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Production of Lipase-catalyzed Structured Lipid from Olive Oil with Omega-3 Polyunsaturated Fatty Acids

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Abstract Acidolysis of olive oil with omega-3 (*n*-3) polyunsaturated fatty acids (PUFAs) was carried out to produce a structured lipid. Novozym 435® from *Candida antarctica* was used as the biocatalyst. Response surface methodology (RSM) was used to determine optimum conditions for lipase-catalyzed enrichment of olive oil. Three factors, 5 levels, central composite design was used. The effects of incubation time, temperature, and substrate mole ratio on incorporation ratio (*n*-3 fatty acids/total fatty acids, %) were investigated. From the evaluation of response surface graphs, the optimal conditions for incorporation of long chain *n*-3 PUFAs into olive oil were 40-60°C for temperature, 30-45 hr for reaction time, and 3:1-5:1 (*n*-3 fatty acids/olive oil) for substrate mole ratio. Experiments conducted under optimized conditions predicted by the model equation obtained from RSM yielded structured lipids with 50.8% *n*-3 PUFAs. This value agreed well with that predicted by the model. Oxidative stability tests showed that the product was more susceptible to oxidation than unmodified olive oil. Antioxidant addition improved the oxidative stability of the product.

Keywords: olive oil, *n*-3 polyunsaturated fatty acid, lipase, structured lipid, response surface methodology

Introduction

Polyunsaturated fatty acids (PUFAs), which are mainly found in fish oils, play an important role in human health and nutrition (1). It has been shown that omega-3 (*n*-3) PUFAs, namely eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), have preventive or healing effects on many diseases such as cancer (2-4), cardiovascular diseases (5,6), diabetes (7,8), immune system diseases (9,10), and depression (11). Long-chain PUFAs are considered as essential for infant growth and development of brain and retina (12-15).

Due to the beneficial effects of *n*-3 PUFAs, there has been a growing interest in enrichment of lipids with these fatty acids in the past few decades. Structured lipids can be produced either by chemical or enzymatic modification. By chemical pathways, randomized products can only be obtained. Enzymatic modification is widely preferred since it is possible to produce regiospecifically modified or restructured triacylglycerols by using lipase (16,17). Studies aiming at modification of several plant lipids with EPA and DHA using lipases originating from *Mucor miehei* or *Candida antarctica* are available (18-23), yet no studies on modifying olive oil with these fatty acids have been published.

Olive oil is known to have positive effects on human health due to its high content of monounsaturated fatty acids (24) and antioxidant properties (25). However, it contains a little amount of *n*-3 PUFAs (24). Berbert *et al.* (26) have shown that olive oil consumption of rheumatoid arthritis patients who also take fish oil supplements had caused clinical and laboratory improvements. This was one of the inspiring studies that led us to design this investigation that aimed to enrich olive oil with *n*-3 PUFAs in order to obtain a functional lipid product. Effects of 3 factors, namely substrate mole ratio, reaction time, and temperature, on incorporation ratio were investigated by using response surface methodology (RSM). Oxidative stability of the resultant product was also investigated.

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Materials and Methods

Materials Olive oil was purchased from a local market. Novozym 435® (immobilized *Candida antarctica* lipase) was from Novozymes (Danbury, CT, USA) and Omega-3 '700'® capsules were from Solgar (Leonia, NJ, USA). Fatty acid composition of olive oil and Omega-3 '700'® capsules are given in Table 1. PUFA standard from menhaden oil was purchased from Supelco (Bellefonte, PA, USA). All solvents and reagents for analysis were of analytical or chromatographic grade and were purchased from Merck (Darmstadt, Germany) and Riedel (Seelze, Germany).

Experimental design A 5-level central composite rotatable design was used for the RSM studies, and the experimental settings determined by Modde® 7.0 (Umetrics, Umeå, Sweden) were generated with 3 factors (27). Temperature, reaction time, and substrate mole ratio were the factors investigated. The ranges for the factors were determined as follows: temperature, 33-57°C; reaction time, 10-38 hr; and substrate mole ratio, 1.8:1-4.2:1 (*n*-3 fatty acids/olive oil); and star points were 25-65°C, 0-48 hr, and 1:1-5:1 mol/mol, respectively. The reaction conditions for each experimental setting are shown in Table 2. Experiments were run randomly.

Acidolysis reaction Olive oil was mixed with *n*-3 fatty acids obtained from Omega-3 '700'® capsules at different

mole ratios (0.88 g olive oil and 0.96 g *n*-3 fatty acids for 1:1 mole ratio) in 30 mL hexane. The amount of immobilized lipase Novozym 435® added to the reaction mixture was maintained at 10%(w/w) of reactants. Reaction was carried out in sealed flasks with magnetic stirring at 400 rpm at different temperatures and for different time periods.

Analysis of product Analysis of product was carried out according to Senanayake and Shahidi (28). The reaction mixture was titrated against a 0.5 N NaOH solution, using a phenolphthalein indicator, to neutralize free fatty acids. The mixture was transferred into a separatory funnel and thoroughly mixed with 25 mL of hexane. The lower aqueous layer was separated and discarded, while the upper hexane layer containing acylglycerols was passed through a bed of anhydrous sodium sulfate. The acylglycerol fraction was subsequently recovered following hexane removal at 45°C using a rotary evaporator. The fatty acid compositions of the triacylglycerol fractions obtained by this method were determined by gas chromatography (GC).

GC analysis The fatty acid composition of substrates and reaction products were determined after methylation with boron trifluoride in methanol (29). The fatty acid methyl esters were analyzed by GC. A gas chromatograph (Trace GC 2000; Thermo Quest, CE Instruments, Milan, Italy) was equipped with a flame ionization detector and a DB-Wax capillary column with 30 m length, 0.32 mm i.d., and 0.25-μm film thickness (J & W Scientific Columns, Folsom, CA, USA). Helium was used as the carrier gas at a flow rate of 1.5 mL/min. The temperatures of the injector and detector were kept at 250 and 270°C, respectively. The initial temperature of the column was 180°C, and it was programmed to increase at a rate of 5°C/min to 225°C. The individual fatty acids were identified by comparing the retention times to standards obtained from Supelco.

Oxidative stability test The oxidative stabilities of *n*-3 fatty acids, olive oil, as such or subjected to process steps in the absence of any enzyme, and reaction product after free fatty acids are removed were determined by a TA Q10 differential scanning calorimetry (DSC, New Castle, DE, USA). The equipment was calibrated by pure indium. Oil samples of 1.0±0.2 mg were weighed into open aluminum pans and placed in the equipment's sample chamber. An empty pan was used as the reference. The isothermal temperature was programmed at 120°C for olive oil, and at 100°C for the rest of the samples. Purified oxygen (99.5%) was passed through the sample enclosure at 100 mL/min.

Oxidative stability improvement with α-tocopherol Reaction products (2 g each) were uniformly mixed with α-tocopherol (100, 200, and 300 ppm). Samples' oxidative stabilities were determined by the same method given above that was used for the original reaction product.

Statistical analysis The regression analyses, statistical significance, and response surfaces were analyzed by means of RSM using the software STATISTICA® 6.0 (StatSoft Inc., Tulsa, OK, USA). Analysis of variance (ANOVA) was carried out at a level of $p<0.05$ to assess the significance of differences among mean values of oxidative stability test results.

Results and Discussion

Fatty acid composition Fatty acid compositions of olive oil, Omega-3 '700'® capsules and reaction product are given in Table 1. Oleic acid was reduced from 76.3 to 25.2% after modification of olive oil. On the other hand, there were no *n*-3 fatty acids in olive oil before the reaction, and this ratio had risen to 47.4% after modification.

Effects of parameters and model fitting Significance of the relationship between linear and quadratic effects of dependent and independent variables as well as their interactions were evaluated by ANOVA (Table 3). ANOVA and regression coefficient calculations were performed by excluding the 11th experiment coded as (0, $-\alpha$, 0; temperature, time, substrate mole ratio) in Table 2 to prevent errors during the observation of the effects of the factors investigated, since no incorporation had occurred at time 0 of reaction. Linear effect of substrate mole ratio was found to be statistically significant ($p<0.01$). The best fit quadratic model was determined for PUFA (EPA plus DHA) incorporation. ($R^2=0.94$). The model equation is as follows:

$$\text{PUFA incorporation }\%=44.72+(0.54\times T)+(0.35\times T^2)+(0.96\times t)+(1.64\times t^2)+(6.14\times m)-(1.62\times m^2)+(0.40\times T\times t)-(0.15\times T\times m)+(0.20\times t\times m) \quad (1)$$

where T is temperature, t is reaction time, and m is substrate mole ratio.

The observed incorporation of PUFAs and the values predicted according to the model were well correlated (Fig. 1). All the statistical results indicated that the model generated represented the actual relationships between reaction parameters.

Table 1. Fatty acid compositions of olive oil, Omega-3 '700'® capsules and olive oil enriched with *n*-3 polyunsaturated fatty acid (PUFA) (%)

Fatty acid	Olive oil	Omega-3 '700'® capsules	Olive oil enriched with *n*-3 PUFA[1)]
16:0	11.9	5.3	6.0
16:1 *n*-7	0.6	1.7	1.3
16:2 *n*-4	-	0.6	-
16:3 *n*-4	-	-	-
18:0	-	6.4	2.6
18:1 *n*-9	76.3	2.2	25.2
18:1 *n*-7	-	-	-
18:2 *n*-6	9.2	1.0	3.0
18:3 *n*-3	0.5	0.6	3.9
18:4 *n*-3	-	1.0	-
20:1 *n*-9	-	1.7	1.4
20:4 *n*-6	-	1.7	2.4
20:4 *n*-3	-	1.5	-
20:5 *n*-3	-	39.2	27.8
22:6 *n*-3	-	25.9	19.6

[1)]Reaction conditions for incorporation: 45°C temperature, 24 hr reaction time, and 3:1 substrate mole ratio (*n*-3 fatty acids/olive oil).

Table 2. Coded and decoded levels of factors and % polyunsaturated fatty acid (PUFA) incorporation into olive oil according to central composite rotatable design

	Temperature (°C)		Time (hr)		Substrate mole ratio, *n*-3 fatty acids/olive oil		Response
	Coded	Decoded	Coded	Decoded	Coded	Decoded	% PUFA incorporation
1	-1	33	-1	10	-1	1.8	38.5
2	+1	57	-1	10	-1	1.8	39.0
3	-1	33	+1	38	-1	1.8	38.5
4	+1	57	+1	38	-1	1.8	40.7
5	-1	33	-1	10	+1	4.2	48.9
6	+1	57	-1	10	+1	4.2	50.2
7	-1	33	+1	38	+1	4.2	49.6
8	+1	57	+1	38	+1	4.2	50.1
9	-1.682	25	0	24	0	3	45.3
10	+1.682	65	0	24	0	3	45.6
11	0	45	-1.682	0	0	3	0
12	0	45	+1.682	48	0	3	46.9
13	0	45	0	24	-1.682	1	27.3
14	0	45	0	24	+1.682	5	52.3
15	0	45	0	24	0	3	47.4
16	0	45	0	24	0	3	44.3
17	0	45	0	24	0	3	41.9

Table 3. Results of analysis of variance

Factor[1)]	Sum of squares	Degrees of freedom	Mean squares	F	*p*
Temperature (L)	3,3389	1	3,3389	0,5901	0,4714
Temperature (Q)	1,3311	1	1,3311	0,2352	0,6448
Time (L)	4,3567	1	4,3567	0,7701	0,4139
Time (Q)	6,0921	1	6,0921	1,0768	0,3394
Substrate mole ratio (L)	420,9862	1	420,9862	74,4136	0,0001[1)]
Substrate mole ratio (Q)	27,6086	1	27,6086	4,8801	0,0692
Temperature × Time	0,7200	1	0,7200	0,1272	0,7334
Temperature × Substrate mole ratio	0,1800	1	0,1800	0,0318	0,8643
Time × Substrate mole ratio	0,1800	1	0,1800	0,0318	0,8643
Error	33,9443	6	5,6574		
Total sum of squares	606,6544	15			

[1)]L, linear; Q, quadratic; Statistically significant ($p<0.01$).

Interaction of parameters on PUFA incorporation The relationship between the response and the parameters were examined by using contour plots. Three contour plots which have been obtained by the interaction of 3 parameters on incorporation of PUFAs into olive oil are given in Fig. 2A-C.

Figure 2A shows the interaction of temperature and reaction time on PUFA incorporation. Within the ranges of both reaction time and temperature, high incorporation of PUFAs was achieved. Considering the conditions valid within the experimental range, incorporation ratio increased with increasing reaction times independent of the temperature.

Interaction of substrate mole ratio with temperature and time appeared as saddle surface in Fig. 2B and 2C. As it can be seen from the Figures, incorporation ratio increased with increasing substrate mole ratio independent of the temperature and time.

In the acidolysis reaction between olive oil and *n*-3 fatty acid concentrate catalyzed by lipase, PUFA incorporation was generally high at higher substrate mole ratios without any significant effects of temperature and reaction time. Among all the experimental results, the highest incorporation ratio (50.2%) was obtained at 57°C, in 10 hr, and at 4.2:1 substrate mole ratio (*n*-3 fatty acids/olive oil). From response surface graphs showing the interactions of different factors, namely temperature, reaction time, and substrate mole ratio, the optimal conditions were found as 40-60°C for temperature, 30-45 hr for reaction time, and 3.5-5.5:1 for substrate mole ratio (*n*-3 fatty acids/olive oil) for incorporation of long chain *n*-3 PUFAs into olive oil.

In this study, substrate mole ratio was the most significant factor on PUFA incorporation. It was observed that incorporation ratio increased with increasing substrate mole ratio independent of the temperature and time. In a

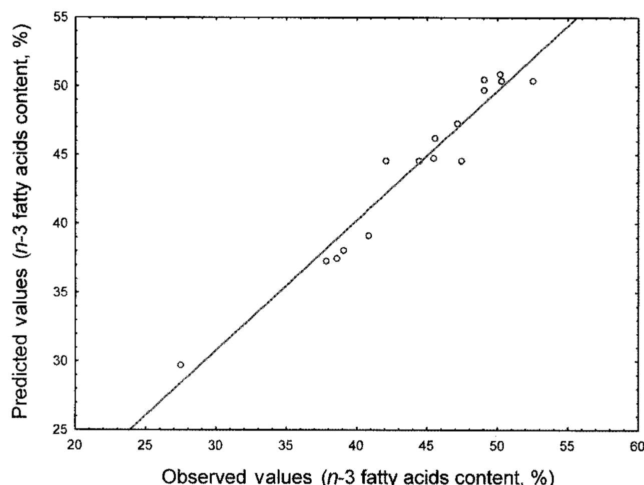

Fig. 1. Relationship between observed and predicted values (n-3 fatty acids content of the product, %).

previous study, incorporation of n-3 PUFAs into hazelnut oil was found to increase by increasing substrate mole ratio, without any significant effect from other factors (30). Other researchers have also reported that increasing substrate mole ratio increased incorporation ratio when incorporating n-3 PUFAs into various seed oils (19,21,28,31). Nevertheless, it was stated that using excess amounts of essential fatty acids may result in inhibition of lipase activity and a decrease in product yield when removing excess fatty acids from the medium (20,32).

Validation of the model and optimization Based on the results of RSM studies, a new set of experiments were carried out under optimized conditions predicted by the model. It was observed that, under optimal conditions, the product contained 50.8% n-3 PUFAs as against the predicted maximum of 50.6%. The verification results indicated that the predicted values from these models were reasonably close to the observed values. Therefore, it is possible to say that when experiments conducted at conditions of temperature of 50°C, over 24 hr and at a substrate mole ratio of 5:1, structured lipid containing 50.8% PUFAs can be obtained.

Oxidative stability of the product Oxidation induction times of n-3 fatty acids, olive oil, as such or subjected to process steps in the absence of any enzyme, and reaction product after free fatty acids are removed with various amounts of α-tocopherol added are shown in Table 4. According to the statistical analysis, there was a significant difference between oxidative stabilities of olive oil and the n-3 enriched product. Olive oil was more stable than the product. When different amounts of α-tocopherol were added, the oxidative stability of the product was improved. Addition of α-tocopherol at a concentration of 400 ppm was the most effective treatment, but still the stability was lower than olive oil.

The differences between oxidative stabilities of olive oil and the enriched product can be partly due to the different tocopherol contents and the amount of incorporated n-3 fatty acids in the product. Tocopherols play an important

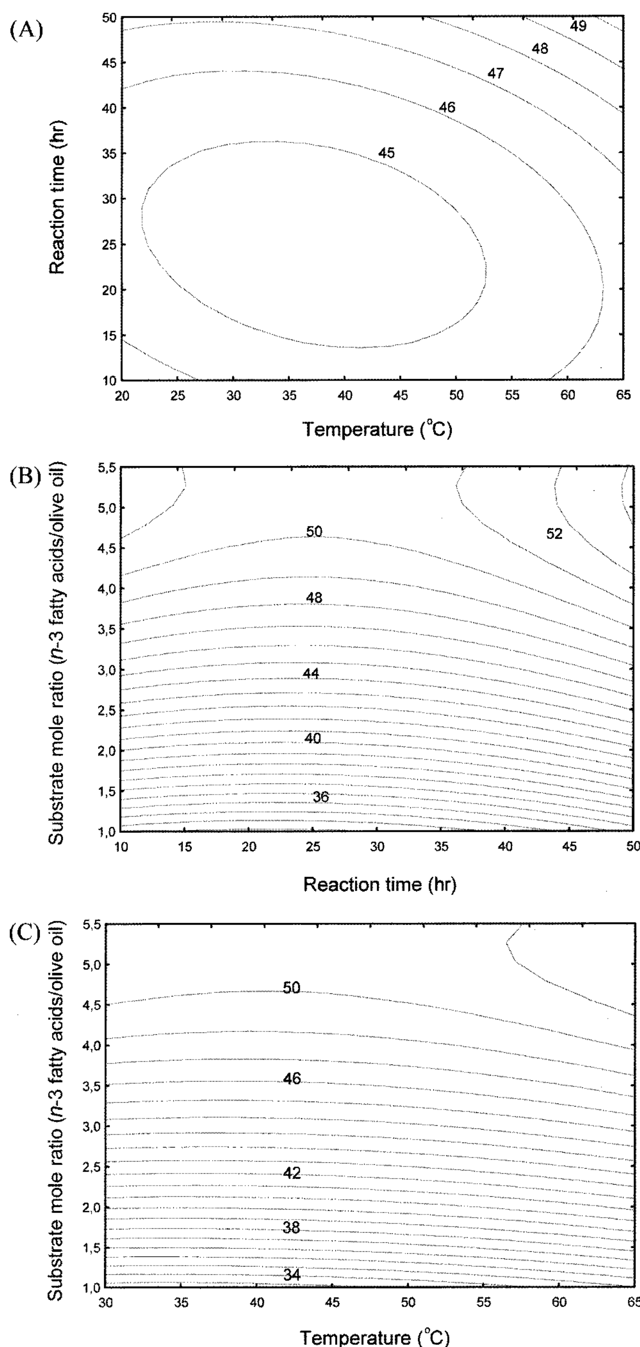

Fig. 2. Contour plots for incorporation of polyunsaturated fatty acid (PUFA) into olive oil. (A) Reaction time and temperature; (B) substrate mole ratio and time; (C) substrate mole ratio and temperature.

role in preventing oxidative reactions in lipids; however, 24 hr of reaction may cause oxidation of tocopherols, which results in a decrease in oxidative stability. This assumption is supported by the low of oxidative stability of mixture of substrates subjected to process conditions without addition of enzyme. Reduction of tocopherol content during structured lipid production was previously observed by Lee *et al.* (33) and Hamam and Shahidi (34). Another reason for the decrease of oxidative stability is the high amount of incorporated n-3 fatty acids in the product. At optimum conditions, 50.8% n-3 PUFAs are incorporated to olive oil.

Table 4. Oxidative induction times of substrates and products

Sample	Oxidative induction time (min)
Omega 3	10.37±0.2
Olive oil	30.64±0.2
Mixture of substrates[1)]	14.63±0.3
Product	8.16±0.2
Product+100 ppm α-tocopherol	9.66±0.1
Product+200 ppm α-tocopherol	10.6±0.1
Product+400 ppm α-tocopherol	11.43±0.4

[1)]Subjected to process steps in the absence of any enzyme.

PUFAs are known to be very sensitive to oxidation due to their double bonds. Therefore, an additional antioxidant should be added into *n*-3 enriched olive oil to retard oxidation.

The *n*-3 PUFA content of olive oil is efficiently increased in this study. This product can be used in nutraceuticals and many healthy, functional food product formulations like salad dressings, bakery products, mayonnaise, etc, aiming to protect against cancer, coronary heart diseases, diabetes, immune system diseases, and depression. Further researches can be done on oxidative stability of such *n*-3 PUFA containing products.

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