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Response surface modelling of the production of ω-3 polyunsaturated fatty acids-enriched fats by a commercial immobilized lipase

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Abstract

The aim of this study was to model the production of fats, enriched with ω-3 polyunsaturated fatty acids (ω-3 PUFA) for nutraceutical purposes, via the response surface methodology. These fats were obtained by transesterification of palm oil stearin (POS) with a concentrate (EPAX 2050TG) of triglycerides enriched with ω-3 PUFA and soybean oil, catalysed by a commercial immobilized *Candida antarctica* lipase ("Novozym 435").

The initial water activity (a_w) of the biocatalyst, POS and EPAX 2050TG concentrations, time and temperature showed a significant effect on the transesterification reaction, as well as on the competing reactions of hydrolysis and lipid oxidation.

Depending on the factors included, the transesterification reaction was described either by first- or second-order models.

The production of free fatty acids, which is ascribed both to the hydrolytic reaction and the mechanism of lipase-catalysed transesterification, showed a second-order dependence on the initial a_w of the biocatalyst.

Keywords: Lipase; Omega-3 polyunsaturated fatty acid; Response surface design; Transesterification

1. Introduction

The functional properties of fats are determined by the distribution pattern of fatty acid radicals in their molecules [1]. The transesterification of natural fats, leading to a modification of that pattern, is a route to improve certain fat properties and to implement their nutritional value [2], namely for applications in the margarine, confectionery and bakery industries, as well as for pharmaceutical purposes. Also, the production of triglycerides (TG) enriched with ω-3 polyunsaturated fatty acids (ω-3 PUFA) for dietary purposes seems to have considerable industrial potential. In fact, diets rich in ω-3 PUFA, especially those containing eicosapentaenoic and docosahexaenoic acids (EPA and DHA), have been shown to have important physiological and pharmacological effects on human health [3–10].

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Nomenclature:

List of symbols

$Abs_{232\ nm}$	UV absorbance at 232 nm
$Abs_{270\ nm}$	UV absorbance at 270 nm
a_w	thermodynamic activity of water
CCRD	central composite rotatable design
DG	diglyceride(s)
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
EPAX 2050TG	concentrate of triglycerides enriched with ω-3 PUFA (c.a. 20% of EPA and 50% of DHA)
FFA	free fatty acid(s)
POS	palm oil stearin
PUFA	polyunsaturated fatty acid(s)
RSM	response surface methodology
R^2	determination coefficient (quadratic correlation coefficient)
R^2_{adj}	adjusted R^2
$SFC_{35°C}$	solid fat content measured at 35°C by nuclear magnetic resonance
TG	triglyceride(s)

Currently, transesterification is carried out at high temperature (higher than 250°C) without catalyst or with an acid, alkaline or metal catalyst (e.g. sodium methoxide), under reduced pressure at lower temperature (70–150°C) [11]. The interchange of acyl groups occurs at random and the composition reached at equilibrium obeys the laws of probability. The final products may remain contaminated by residual catalyst. The lack of specificity of chemical catalysts may lead to the formation of considerable amounts of side products (soaps as sodium salts of fatty acids, mono- and diglycerides) with a subsequent decrease in yield [11].

During the last decade, the oleochemical industry has faced the challenge of replacing chemical catalysts by lipases (acylglycerol acylhydrolases, EC 3.1.1.3.). Lipase-catalysed reactions are carried out under milder conditions (temperature lower than 70°C, atmospheric pressure) and with higher selectivity than their chemical counterparts. In vivo, these enzymes catalyse the hydrolysis of glycerides at oil/water interfaces. In organic media with low water activity, they catalyse esterification [12] and also interesterification of TG via alcoholysis, acidolysis, and transesterification.

A considerable number of papers has been published on lipase-catalysed interesterifications [13–38] and a number of procedures patented [39–41].

Most of these works are kinetic studies on model reactions of acidolysis [16,22,24,26–28] at lab-scale, and frequently in the presence of organic solvents [16–19,26]. The bioproduction of TG enriched with ω-3 PUFA has also been attempted via acidolysis [29–36]. In these systems, the recovery of the modified TG poses a separation problem. Therefore, for industrial purposes, lipase-catalysed transesterification (ester interchange) seems to be a more adequate route than acidolysis. With this aim, several studies have been carried out, either in the presence of organic solvents [18,19,21] or in solvent-free media [14,20,37,38].

Nevertheless, scale-up has been carried out only in the production of cocoa butter substitutes. To our knowledge, the enzymatic route is being used at small industrial scale by Unilever and also by two Japanese companies, Kao and Fuji Oil. Large-scale

industrial processes for enzymatic interesterification of TG have not yet been implemented.

The aim of this study was to model the production of fats enriched with ω-3 PUFA by lipase-catalysed transesterification, via the response surface methodology (RSM). These fats were obtained by transesterification of palm oil stearin (POS) with a concentrate of TG enriched with ω-3 PUFA and soybean oil, catalysed by a commercial immobilised *Candida antarctica* lipase ("Novozyme 435") in solvent-free media.

In a first stage, a full factorial design was used to investigate the effect of the following parameters: (i) initial water activity (a_w) of the biocatalyst, (ii) composition of the reaction medium, (iii) time and (iv) temperature, on the transesterification reaction, as well as on the competing reactions of hydrolysis and lipid oxidation. Temperature is a key variable in the process. On one hand, a temperature higher than 55°C has to be used to prevent medium solidification; on the other hand, the thermal oxidation of lipids should be minimised [42].

In addition, as an attempt to fit second-order models to the experimental data points, a central composite rotatable design (CCRD) was used. Only the variables having significant effect on the transesterification reaction and/or on hydrolysis and oxidation were considered.

2. Materials and methods

2.1. Materials

Refined, bleached and deodorised POS, i.e. the obtained fraction of palm oil rich in saturated fatty acids, and soybean oil were supplied by FIMA, Produtos Alimentares, Portugal. The EPAX 2050TG, a concentrate of TG containing about 80% of ω-3 PUFA (c.a. 20% of EPA and 50% of DHA), was from Pronova Biocare, Norway.

The commercial preparation of the *C. antarctica* lipase, "Novozym 435", immobilised on a macroporous acrylic resin (bead-shaped particles with 0.3–0.9 mm diameter) was kindly donated by Novo Nordisk, Denmark. It is a thermostable lipase preparation with a maximum activity in the range 70–80°C. However, working temperatures in the range of 40–60°C are recommended for high operational stability. The positional specificity of this lipase towards the fatty acids in glycerides depends on the substrates used (Novo Nordisk, Product sheet).

2.2. Methods

2.2.1. Initial water activity of the biocatalyst

The influence of the initial a_w of the "Novozym 435" on transesterification kinetics was investigated, since high a_w values promote the competing hydrolysis reaction [13,25,43,44]. The biocatalyst was either (i) dried under vacuum at 40°C for 30 min at 10 kPa (final $a_w = 0.10$) or (ii) equilibrated, for 4–5 days at 30°C, with the vapour phase of saturated salt solutions of known a_w: KCH_3COO ($a_w = 0.23$); KI ($a_w = 0.68$) and NaCl ($a_w = 0.75$) [45]. The final a_w was measured in a ROTRONIC HYGROSKOP DT humidity sensor (DMS-100H). The immobilised lipase at an initial pre-established a_w value was used in batch transesterification reactions.

2.2.2. Transesterification reaction

The immobilised biocatalyst (5%, w/w) was added to the reaction medium (50 g) composed of POS, soybean oil and EPAX 2050TG in various ratios. The reactions were carried out inside small magnetically stirred thermostated cylindrical glass reactors (100 ml) at the desired temperature (higher than 55°C to prevent medium solidification). The initial a_w of the biocatalyst, reaction medium formulation, as well as temperature and reaction time, varied according to the experimental design followed (cf. Section 2.2.3). Experiments were run randomly.

At different reaction times, 5-ml samples were taken, paper-filtered in an oven at approximately 80°C to remove biocatalyst particles, and assayed for $SFC_{35°C}$, FFA and oxidation products (cf. Section 2.2.4).

2.2.3. Experimental design

The best reaction conditions for batch transesterification reaction were established via the RSM. It consists of a set of mathematical and statistical methods developed for modelling phenomena and finding combinations of a number of experimental factors (variables) that will lead to optimum responses [46–49]. With RSM, several variables are tested simultaneously with a minimum number of trials, according to special experimental designs based on factorial

designs [46–49]. This methodology has the advantage of being less expensive and time-consuming than the classical methods.

In a first stage, a full factorial design 2^5 (two levels and five factors) was followed to investigate the effect of the initial a_w of the biocatalyst (a_w; 0.1 vs. 0.7), the concentrations of POS (50% vs. 80%) and of concentrate (EPAX 2050TG; 0 vs. 18%), time (t; 60 vs. 120 min) and temperature (T; 60°C vs. 80°C) on the transesterification reaction, as well as on the competing reactions of hydrolysis and lipid oxidation [42]. When factorial designs are used, the modelling of reaction conditions is achieved by first-order polynomials and, therefore, described by flat surfaces.

In a second stage, 29 experiments were carried out according to a CCRD, as a function of the variables previously considered in the 2^5 factorial design. This design was composed by a $2^{(5-1)}$ fractional factorial design (16 experiments coded as ± 1) with 10 star or axial extra-points (levels coded as ± 2) and three centre points (coded levels as 0). The levels of the factorial design represent the vertices of a 5-dimensional cube centred at the origin of the coded system of reference [48]. The repetition of the centre point provides an estimation of the variance of the experimental error, which is assumed to be constant along the experimental domain [47–49].

In CCRD, the use of five levels for each factor enables the fitting of second-order polynomials to the experimental data points and, therefore, to fit curved surfaces to the experimental data. In addition, partial differentiation is used to find the optimum of a multivariate function. These solutions are called stationary points. Usually, for most practical applications, the identification of the regions of independent variables corresponding to optimal responses may be directly obtained by visual examination of the response surfaces [47].

The following levels were considered in the factorial design part of CCRD: a_w (0.28 vs. 0.62); concentrations of POS (57.5% vs. 72.5%) and EPAX 2050TG (4.5% vs. 13.5%); temperature (65°C vs. 75°C) and time (75 vs. 105 min). The original a_w of "Novozym 435" is 0.45 and was used as the centrepoint. The star levels correspond to the low and high levels considered in the full factorial design previously tested in the first stage of this work.

The maximum concentration of ω-3 PUFA used (18%) was based on the following assumptions: (i) the transesterified fat is to be blended in a ratio of 1:4 with other fats, (ii) the maximum allowed daily ingestion of ω-3 PUFA is 2.8 g [50] and (iii) the maximum daily intake of blended fat is 40 g.

2.2.4. Analytical methods

2.2.4.1. Solid fat content assay. The efficiency of the transesterification reaction may be indirectly evaluated by the decrease in the extent of fat crystallisation at a given temperature [51]. This was assayed by nuclear magnetic resonance (NMR) in a pulsed NMR spectrophotometer (Minispec P-20i, IBM) at 35°C, solid fat content ($SFC_{35°C}$), which is related to the rheological behaviour of fats at consumption temperature. The $SFC_{35°C}$ of the transesterified fats should be lower than that of their original counterparts, to prevent a sandy and coarse texture. Each sample was melted at 60°C for 5 min, followed by 60 min at 0°C and, at last, 30 min at 35°C.

2.2.4.2. Free fatty acids (FFA). Due to the reaction mechanism of lipase-catalysed interesterification [13,19,43,52], there will always be FFA present in the reaction media. Plus, hydrolysis of glycerides has been shown to occur even in low a_w [44,53,54] environments. The FFA were assayed by titration with a 0.1N sodium hydroxide aqueous solution. Their percentage (w/w) was calculated on the basis of the molecular weight of oleic acid [55,56].

2.2.4.3. Oxidation products. The time course of thermal oxidation of the fat was indirectly evaluated by the UV absorbance at 232 nm, $Abs_{232\ nm}$ (related with the presence of initial products of oxidation, i.e. conjugated hydroperoxides) and at 270 nm, $Abs_{270\ nm}$ (final oxidation products, i.e. FFA, aldehydes and ketones) of the solutions of 1% (w/v) of fat in *iso*-octane [55,56].

2.2.5. Statistical analysis

The results of the full factorial experiments and CCRD were analysed using the software "Statistica™", version 5, from Statsoft, USA. The linear and quadratic effects of each of the five factors under study, as well as their linear interac-

tions, on transesterification, hydrolysis and oxidation kinetics were calculated. Their significance was evaluated by analysis of variance. A surface, described by a first- or a second-order polynomial equation was fitted to each set of experimental data points ($SFC_{35°C}$, FFA, $Abs_{232\ nm}$, and $Abs_{270\ nm}$). First- and second-order coefficients were generated by regression analysis. The results of the full factorial experiments were used to establish first-order models. Second-order polynomials were fit to the experimental data of the CCRD. The goodness of fit of the models was evaluated by the determination (R^2) and adjusted R^2 (R^2_{adj}) coefficients [57] complemented by the graphic plot of predicted values by the model vs. observed experimental values [58]. High values of both R^2 and R^2_{adj} suggest a good fit of the model to the experimental data points.

2.2.6. Validation of the transesterification model

After the selection of the most adequate model, six experiments were carried out at different values for each factor, inside the technological space considered in the model. The obtained experimental results were compared to the theoretical values predicted by the model.

3. Results and discussion

3.1. Full factorial design

In a first stage, the transesterification experiments were carried out according to a full factorial design 2^5. The obtained results ($SFC_{35°C}$, FFA, $Abs_{232\ nm}$ and $Abs_{270\ nm}$) were used to calculate the significant linear effects of each factor and their interactions on the transesterification reaction and on the hydrolysis and lipid oxidation, respectively (Table 1).

All the variables considered in this study showed a significant effect on $SFC_{35°C}$ and, thus, on the transesterification reaction. However, no significant effects of interactions between factors were found. As to the formulation of fat blends, an increase in the amount of POS and a decrease in EPAX 2050TG content in the starting material led to an increase in the final value of $SFC_{35°C}$ of the transesterified product. Since the extent of transesterification is related to a decrease in $SFC_{35°C}$, factors with significant negative effect on $SFC_{35°C}$ promote the transesterification. In fact, a temperature increase from 60°C to 80°C improves the activity of the biocatalyst.

Table 1
Full factorial design-effects and respective significance levels (α) of the tested variables (factors) and interactions on transesterification, hydrolysis and oxidation reactions, followed by $SFC_{35°C}$, FFA and oxidation products (Abs at 232 and 270 nm), respectively

Factor	$SFC_{35°C}$	FFA	$Abs_{232\ nm}$	$Abs_{270\ nm}$
a_w	-0.611^{**}	1.249^{**}	(a)	(a)
[EPAX 2050TG]	-1.169^{***}	(a)	0.130^{***}	0.274^{***}
[POS]	10.810^{***}	(a)	-0.036^{***}	-0.047^{*}
Temperature (°C)	-2.840^{***}	(a)	(a)	(a)
Time (min)	-1.592^{***}	(a)	-0.023^{**}	-0.084^{***}
(a_w) × [EPAX 2050TG]	(a)	(a)	(a)	(a)
(a_w) × [POS]	(a)	(a)	(a)	(a)
(a_w) × (temperature)	(a)	(a)	(a)	(a)
(a_w) × (time)	(a)	(a)	(a)	(a)
[EPAX 2050TG] × [POS]	(a)	(a)	(a)	(a)
[EPAX 2050TG] × (temperature)	(a)	(a)	(a)	(a)
[EPAX 2050TG] × (time)	(a)	(a)	(a)	(a)
(POS) × (temperature)	(a)	(a)	(a)	(a)
(POS) × (time)	(a)	(a)	(a)	0.040^{*}
(Temperature) × (time)	0.550^{*}	(a)	(a)	0.071^{**}

(a) = Not significant effects. [EPAX 2050TG] and [POS] are EPAX 2050TG and POS concentrations (%, w/w), respectively.
*At $\alpha < 0.05$.
**At $\alpha < 0.01$.
***At $\alpha < 0.001$.

In addition, longer reaction times are beneficial. An increase in the initial water activity of "Novozym 435", from 0.1 to 0.7, promotes the decrease in the $SFC_{35°C}$ of the transesterified fat. In a previous work on the transesterification of POS with palm kernel oil catalysed by the immobilised lipase from the *Rhizomucor miehei* ("Lipozyme IM"), a similar relationship between the a_w of the biocatalyst and $SFC_{35°C}$ was observed [25]. The decrease in the melting point of the transesterified fat might be ascribed to (i) a change in the TG crystallisation pattern [1] or to (ii) the presence of partial glycerides and FFA. The presence of FFA and partial glycerides may result from the hydrolysis reaction, which is promoted in high water activity environments. Accordingly, only a significant and positive effect of the a_w of the biocatalyst was observed on FFA production. The use of "Novozym 435" at low a_w seems to be a way to prevent the competing hydrolysis reaction. However, even when "Novozym 435" was used at an a_w value of 0.1, the amount of FFA was still not negligible (1.9–3.9%, w/w). In "Lipozyme IM"-catalysed transesterification of POS with palm kernel oil in solvent-free media, the production of 5–7% and 2.7% FFA was also reported, respectively, when the biocatalyst was at an a_w of 0.1 [25] or when molecular sieves were added to the reaction medium [38]. Higher levels of FFA (8%) were observed during the transesterification of palm olein in water-saturated hexane catalysed by *C. rugosa* lipase immobilised in celite and lyophilised for 4 h [18].

The FFA may be formed during the first step of lipase-catalysed interesterification in which fatty acids are released from glycerides to the reaction medium [13,19,31,43,52,53].

The sensitivity of a fat to oxidation increases with its content in PUFA [42]. In fact, an increase in the EPAX 2050TG concentration and a decrease in POS showed to be positively related to the oxidation (both to the initial and final oxidation products). A positive interaction (temperature) × (time) is also significant in the final stages of the oxidation process (related to the $Abs_{270\ nm}$). A significant negative effect of time on the production of conjugated hydroperoxides (related to the $Abs_{232\ nm}$) was obtained. This is in agreement with Sonntag [42] who reported that the rate of degradation of these molecules in the latter stages of oxidation is higher than their rate of formation.

In addition, a multiple regression analysis was carried out to fit first-order polynomial equations to the experimental data points (Table 2). Only significant effects were maintained in the final model equations. With respect to FFA production, the experimental results didn't entirely fit the first-order model. The high values of R^2 and R^2_{adj} [57], observed for first-order models, describing the evolution of $SFC_{35°C}$, $Abs_{232\ nm}$ and $Abs_{270\ nm}$, suggest the applicability of these models. In fact, a linear relationship between the predicted and the experimental values was observed for $SFC_{35°C}$. However, a certain scattering was obtained for both $Abs_{232\ nm}$ and $Abs_{270\ nm}$.

3.2. Central composite rotatable design

Since the five experimental factors tested in the factorial design had a significant effect on $SFC_{35°C}$ and thus on the transesterification reaction, all of

Table 2
First-order model equations for the response surfaces fitted to the experimental data points, as a function of the initial a_w of "Novozym 435", composition of the reaction medium (concentration of POS and EPAX 2050TG, %, w/w), temperature (T, °C) and reaction time (t, min), and respective R^2 and R^2_{adj}

Response	Model equations	R^2	R^2_{adj}
$SFC_{35°C}$	$SFC_{35°C} = 6.22 - 1.019a_w - 0.065[EPAX\ 2050TG] + 0.360[POS] - 0.225T - 0.091t + 0.001(T \times t)$	0.993	0.991
$Abs_{232\ nm}$	$Abs_{232\ nm} = 0.259 + 0.007[EPAX\ 2050TG] - 0.001[POS] - 0.0004(t)$	0.935	0.928
$Abs_{270\ nm}$	$Abs_{270\ nm} = 1.143 + 0.015[EPAX\ 2050TG] - 0.006[POS] - 0.005(t) + 0.0001[POS] \times (t) + 0.00001(T \times t)$	0.929	0.916

Table 3
Central composite rotable design-effects and respective significance levels (α) of the tested variables (factors) and interactions on transesterification, hydrolysis and oxidation reactions, followed by $SFC_{35°C}$, FFA and oxidation products ($Abs_{232\ nm}$ and $Abs_{270\ nm}$), respectively

Factor	$SFC_{35°C}$	FFA	$Abs_{232\ nm}$	$Abs_{270\ nm}$
a_w (linear term)	(a)	1.055^{***}	(a)	(a)
a_w (quadratic term)	(a)	0.895^{***}	(a)	-0.076^{*}
[EPAX 2050TG] (linear term)	-0.697^{*}	(a)	(a)	(a)
[EPAX 2050TG] (quadratic term)	(a)	(a)	(a)	-0.077^{*}
[POS] (linear term)	5.542^{***}	(a)	(a)	(a)
[POS] (quadratic term)	0.805^{**}	(a)	(a)	-0.087^{*}
Temperature (°C; linear term)	-1.855^{***}	(a)	(a)	(a)
Temperature (quadratic term)	(a)	(a)	(a)	-0.075^{*}
Time (min, linear term)	-0.948^{***}	(a)	(a)	(a)
Time (quadratic term)	(a)	(a)	(a)	(a)
(Temperature) × (time)	(a)	(a)	(a)	0.086^{*}
Other interactions	(a)	(a)	(a)	(a)

(a) = Not significant effects. [EPAX 2050TG] and [POS] are EPAX 2050TG and POS concentrations (%, w/w), respectively.
*At $\alpha < 0.05$.
**At $\alpha < 0.01$.
***At $\alpha < 0.001$.

them were considered in the CCRD. Here, five levels were tested for each factor, while only two levels were used to establish the first-order models.

The linear and quadratic significant effects of each factor and their interactions on the transesterification reaction and on the hydrolysis and lipid oxidation are shown in Table 3. Some differences were observed between these results and those obtained from the first factorial design. In the CCRD, a narrower variation range was considered in the factorial part of the design, for each factor tested. In addition, for each factor, eight experiments were carried out at the level (-1), eight at the level $(+1)$ and three in the centre of the cube defined by these factorial points; the levels ± 2 were tested only once. The weight of the fractional factorial design and of the repetition of the centre point is higher than that of the axial points on the second-order model.

In fact, when the initial water activity of "Novozym 435" was between 0.28 and 0.62 (factorial levels in CCRD), no significant effect was observed on $SFC_{35°C}$, and thus on the transesterification reaction. This is a very important result since, in future work, "Novozym 435", with an original a_w of about 0.45, can be used as such. A lower dependence of "Novozym 435" on the a_w of the reaction medium, as compared to "Lipozyme IM", during the glycerolysis of a vegetable oil in organic medium was suggested [59]. Similarly, this behaviour may be explained by the relatively hydrophobic character of the immobilisation matrix in "Novozym 435" [59], which promotes the access of TG to the microenvironment while keeping away the water molecules. The POS concentration showed a positive (linear and quadratic) effect on the $SFC_{35°C}$ of the transesterified fat; only linear effects were observed for the remain-

Table 4
Second-order model equations for the response surfaces fitted to the experimental data points, as a function of the initial a_w of "Novozym 435", composition of the reaction medium (concentration of POS and EPAX 2050TG), temperature (T, °C) and reaction time (t, min), and respective R^2 and R^2_{adj}

Response	Model equations	R^2	R^2_{adj}
$SFC_{35°C}$	$SFC_{35°C} = 33.79 - 0.077\,[EPAX\ 2050TG] - 0.608[POS] + 0.008[POS]^2 - 0.186\,(T) - 0.032(t)$	0.962	0.954
FFA (%, w/w)	$FFA = 4.565 - 10.827a_w + 15.478a_w^2$	0.566	0.532

ing factors. The time course of the fat $SFC_{35°C}$ during the transesterification can be well described by a second-order model as a function of POS and EPAX 2050TG concentrations, time and temperature (Table 4). The high values of R^2 and R^2_{adj} indicate a good fit of this model to the experimental data points, which is confirmed by the linear relationship obtained between the observed and predicted $SFC_{35°C}$ values. According to this model, transesterification is promoted at high temperatures and prolonged reaction times. A high percentage of ω-3 PUFA concentrate leads to a final interesterified product with lower SFC at 35°C. The $SFC_{35°C}$ is described by a 5-dimensional surface.

For $SFC_{35°C}$, similar R^2 and R^2_{adj} values were obtained both with first- and second-order models. However, the second-order model is preferred since only four factors are included instead of five, and a uniform distribution of the experimental data points along the line "observed values vs. predicted values" was obtained (data not shown).

The production of FFA is strongly dependent on the initial a_w of the biocatalyst and independent from the other factors (Table 3) as already observed with the results from the full factorial design. Also low values for both R^2 and R^2_{adj} (Table 4) were obtained for the second-order model. This indicates that the model does not fit the experimental data.

The first stage of lipid oxidation was independent of the variation in the five factors tested, within the observed ranges (Table 3). On the contrary, the final oxidation products depended on the initial a_w of the biocatalyst, the medium composition and temperature (quadratic terms), as well as on the interaction (temperature) × (time). However, no fitting was obtained with the second-order model ($R^2 = 0.41$; $R^2_{adj} = 0.26$). The effect of the initial a_w of the biocatalyst on the $Abs_{270\ nm}$ may be explained by the presence of FFA that absorb at this wavelength [55,56].

3.3. Validation of the second-order model for $SFC_{35°C}$

To investigate the applicability of the second-order model describing $SFC_{35°C}$ (Table 4), six additional transesterification experiments were carried out (Table 5). Since the main purpose is to obtain ω-3 PUFA enriched transesterified fat with low values of $SFC_{35°C}$, POS at low concentration (coded level −2) and the maximum amount of EPAX 2050TG (18%, w/w) were tested. In addition, temperature was taken at the lowest value tested (60°C), so as to enhance the operational stability of the enzyme. The biocatalyst was tested at a_w values of 0.45 and 0.1.

The obtained values were compared with the theoretical $SFC_{35°C}$ values predicted by the model and a linear relationship was obtained ($R^2 = 0.9909$). The experimental results of $SFC_{35°C}$ were slightly lower than those predicted by the model and thus advantageous. These experiments were run under experimental conditions in the border zone of the technological space considered in the CCRD. This may explain the slight deviation observed.

As predicted by the model, the use of "Novozym 435" at the original water activity value (0.45) or at 0.11 (cf. Section 2.2.1), has an almost negligible effect on the final $SFC_{35°C}$ of the transesterified fat.

Table 5
Reaction conditions of experiments carried out to investigate the applicability of the second-order model for $SFC_{35°C}$, when 18% (w/w) of EPAX 2050TG was used, and respective experimental results and predicted values, after 120 min reaction time

Experiment	a_w		POS (%, w/w)		Temperature (°C)		Experimental value	Predicted value
	Cod.	Decod.	Cod.	Decod.	Cod.	Decod.		
1	0	0.45	0	65	−2	60	10.29	11.68
2	0	0.45	−2	50	−2	60	5.08	7.00
3	−2	0.10	−2	50	−2	60	5.09	7.00
4	0	0.45	0	65	0	70	8.47	9.82
5	0	0.45	−2	50	0	70	3.01	5.14
6	−2	0.10	−2	50	0	70	3.72	5.14

cod. = Coded values; decod. = decoded values.

4. Conclusions

The modelling of the transesterification of POS with soybean oil and a concentrate of triglycerides enriched with ω-3 PUFA, catalysed by "Novozym 435", was attempted by RSM. The reaction was well described either by first- or second-order models. The second-order model was considered more adequate for the reasons previously discussed.

A second-order dependence of FFA production on the initial a_w of the biocatalyst was observed. However, no good correlation was verified between the experimental results and the model. In fact, whatever the experimental conditions were, values between 2% and 6% (w/w) of FFA were obtained. The production of FFA, which is related to the mechanism of lipase-catalysed transesterifications, is worthy of attention before the scaling up of the system is attempted. Aspects related to transesterification kinetics might have to be considered in further modelling.

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