lipase was a commercially available food--grade enzyme, previously selected as suitable to mediate this kind of reaction (*11*). The carrier SiO_2-PVA has been used to immobilize lipases from different sources with promising results in the synthesis of monoacylglycerols, biodiesel and structured lipids (*12*,*13*). Considering the nutritional properties, canola oil was used because it contains a suitable proportion of essential fatty acids ω-6 and ω-3 (2:1), respectively (*14*).

Material and Methods

Materials

A commercial food-grade lipase from *Rhizopus oryzae* (L036P, Biocatalysts, Cardiff, UK) in a crude form was used without further purification.

Milk fat was obtained from commercial butter (Aviação™ without salt, purchased in a local market, Brazil). The butter was totally melted at 50–60 °C in a microwave oven, followed by centrifugation (1900×*g* for 10 min) to separate the aqueous phase. Commercial canola oil (Liza, purchased in a local market, Brazil) was used. Tetraethoxysilane (TEOS) and polyvinyl alcohol (PVA, M_r= 88 000) were acquired from Aldrich Chemical Co. (Milwaukee, WI, USA). Hydrochloric acid (minimum 36 %), ethanol (minimum 95 %) and polyethylene glycol (PEG 1500 g/mol) were supplied by Synth (São Paulo, Brazil). All the other reagents were of analytical grade.

Support synthesis, activation and immobilization procedure

The polysiloxane-polyvinyl alcohol (SiO_2-PVA) hybrid matrix was prepared by sol-gel technique (*11*). The resulting SiO_2-PVA particles were neutralized with 0.1 M KOH solution at 1:15 (solid:liquid) ratio, under agitation for 30 min. The material was then washed with distilled water and phosphate buffer (0.1 M, pH=7.0). Then, the particles were dried at 60 °C for 24 h and used to immobilize *Rhizopus oryzae* lipase by physical adsorption according to the methodology described by de Paula *et al*. (*11*). The immobilized derivative displayed average hydrolytic activity of (4890±322) U/g and moisture content of about 10 % by mass. One activity unit (U) was defined as the mass of enzyme (dry basis) that liberates 1 μmol of free fatty acid per minute under assay conditions, using olive oil as a substrate, as described elsewhere (*11*).

Interesterification of milk fat with canola oil

Batch interesterification reactions were performed under inert nitrogen atmosphere in cylindrical glass reactors (80 mL), containing 40 g of milk fat and canola oil blends. The reaction medium was incubated with immobilized derivative on SiO_2-PVA at fixed loading of 500 U/g of reaction medium. All assays were carried out under magnetic stirring (200 rpm) for 48 h.

Statistical design

The mass fractions of raw materials in the blend and temperatures were adjusted according to the central composite design (CCD) as shown in Table 1. The response variables were interesterification degree (ID) and consistency (at 10 °C).

Table 1. Experimental ranges and levels of the independent variables according to the central composite design

Independent variable	Symbol	Range and levels		
		–1	0	+1
Temperature/°C	X_1	45	55	65
w(milk fat)/%	X_2	50	65	80

A multiple regression model was applied to the response variables. The softwares STATISTICA (v. 5.0, StatSoft, Inc., Tulsa, OK, USA) and Design–Expert (v. 6.0.6, Stat-Ease, Inc., Minneapolis, MN, USA) were used to evaluate the effects of the independent variables (ID and consistency), to generate coefficients for the models and to assess their significant levels, determination coefficients and variance analysis.

Triacylglycerol (TAG) composition

TAGs of the non-interesterified (NIE) blends and enzymatically interesterified (EIE) products were analyzed by gas chromatography using a Varian CP 3800 chromatograph (Varian, Inc., Palo Alto, CA, USA) equipped with a 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 to 210 °C, which was kept constant for 1 min. After that, the column temperature was programmed at 6 °C/min to 340 °C and kept constant for 2 min. The injection volume was 1 μL. Before injection, samples were diluted in hexane, and tetradecane was used as internal standard. The chromatograms were processed using the software Galaxie Chromatography Data System, v. 1.9 (Agilent Technologies, Inc., Santa Clara, CA, USA). For the determination of TAG calibration curve, milk fat standard of the Community Bureau of Reference Materials (*15*) was used. The groups of TAGs were

identified by carbon number (CN), *i.e.* the total number of acyl-C atoms within the triglyceride.

Interesterification degree

The interesterification degree (ID) was defined according to the following equation (*16*):

$$\mathrm{ID}/\% = \frac{\sum(\mathrm{TAG}_{I_t} - \mathrm{TAG}_{I_0})}{\sum \mathrm{TAG}_{D_0}} \qquad /1/$$

where TAG_I is the fraction (% by mass) of triacylglycerols which increased during the reaction, TAG_D is the fraction (% by mass) of triacylglycerols, which decreased during the reaction, subscripts t and 0 represent the fractions of TAG at a given reaction time and at the beginning of the reaction, respectively.

Consistency tests

The consistency of the NIE blends and EIE products was determined using a texture analyzer (model QTS--25) controlled by the TexturePro software (v. 2.1, Brookfield, Middleboro, MA, USA). Samples were heated in a microwave oven (50–60 °C) for the fusion of the crystals, and conditioned in cubic silicone moulds (15.6 mL) for 48 h at 10 °C. For the consistency analysis a probe TA15 was used corresponding to an acrylic cone with the angle of 45° (*16,17*). The consistency tests were performed under the following conditions: total of cycles 1; distance 10 mm, speed 120 mm/min, time 5 s, determination of the force in compression. Consistency was calculated as yield value according to the equation proposed by Haighton (*18*) and the results were expressed in Pa.

Results and Discussion

The interesterification degree (ID) and consistency values for EIE products are shown in Table 2. The ID varied from 16.78 to 24.77 % and the highest value was obtained for the medium containing the lowest milk fat fraction (MF:CNO=50:50 blend), while the lowest value was obtained using MF:CNO=80:20 blend. Concerning the values of consistency, which are inversely associated with the spreadability perception, the highest (188 091.5 Pa) and lowest (0 Pa) were obtained for MF:CNO=80:20 and 50:50 blends, respectively. Values equal to zero (runs 3 and 4) indicated that this property of EIE product could not be detected since the samples were fluid at the analyzed temperature (10 °C).

In comparison with NIE blends, the highest reductions in the consistency values of 88 % (run 10) and 100 % (runs 3 and 4) were verified for interesterified products obtained using the MF:CNO=50:50 blend, while the product obtained using the MF:CNO=80:20 blend showed the lowest decrease in this response variable, from 26 % (run 8) to 43 % (run 2). For products using the MF:CNO= 65:35 blend, the decrease varied from 32 % (run 11) to 80 % for runs at centre points (runs 5 and 7). Similar behaviour was observed by de Paula *et al.* (*13*) in the interesterification of different milk fat and soybean oil blends.

As shown in Table 3, only the main effects of the independent variables were significant for ID. The positive and negative sign of the effects indicated that the value of this response variable was high for higher temperatures and lower fractions of milk fat. Regarding the consistency, the main and quadratic effects for the fractions of milk fat were significant and this response value was not significantly affected by the temperature. The positive and negative sign of the effects indicate that the value of this response variable was high for higher fractions of milk fat.

To optimize the interesterification reactions, regression analysis was performed to fit the response variables as a function of the experimental data. The models shown in Eqs. 2 and 3 represent the ID (Y_1) and consistency (Y_2) as a function of X_1 (coded value for temperature) and X_2 (coded value for mass fraction of milk fat), respectively. Only coefficients correspondent to significant effects were considered.

$$Y_1 = 20.42 + 1.92 \cdot X_1 - 2.04 \cdot X_2 \qquad /2/$$

$$Y_2 = 49229.38 + 84010.17 \cdot X_2 + 35761.45 \cdot X_2^2 \qquad /3/$$

Table 2. Experimental matrix for interesterification degree (ID) and consistency (at 10 °C) with coded and actual values of independent variables temperature (X_1) and mass fraction of milk fat (X_2) according to the central composite design

Run	Coded variables		Experimental conditions		ID	Consistency/Pa		Decrease in consistency value after reaction/%
	X_1	X_2	Temperature / °C	*w*(milk fat) / %	/ %	Initial value (NIE blend)	EIE product	
1	–	+	45	80	16.78	254874.80	174068.0	32
2	+	+	65	80	20.39	254874.80	144844.0	43
3	–	–	45	50	19.49	23928.23	0	100
4	+	–	65	50	24.77	23928.23	0	100
5	0	0	55	65	21.73	121602.50	22849.5	81
6	0	0	55	65	18.00	121602.50	73844.1	39
7	0	0	55	65	19.19	121602.50	23928.2	80
8	0	+	55	80	17.24	254874.80	188091.5	26
9	+	0	65	65	23.62	121602.50	42658.9	65
10	0	–	55	50	22.38	23928.23	2942.0	88
11	–	0	45	65	21.01	121602.50	82866.2	32

Table 3. Estimated effects, standard errors (S.E.), *t*-test and p-values for interesterification degree (ID) and consistency of the products of interesterification of the blends of milk fat and canola oil catalyzed by *Rhizopus oryzae* lipase immobilized in SiO_2-PVA, according to the central composite design

Response variables	Independent variable and interaction	Estimated effects	S.E.	*t*	p
ID	X_1	3.83	1.27	3.02	0.0294*
	X_1^2	3.11	1.95	1.59	0.1723
	X_2	–4.08	1.27	–3.21	0.0237*
	X_2^2	–1.90	1.95	–0.97	0.3756
	$X_1 \cdot X_2$	–0.83	1.55	–0.54	0.6143
Consistency	X_1	–23143.8	20214.61	–1.14	0.3041
	X_1^2	4748.7	31109.63	0.15	0.8846
	X_2	168020.3	20214.61	8.31	0.0004*
	X_2^2	70256.6	31109.63	2.26	0.0735**
	$X_1 \cdot X_2$	–14612.0	24757.74	–0.59	0.5807

*p<0.05, **p<0.10; X_1=temperature, X_2=milk fat mass fraction

These models were validated by the corresponding analysis of variance (ANOVA) as displayed in Table 4. The statistical analysis of the models fitted for ID and consistency indicated that they were significant at 95 % confidence level, without significant lack of fit (p>0.10). Moreover, the R^2 value indicated that the models can explain more than 70 % of the experimental variability for ID and more than 91 % experimental variability for consistency.

Fig. 1 shows the response surfaces with the results predicted by the adjusted models for: a) ID and b) consistency values. As can be observed, the highest value for ID (about 25 %) can be obtained using the MF:CNO= 50:50 blend, while the lowest value for ID (about 15 %) can be achieved using the MF:CNO=80:20 blend. The influence of the medium composition on the ID can be explained by the specificity of *Rhizopus oryzae* lipase for C18 fatty acids (*19*), as found in a substrate having higher amount (or fraction) of canola oil. These results are comparable to those described by de Paula *et al.* (*13*) where interesterification of milk fat and soybean oil blends was catalyzed by *Rhizopus oryzae* lipase immobilized on SiO_2-PVA.

Fig. 1a also shows that increased temperatures resulted in increased ID values. This behaviour can be related to the temperature effect on the medium viscosity; higher temperatures allow better medium agitation and consequently a higher reaction rate can be achieved.

Regarding the consistency values (Fig. 1b), a quadratic decline was observed with a decrease of the milk fat mass fraction in the blend. Blends between MF:CNO=60:40 and 70:30 resulted in products with consistency values

Table 4. Analysis of variance (ANOVA) of the fitted models for interesterification degree (ID) and consistency of the products obtained in the interesterification of milk fat and canola oil blends catalyzed by *Rhizopus oryzae* lipase immobilized on SiO_2-PVA

Response variables	Factor	Sum of square	Degrees of freedom	Mean square	F	p
ID	Model	46.97	2	23.49	9.54	0.0076*
	X_1	22.04	1	22.04	8.95	0.0193*
	X_2	24.93	1	24.93	10.13	0.0130*
	Residual error	19.69	8	2.46		
	Lack of fit	12.43	6	2.07	0.57	0.7483
	Pure error	7.26	2	3.63		
	R^2	0.7046				
	Adj R^2	0.6307				
Consistency	Model	$4.58 \cdot 10^{10}$	2	$2.29 \cdot 10^{10}$	44.76	<0.0001*
	X_2	$4.24 \cdot 10^{10}$	1	$4.24 \cdot 10^{10}$	82.71	<0.0001*
	X_2^2	$3.49 \cdot 10^{9}$	1	$3.49 \cdot 10^{9}$	6.81	0.0311*
	Residual error	$4.10 \cdot 10^{9}$	8	$5.12 \cdot 10^{8}$		
	Lack of fit	$2.40 \cdot 10^{9}$	6	$3.99 \cdot 10^{8}$	0.47	0.7993
	Pure error	$1.70 \cdot 10^{9}$	2	$8.49 \cdot 10^{8}$		
	R^2	0.9180				
	Adj R^2	0.8975				

*p<0.05; X_1=temperature, X_2=mass fraction of milk fat

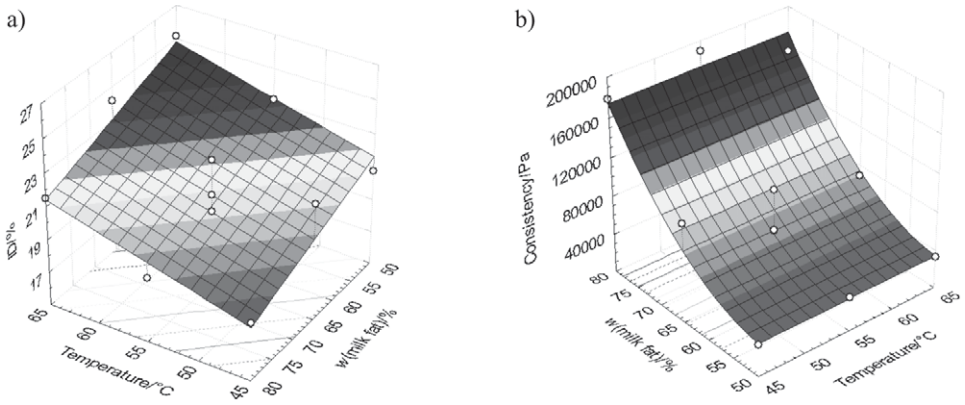

Fig. 1. Response surface fitted to the experimental data points corresponding to: a) the interesterification degree (ID) and b) consistency of the products obtained from the interesterification of milk fat and canola oil blends catalyzed by *Rhizopus oryzae* lipase immobilized in SiO_2-PVA

in the range from 19 613 to 78 453 Pa, with satisfactory plasticity and spreadability properties to be used at refrigerated temperatures, according to Haighton criteria (*18*). Thus, the MF:CNO=65:35 blend was selected. In relation to the temperature, the lowest value (45 °C) was chosen because high temperatures can adversely affect butter flavour, demand high energy cost and when used for a long time, they can inactive the enzyme. Moreover, the effect of temperature was not significant on the product consistency and it was observed that the variation of temperature in the studied range resulted in a variation of the ID value from 16 to 25 %, which was not considered enough to justify the use of temperatures higher than 45 °C. To confirm the models fitted for ID and consistency, a reaction was performed under the selected conditions (MF:CNO=65:35 blend and 45 °C), and the following values were obtained: ID=19.77 % and consistency=56 290 Pa. The experimental results are in agreement with the predicted values since the values were within the range predicted by the models at 95 % confidence level.

Conclusions

The potential of *Rhizopus oryzae* lipase immobilized on SiO_2-PVA to mediate the interesterification of milk fat and canola oil (MF:CNO) blends in a solvent-free system was demonstrated. The highest interesterification degree (ID) and reduction of the consistency value were obtained for the blend with the lowest MF fraction (50 %). The statistical analyses showed that the mass fraction of MF in the blend and temperature were significant for ID, while for consistency, only the MF mass fraction was significant. Empiric models for ID and consistency were obtained and the most suitable conditions were established: 65 % of milk fat and 35 % of canola oil, and temperature of 45 °C. This process is a promising approach to obtain a product incorporating a portion of unsaturated and essential fatty acids of canola oil with the desirable flavour of milk fat and better spreadability under refrigerated conditions.

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