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Lipase-catalysed acylglycerol synthesis of glycerol and $n-3$ PUFA from tuna oil: Optimisation of process parameters

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Abstract

Enzymatic synthesis of acylglycerols from glycerol and $n-3$ polyunsaturated fatty acids concentrates, prepared from tuna oil, was optimised by response surface methodology. Acylglycerol synthesis was performed using Lipozyme Novo 435 under different reaction conditions according to the experiment design. Five factors were chosen to optimise the process parameters of lipase-catalysed synthesis. The results indicated that the esterification degree was above 90% under the following conditions: glycerol – 2.5 g, hexane – 5 ml, the initial water content – 0.60%, temperature – 40 °C, and molecular sieves – 1 g. The product was a bright orange and transparent liquid, with no fishlike smell and high mobility. The contents of DHA and EPA were 73.4% and 13.5%, respectively. The reaction product was composed of monoacylglycerols, diacylglycerols and triacylglycerols, the contents of which were 12.1%, 56.1% and 31.3%, respectively.

Keywords: Tuna oil; Polyunsaturated fatty acids; Lipase-catalysed synthesis; Process optimisation

1. Introduction

It is well established that the polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are beneficial to health. EPA is the precursor of prostaglandins, thromboxanes and leukotrienes, which are effective anti-aggregatory substances (Simopoulos, 1996).

DHA is a component of membrane phospholipids of brain and retina cells, and consequently is essential for human health (Simopoulos, 1996). Some studies indicate that PUFA concentrates, devoid of more saturated fatty acids, are much better than marine oils themselves, since they allow the daily intake of total lipid to be kept as low as possible (Haagsma, Gent, Luten, Jong, & Doorn, 1982). For medical or dietetic purposes, PUFA may be administered in different forms: as free fatty acids (FFA), as ethyl esters, or as acylglycerols. Some studies indicate that PUFA are most promptly absorbed from the intestines when free fatty acid is given orally, moderately absorbed as acylglycerols and poorly absorbed as $n-3$ PUFA ethyl ester. However, free $n-3$ PUFA is oxidised most easily and, moreover, free $n-3$ PUFA is unacceptable as a food. Therefore, acylglycerols are considered to be the most desirable chemical form as a food (Tsuneo, Tomomasa, Youko, Line, & Tamotsu, 1992). Lipases are known to catalyse mild esterification reactions with the formation of specific compounds. Lipase-catalysed esterification has been shown as a good tool for obtaining PUFA concentrates as acylglycerols. There appears to be great potential for using lipases for the synthesis of acylglycerols for food, health and pharmaceutical use (Cerdan, Medina, Gimenez, Ibanez, & Grima, 1998; He & Shahidi, 1997; Li & Ward, 1993; Linder, Kochanowski, Fanni, & Parmentier, 2005; Medina, Cerdan, Gimenez, Ibanez, & Grima, 1999; Yoshitsugu & Naoki, 1994).

Enzymatic reactions are influenced by experimental conditions. Many factors affect the esterification degree. The results of one-factor-at-a-time experiments do not reflect actual changes in the environment as they ignore interactions between factors that are present simultaneously. Therefore, these factors may be collectively studied to

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validate the optimal reaction conditions. In this study, five-factor response surface methodology (RSM) is employed to optimise the reactive conditions of lipase-catalysed synthesis, in order to obtain a maximum degree of esterification and acylglycerols rich in $n-3$ PUFA. This method is the preferred experimental design for fitting polynomial models to analyse the response surfaces of multi-factor combinations. The method of process optimisation by the response surface methodology is a faster and more economical method for gathering research results than classical one-variable-at-a-time or full-factorial experimentation. This method has been successfully used in many optimisation studies (Namal & Shahidi, 2002; Shieh, Akoh, & Koehler, 1995; Wannasundara & Shahidi, 1996).

2. Materials and methods

2.1. Chemicals and materials

Lipozyme Novo 435 (Novo Nordisk, Bagsvaers, Denmark) from *Aspergillus oryzae* was used for acyglycerol synthesis. All solvents were of analytical grade. Crude tuna (*Thunnus albacares*) oil was obtained from tuna head by protease hydrolysis. Refining (R), bleaching (B), and deodorising (D) of the tuna oil was carried out according to recommended procedures for fish oil (Bimbo, 1998). RBD oil was stored under nitrogen at −20 °C until used. The $n-3$ PUFA concentrates were prepared by urea complexation (Liu, Zhang, Hong, & Ji, 2006).

2.2. Esterification reaction

Reaction mixtures for acylglycerols synthesis were carried out according to the experimental design. Conical flasks (50 ml) were blanketed with nitrogen and kept sealed throughout the reaction for 24 h. The vessels were stirred in an orbital shaker at 150 rpm at different temperatures. The amounts of $n-3$ PUFA concentrates were 0.4 g. The amount of Lipozyme Novo 435 was 25 mg. Molecular sieve was added after the reaction (1 h), to remove the water formed during the reaction. The reaction was stopped by addition of 20 ml acetone:ethanol mixture (1:1, v/v), and free fatty acid was titrated with 0.025 mol/L NaOH. The esterification degree (%) represents the percent of initial fatty acids consumed in the reaction mixture. The five major factors, affecting the esterification degree, such as temperature, the amount of glycerol, the initial water content, the amount of molecular sieve and the amount of hexane, were examined.

2.3. Separation of reaction product

The lipase and molecular sieve were removed by filtration of the media through a bed of anhydrous sodium sulfate. The samples were placed in 250 ml conical flasks and 20 ml acetone:ethanol (1:1, v/v) was added. The reaction mixture was titrated against a 0.025 mol/L NaOH solution (using a phenolphthalein indicator), in order to neutralise free fatty acids. The mixture was transferred into a separating funnel and thoroughly mixed with 25 ml hexane. The lower aqueous layer was separated and discarded. The upper hexane layer, containing acylglycerols, was passed through a bed of anhydrous sodium sulfate. The acylglycerols fraction was subsequently recovered, following hexane removal at 45 °C, using a rotary evaporator. The fatty acid compositions of the acylglycerols were determined by gas chromatography.

The different acylglycerols in the reaction product were separated by column chromatography, using a silica gel column prepared with petroleum ether (Paquot & Hantfenne, 1987). One gram of the reaction mixture was accurately weighed into a 100 ml beaker, and 15 ml of chloroform was added to effect solution. The sample solution was added to the prepared column and eluted at 2 ml/min. Benzene (200 ml) was then added as eluent: this fraction contained the triacylglycerol. Subsequently, 200 ml of a (1:9) (v/v) mixture of diethyl ether and benzene was added. As eluant this fraction contained the diacylglycerols and free fatty acids fraction. Diethyl ether (200 ml) was then used to elute the monacylglycerol fraction. Free fatty acids eluted with the diacylglycerol fraction. To determine the free fatty acid content of the separated diacylglycerol, a weighed amount of fraction was dissolved in 25 ml of warm neutral ethanol and titrated with sodium hydroxide solution in the presence of phenolphthalein. The percentages (m/m) of tri-, di- and monoacylglycerols are given by the formulae:

$$\text{Percentage (m/m) of triacylglycerols} = \frac{m_1}{m} \times 100,$$

$$\text{Percentage (m/m) of diacylglycerols} = \frac{m_2}{m} \times (100 - A),$$

$$\text{Percentage (m/m) of monoacylglycerols} = \frac{m_3}{m} \times 100,$$

where m_1 is the mass (g), of the fraction eluted with benzene, m_2 is the mass (g), of the fraction eluted with diethyl ether–benzene mixture, m_3 is the mass (g), of the fraction eluted with diethyl ether, m is the mass (g), of the test portion, and A is the percentage (m/m) of free fatty acids (as oleic acid) in the diacylglycerol fraction. A is calculated using the equation:

$$A = \frac{V \times T \times 282}{10 \times m_4}$$

where V is the number of ml of sodium hydroxide solution used, T is the exact normality of sodium hydroxide solution used, and m_4 is the mass (g) of the diacylglycerol fraction taken for analysis.

2.4. Optimisation procedure for acylglycerol synthesis

A five-factor response surface methodology (RSM) was employed to study the esterification degree (Y-variable) by lipase-catalysed synthesis of glycerol and $n-3$ concentrates from tuna oil. Temperature (X_1), the amount of glycerol (X_2), the initial water content (X_3), the amount of molecular sieve (X_4) and the amount of hexane (X_5) were independent

Table 1
Variables (factors) used for response surface methodology

Variable levels	+1	0	−1
X_1 (temperature, °C)	50	40	30
X_2 (glycerol, g)	3	2	1
X_3 (water, %)	1.5	1	0.5
X_4 (molecular sieve, g)	1.5	1	0.5
X_5 (hexane, mL)	4	6	8

variables studied to optimise the *Y*-variable (Table 1). Duplicate reactions were carried out at all designed points except at the central point (0, 0, 0, 0, 0), where three replications were performed, to allow the estimation of the "pure error". All experiments were carried out in a randomised order to minimise the effect of unexplained variability in the observed responses, due to extraneous factors.

A quadratic polynomial regression model was assumed for predicting the *Y*-variable. The model proposed for each response of Y was

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \sum \beta_{ij} X_i X_j$$

where β_0, β_i, β_{ii}, β_{ij} are intercept, linear, quadratic and interaction regression coefficient terms, respectively, and X_i and X_j are independent variables (Mason, Gunst, & Hess, 1989). The statistical analytical system (SAS) was used for multiple regression analysis, analysis of variance (ANOVA) and canonical analysis of data in the response surface regression procedure.

2.5. Chemical and physical analysis

Physical and chemical indices, such as relative density d_{15}^{15}, refractive index, apparent, colour, water and volatile matter, acid value, peroxide value, saponification value, unsaponifiable matter, iodine value, insoluble impurities, were determined using IUPAC (Paquot & Hantfenne, 1987) methods.

2.6. Fatty acid analysis

Fatty acid profiles were determined by preparation of methyl esters as described by IUPAC (Paquot & Hantfenne, 1987). The fatty acid methyl esters were identified by gas chromatography, using a Shimadzu GC-14B (Tokyo, Japan), equipped with a flame ionisation detector and integrator. A capillary column (30 m × 0.25 mm × 0.25 mm, FFAP) was purchased from the Dalian Institute of Chemical Physics, with a stationary phase of polyethylene glycol. The temperature for injector and detector were 250 °C. The oven temperature was hold at 190 °C for 15 min, then increased to 230 °C at 5 °C/min and held at 230 °C for 15 min. Nitrogen was used as carrier gas at a pressure of 500 kPa. The fatty acid methyl esters were identified by comparison with standards and were quantified as the area percentage of each fatty acid methyl ester. EPA methyl ester and DHA methyl ester standards were purchased from Sigma Chemical Co., St. Louis, USA.

3. Results and discussion

3.1. Analysis of model

Experimental results are given in Table 2.

Analysis of variance of the factors studied for the degree of esterification degree are in Table 3. Temperature, the amount of glycerol, the initial water content and the amount of molecule sieves affected the degree of esterification (Y) highly significantly. The amount of hexane affected the degree of esterification significantly.

Multiple regression coefficients, obtained by employing a least squares technique to predict a quadratic polynomial model for the esterification degree, are summarised in Table 4. For the degree of esterification, examination of these coefficients with the *t*-test indicated that linear terms of temperature and the amount of glycerol were highly significant ($P < 0.01$), linear term of the amount of hexane was significant ($0.01 < P < 0.05$), quadratic terms of four factors (temperature, the amount of glycerol, the amount of molecular sieve and the amount of hexane) were highly significant ($P < 0.01$); quadratic term of the initial water content was significant ($0.01 < P < 0.05$), there was significant ($0.01 < P < 0.05$) interaction between the initial water content and the amount of molecular sieve. Therefore, these results suggest that linear, quadratic and/or interaction effects may be the primary determining factors affecting the degree of esterification.

The coefficients of independent variables determined for the quadratic polynomial model for the esterification degree are given below:

$$\begin{aligned} Y = {} & -186.09 + 7.73X_1 + 62.04X_2 - 16.71X_3 + 31.43X_4 \\ & + 16.88X_5 - 0.10X_1^2 - 0.07X_1X_2 - 13.27X_2^2 \\ & + 0.08X_1X_3 + 1.94X_2X_3 - 18.16X_3^2 + 0.37X_1X_4 \\ & + 2.84X_2X_4 + 21.11X_3X_4 - 30.27X_4^2 - 0.04X_1X_5 \\ & - 0.02X_2X_5 + 1.88X_3X_5 - 0.88X_4X_5 - 1.52X_5^2 \end{aligned}$$

The analysis of variance and error for the degree of esterification model are given in Tables 5 and 6. These results show that the model predicted was adequate as indicated by error analysis that showed non-significant lack-of-fit. The regression model for the esterification degree was highly significant, with a satisfactory coefficient of determination ($R^2 = 0.96$). The model indicated that linear, quadratic and/or interaction effects in the model are all determining factors for the degree of esterification. The contributions of linear, quadratic and interaction terms to the model were 0.40, 0.50 and 0.06, respectively.

3.2. Analysis of the stationary point

The stationary point of the model was obtained mathematically. If the stationary point of a function exists, it should make partial derivative zero to each variable,

Table 2
Scheme and results of five factors response surface experiment

No.	Independent variable					Experimental value, Y	Predicted value, Y
	X_1	X_2	X_3	X_4	X_5		
1	30	1	0.5	0.5	8	47.51	44.37
2	50	1	0.5	0.5	4	50.62	48.30
3	30	3	0.5	0.5	4	68.43	70.98
4	50	3	0.5	0.5	8	49.05	51.62
5	30	1	1.5	0.5	4	25.56	22.08
6	50	1	1.5	0.5	8	17.99	14.85
7	30	3	1.5	0.5	8	41.72	43.12
8	50	3	1.5	0.5	4	35.78	38.33
9	30	1	0.5	1.5	4	48.22	44.57
10	50	1	0.5	1.5	8	35.41	32.10
11	30	3	0.5	1.5	8	55.11	56.34
12	50	3	0.5	1.5	4	66.07	68.45
13	30	1	1.5	1.5	8	35.05	30.57
14	50	1	1.5	1.5	4	43.07	39.42
15	30	3	1.5	1.5	4	61.52	62.73
16	50	3	1.5	1.5	8	55.05	56.28
17	20	2	1	1	6	48.90	50.13
18	60	2	1	1	6	44.88	43.78
19	40	0	1	1	6	0.00	12.08
20	40	4	1	1	6	66.93	54.98
21	40	2	0	1	6	83.21	82.11
22	40	2	2	1	6	53.54	54.77
23	40	2	1	0	6	50.66	49.23
24	40	2	1	2	6	61.87	63.44
25	40	2	1	1	2	70.35	70.57
26	40	2	1	1	10	54.26	54.17
27	40	2	1	1	6	85.17	86.61
28	40	2	1	1	6	88.31	86.61
29	40	2	1	1	6	88.87	86.61

Table 3
Analysis of variance of the factors studied for the degree of esterification

Independent variables	Degrees of freedom	Sums of squares	Mean squares	F-value	P-value[a]
X_1	6	2550	425	7.96	0.005
X_2	6	7260	1210	22.7	0.000
X_3	6	2150	358	6.71	0.009
X_4	6	2250	375	7.04	0.007
X_5	6	1350	226	4.23	0.032

[a] $P < 0.01$, highly significant; $0.01 < P < 0.05$, significant; $P > 0.05$, not significant.

respectively, so simultaneous equations can be set up. Utilizing the method, the coordinates of the stationary point of the mathematics model are 39.10, 2.39, 0.60, 1.00, 5.11; the predicted value is 92.90% of the stationary point.

Canonical analysis was performed on the predicted quadratic polynomial model to examine the overall shape of the response surface curves and used to characterise the nature of the stationary point. Canonical analysis is a mathematical approach used to locate the stationary point of the response surface and to determine whether it represents a maximum, minimum or saddle point. Thus, to determine the nature of the stationary point, canonical analysis was carried out on the second order polynomial model. The canonical form of the equation demonstrating the nature of the response surface was:

$$Y = 92.90 - 11.23\omega_1^2 - 23.29\omega_2^2 - 36.16\omega_3^2 - 41.03\omega_4^2 - 53.69\omega_5^2$$

where ω_1, ω_2, ω_3, ω_4, ω_5 are the axes of the response surface. It is evident that all the eigen-values were negative, indicating that the stationary point was, in fact, a maximum for the esterification degree.

Lastly, temperature at 40 °C, glycerol at 2.5 g, the initial water content at 0.6%, molecular sieve at 1 g and hexane at 5 ml may be suitable considering the factors involved. The adequacy of the predictive models was examined by performing independent experiments at the optimal conditions. Verification results revealed that the predicted values from these models were reasonably close to observed values. Under the optimal conditions, the esterification degree was above 90%.

Table 4
Regression coefficients of predicted second-order polynomial model for response variables

Variables	Coefficients (β)	Standard error	P-value[a]
Intercept	−186	53.08	0.008
Linear			
X_1	7.73	1.45	0.001
X_2	62.0	12.1	0.001
X_3	−16.7	24.3	0.510
X_4	31.4	24.3	0.231
X_5	16.9	6.59	0.034
Quadratic			
X_{11}	−0.10	0.01	0.000
X_{22}	−13.3	1.47	0.000
X_{33}	−18.2	5.90	0.015
X_{44}	−30.3	5.90	0.001
X_{55}	−1.52	0.37	0.003
Interaction			
X_{12}	−0.07	0.18	0.702
X_{13}	0.08	0.37	0.838
X_{14}	0.37	0.37	0.343
X_{15}	−0.04	0.09	0.652
X_{23}	1.94	3.65	0.610
X_{24}	2.84	3.65	0.460
X_{25}	0.02	0.91	0.983
X_{34}	21.1	7.30	0.020
X_{35}	1.88	1.83	0.332
X_{45}	−0.88	1.83	0.641
R^2	0.96		

[a] $P < 0.01$, highly significant; $0.01 < P < 0.05$, significant; $P > 0.05$, not significant.

Table 5
Analysis of variance of regression parameters for the response surface model

Regression	Degree of freedom	Sum of squares	R^2	F-value	P-value[a]
Linear	5	4740	0.40	17.8	0.000
Quadratic	5	5840	0.50	21.9	0.000
Interaction	10	639	0.06	1.20	0.407
Total	20	11,200	0.96	10.5	0.001

[a] $P < 0.01$, highly significant; $0.01 < P < 0.05$, significant; $P > 0.05$, not significant.

3.3. Physical and chemical indices of the reaction product

The physical and chemical indices of the reaction product are given in Table 7. The reaction product is bright orange and transparent liquid with no fishlike smell and good mobility. The physical and chemical indices were in agreement with those of the standard edible oil.

Table 7
Physical and chemical indices of the reaction product

Index	Value
Relative density, d_{15}^{15}	0.947
Refractive index, n_d^{40}	1.50
Apparent	Clarification, transparency
Color	Bright orange
Water and volatile matter (%)	0.10
Acid value (mg KOH/g)	0.27
Peroxide value (mmol/kg)	7.86
Saponification value (mg KOH/g)	150
Unsaponifiable matter (%)	0.06
Iodine value (I_2 g/100 g)	349
Insoluble impurities (%)	0.05

3.4. Fatty acid composition of the reaction product

Table 8 and Fig. 1 show the fatty acid compositions of the product by preliminary separation. The fatty acids in the product were mainly $n-3$PUFA. The contents of DHA and EPA were 73.4% and 13.5%, respectively, the total content being above 85%. Table 9 shows the content of the separated components in the product. Diacylglycerols comprised of the product, 56.1%; triacylglycerol, 31.3%; mononacylglycerol, 12.1%; and free fatty acid only 0.39%. The proportions of the components in the product were related to with the lipase-catalysed esterification mechanism. Previous studies have shown that, initially, almost equimolar amounts of monoacylglycerol and diacylglycerol were produced as PUFA decreased. Formation of triacylglycerol was much less than that of monoacylglycerol and diacylglycerol. Production of triacylglycerol was likely to have resulted from acyl migration to the 2-position in 1- or 3-monoacylglycerol or 1,3-diacylglycerol, followed by further enzymatic esterification, because lipases PS-30 and IM-60 are 1,3-specific (Li & Ward, 1993). Lipase IM showed a high esterification degree, but a low percentage of triacylglycerol; lipase PS produced a lower esterification degree but a higher percentage of triacylglycerols in lipase. Novo 435 had the highest esterification degree and triacylglycerol yield. Lipase Novo 435 produced monoacylglycerol, diacylglycerol and triacylglycerol in proportions that depended on reaction conditions, because it was not 1,3-specific in the reaction. Triacylglycerol was synthesised faster by esterification in positions 1, 2, and 3 (Cerdan et al., 1998). Some researchers think that they used 1, 3-specific lipase to esterify glycerol, making migration of the acyl group from position 1 or 3 or 2 necessary for triacylglycerol formation. This isomerisation reaction was the limiting

Table 6
Analysis of variance for second-order polynomial model fitted to response surface

Source	Degree of freedom	Sum of squares	Mean squares	F-value	P-value[a]
Lack of fit	6	419	69.8	17.6	0.055
Pure error	2	7.95	3.98		
Total error	8	427	53.3		

[a] $P < 0.01$, highly significant; $0.01 < P < 0.05$, significant; $P > 0.05$, not significant.

Table 8
Fatty acids composition of the reaction product

Fatty acid	16:1	16:2	20:4	20:5 ($n-3$)	22:3	22:4	22:5	22:6 ($n-3$)	others
Content (%)	1.28	1.14	4.39	13.5	0.36	2.36	1.71	73.4	1.91

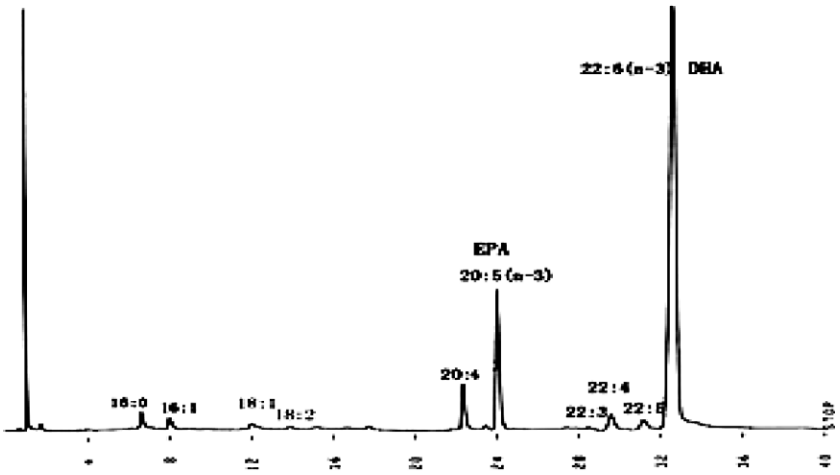

Fig. 1. Gas chromatogram of the reaction product.

Table 9
The content of the separated components in the reaction product

	Component			
	Triacylglycerol	Diacylglycerol	Monoacylglycerol	Free fatty acid
Content (%)	31.3	56.1	12.1	0.39

step of triacylglycerol synthesis (Castillo, Dossat, Marty, & Combers, 1997).

4. Conclusion

The acylglycerols rich in $n-3$ PUFA were successfully synthesised by lipase Novo 435 in hexane. The mathematic model between the degree of esterification and affecting factors was obtained by RSM optimisation. The optimal process parameters were as follows: $n-3$ PUFA at 0.4 g, glycerol at 2.5 g, hexane at 5 ml, the content of initial water at 0.60%, temperature at 40 °C, 1 g molecular sieve added after 1 h, reaction time of 24 h and shaking frequency of 150 rpm. Under these conditions, the degree of esterification was above 90%. The content of DHA and EPA in the reaction product was 73.4% and 13.5%, respectively. The reaction product was composed of monoacylglycerol, diacylglycerol and triacylglycerol, the contents of which were 12.1%, 56.1% and 31.3%, respectively. These results will provide a reference for the development of fish oil health products.

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