JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Process Optimization Using Response Surface Design and Pilot Plant Production of Dietary Diacylglycerols by Lipase-Catalyzed Glycerolysis

JANNI BROGAARD KRISTENSEN,*,† XUEBING XU,‡ AND HUILING MU†

Biochemistry and Nutrition and Food Biotechnology and Engineering Groups, BioCentrum-DTU, Technical University of Denmark, DK-2800 Lyngby, Denmark

Two approaches to shift the acylglycerol equilibrium were tested as follows: addition of monoacylglycerols and lowering of the temperature. None of these approaches were able to shift the equilibrium toward higher diacylglycerol (DAG) contents. The glycerolysis reaction was optimized with five factors using response surface methodology. Evaluation of the resulting model enabled the determination of optimal reaction conditions for glycerolysis aiming at high DAG yield. However, verification of the model showed that the model was unable to take the molecular equilibrium into account but it provided good insight in how process settings can be chosen to, for example, minimize production costs. Optimal conditions were found to be the following: no extra water, low content of glycerol (molar ratio of 2), temperature of 60−65 °C, 4−5 h reaction time, and only 5 wt % lipases. Up scaling of the glycerolysis process was performed and revealed that scale-up to a 20 kg production in a pilot plant batch reactor was possible with a similar DAG yield (60 wt %) as in lab scale. Purification of DAG oil using batch deodorization and short path distillation yielded 93 wt % pure DAG oil.

KEYWORDS: Diacylglycerol; glycerolysis; Novozym 435; optimization; response surface methodology; pilot plant production; purification; short path distillation

INTRODUCTION

Diacylglycerols (DAGs) are esters of glycerol where two of the hydroxyl groups are esterified with fatty acids (FA). They are found as a minor natural component of various edible oils (*1*). DAG exists in two isomeric forms, *sn*1,2(2,3)-DAG and *sn*1,3-DAG, with a natural isomeric ratio of approximately 3:7 (*2*). Today, a functional DAG cooking oil containing approximately 80 wt % DAG with the rest being triacylglycerol (TAG) is manufactured (*3*). The DAG cooking oil has been on the Japanese market since 1999 and was launched nationwide in the United States in January 2005 after a test period on parts of the American market to evaluate the consumers' interest in the product.

Recent studies on the nutritional properties and dietary effects of DAG oil have claimed that it has some beneficial effects as compared to traditional TAG oil. Both studies in animals and humans have shown a decrease in postprandial TAG levels in serum and a suppressed accumulation of body fat and liver TAG levels after intake of DAG oil as compared to TAG oil (*4*−*7*). DAG oil has a similar energy value and digestibility as traditional TAG oil (*8*). The 1,3-DAG isomer is suggested to be responsible for the observed beneficial effects regarding obesity and lipemia due to a different metabolic pathway as compared with TAG and 1,2-DAG (*4*).

A mixture of monoacylglycerol (MAG) oil and DAG oil can be produced chemically (*9*). However, this process requires high temperatures (220−260 °C) and the use of an inorganic catalyst, such as sodium, potassium, or calcium hydroxide (*10*). Another approach is to use lipase-catalyzed reactions for the synthesis of partial acylglycerols with either MAG (*11*−*13*) or DAG as the primary target. With respect to DAG synthesis, esterification reactions with and without the use of an organic solvent (*14*−*17*) but also glycerolysis reactions have been studied (*18*−*21*). Another method reported uses commercial preparations of MAGs in interesterification with rapeseed oil or in esterification with rapeseed oil FA (*22*). The most recent method for production of high-purity 1,3-DAG in the industry involves two reactions: the partial hydrolysis of a fat or oil followed by the 1,3-specific lipase-catalyzed esterification of the free fatty acid (FFA) in the hydrolysate with glycerol (*23*).

In the present work, glycerolysis was chosen as the reaction process because it is a simple process where oil can be used directly together with glycerol without the need to first liberate FFA. In glycerolysis, DAG can be formed both by removal of an acyl moiety from TAG and by acylation of MAGs formed during the reaction. The aim of this work was to optimize the DAG yield and reaction conditions in a solvent-free glycerolysis reaction. The lipase of choice for the optimization was Novozym 435 since this lipase in our previous work, where several lipases

* To whom correspondence should be addressed. Tel: (+45)45252747. Fax: (+45)45886307. E-mail: jbk@biocentrum.dtu.dk.
† Biochemistry and Nutrition Group.
‡ Food Biotechnology and Engineering Group.

10.1021/jf0507745 CCC: $30.25
Published on Web 08/05/2005

Table 1. DAG Yield in Reactions Run to Shift the Glycerolysis Equilibrium

	glycerolysis reaction where MAG (9 wt % of substrate) was added after 5 h				glycerolysis reaction where the temperature was lowered to 5 °C after 5 h			
hours of incubation	5[a]	6	10	24	5[a]	24	48	74
DAG (wt %)	55	54	57	56	61	52	62	65
TAG (wt %)	24	23	22	23	19	20	16	13
MAG (wt %)	21	23	21	20	20	28	22	22

Reaction conditions were as follows: EnzL = 10 wt %, R_{temp} = 75 °C, WatC = 0%, SubR = 1. [a] After 5 h either MAG was added or the temperature lowered. The measurement equals the value prior to MAG addition or temperature lowering. Abbreviations: EnzL = enzyme load (wt % of oil mass), R_{temp} = reaction temperature (°C), WatC = water content (wt % of glycerol mass), and SubR = substrate molar ratio (oil/glycerol).

were screened, was found suitable for solvent-free glycerolysis (*21*). Novozym 435 yielded DAG in an acceptable amount with an isomeric ratio similar to the naturally occurring ratio of DAG isomers. No optimization has been carried out so far for glycerolysis aiming at high DAG yield using Novozym 435.

For the optimization, we used response surface methodology (RSM) as a tool since it enables the evaluation of multiple parameters alone or in combination on response variables. Moreover, it allows a reduced number of experiments to be run to provide sufficient information for statistically acceptable results (*24*). The factors, which were optimized with respect to DAG yield in this work, were as follows: reaction time, enzyme load, reaction temperature, water content, and substrate molar ratio (oil:glycerol).

To produce DAG oil by glycerolysis in large-scale might give different yields and cause difficulties not experienced in small-scale production. To find out if the optimized reaction conditions can be applied in a large-scale, pilot plant productions were run with rapeseed oil and sunflower oil. The obtained reaction products were purified by means of batch deodorization and short path distillation (SPD), and the two DAG oils [DAG oil (rapeseed) and DAG oil (sunflower)] were characterized.

MATERIALS AND METHODS

Materials. Rapeseed oil and sunflower oil were provided by Aarhus United (Aarhus, Denmark). Glycerol (99%) was supplied by BHD Laboratory Suppliers (Poole, England). Purified MAG (100%) was obtained from our own pilot plant production. The lipase used in all reactions was Novozym 435 (*Candida antarctica* lipase B immobilized on a macroporous acrylic resin), which was generously supplied by Novozymes A/S (Bagsvaerd, Denmark). All other chemicals used were of analytical grade and obtained from various sources.

General Experimental Setup. All lab-scale reactions were performed with 30 g of rapeseed oil in 200 mL Erlenmeyer flasks with glass slip stoppers and magnetic stirring (≈250 rpm). Heating to the desired temperature was achieved using a water bath. The lipases were added after the substrate had gained the desired temperature.

Experimental Setup for Equilibrium Shifting. To test if the glycerolysis equilibrium could be shifted toward a higher DAG content, approaches were tested as follows: MAG (9 wt %, 8.5 mmol) was added to the reaction mixture after 5 h of incubation (**Table 1**), and samples were withdrawn at 5, 6, 7, 10, and 23.5 h. Moreover, the temperature was lowered to 5 °C after 5 h of incubation (**Table 1**) and samples were withdrawn at 24, 48, 75, and 96 h.

Experimental Design. A five factor fractional factorial design (central composite face design design) according to the principles of RSM was used in this work (*24*, *25*). The five factors and their levels were reaction time (R_{time}, 3−14 h), enzyme load (EnzL, 3−15 wt % of oil mass), reaction temperature (R_{temp}, 40−75 °C), water content (WatC, 0−6 wt % of glycerol mass), and substrate molar ratio, oil/glycerol (SubR, 0.25−2.00). This design generated 29 experimental settings by the use of the software Modde 7.0, Umetrics (Umeaa, Sweden) (**Table 2**).

Table 2. Factor Levels Generated by Modde for Response Surface Method Analysis and the Comparison between Observed Responses and Predicted Results for Total DAG Yield by the Model

	factors[a]					responses (DAG, wt %)	
no.	R_{time}	EnzL	R_{temp}	WatC	SubR	observed	error[b]
1	3	3	40	0	2.00	9.75	0.12
2	14	3	40	0	0.25	29.33	0.45
3	3	15	40	0	0.25	6.02	0.42
4	14	15	40	0	2.00	54.54	0.22
5	3	3	75	0	0.25	34.28	0.63
6	14	3	75	0	2.00	51.15	0.10
7	3	15	75	0	2.00	53.46	1.34
8	14	15	75	0	0.25	65.98	1.23
9	3	3	40	6	0.25	8.86	0.55
10	14	3	40	6	2.00	26.11	0.13
11	3	15	40	6	2.00	38.53	0.16
12	14	15	40	6	0.25	26.76	0.21
13	3	3	75	6	2.00	37.29	1.21
14	14	3	75	6	0.25	52.42	1.54
15	3	15	75	6	0.25	25.85	1.78
16	14	15	75	6	2.00	55.97	1.81
17	3	9	57.5	3	1.13	45.25	2.21
18	14	9	57.5	3	1.13	61.16	0.09
19	8.5	3	57.5	3	1.13	44.35	1.79
20	8.5	15	57.5	3	1.13	52.50	0.33
21	8.5	9	40	3	1.13	39.70	1.24
22	8.5	9	75	3	1.13	61.34	0.89
23	8.5	9	57.5	0	1.13	57.07	1.41
24	8.5	9	57.5	6	1.13	52.33	1.98
25	8.5	9	57.5	3	0.25	52.24	0.45
26	8.5	9	57.5	3	2.00	57.31	3.64
27	8.5	9	57.5	3	1.13	58.61	2.21
28	8.5	9	57.5	3	1.13	56.91	0.51
29	8.5	9	57.5	3	1.13	56.00	0.40

[a] R_{time} = reaction time (h), EnzL = enzyme load (wt % of oil mass), R_{temp} = reaction temperature (°C), WatC = water content (wt % of glycerol mass), and SubR = substrate molar ratio (oil/glycerol). [b] Absolute prediction error = |observed − predicted|.

Glycerolysis Reactions for Optimization. To optimize the DAG yield in glycerolysis reactions, 29 reactions were run (**Table 2**). After the optimization, a set of optimal reaction conditions were predicted on the basis of the contour plots in **Figure 2** and a reaction was run to test the model. Samples were taken from optimization reactions when the reaction was finished by withdrawing approximately 2 mL of the reaction mixture followed by immediate centrifugation (2900 G/15 min) to separate the oil phase and the enzyme/glycerol phase. Two separate drops of the oil phase were used for analysis.

Pilot Plant Production and Purification. Five pilot plant productions were conducted (**Table 5**) in a 45 L pilot refining vessel. The equipment is described by Xu et al. (*26*). The process was carried out under slightly reduced pressure (≈0.5 bar) using nitrogen. Settings for the production were as follows: R_{temp} = 65 °C, R_{time} ≈ 18 h, SubR = 0.9 (oil/glycerol), and WatC = zero in the first run; thereafter, approximately 2 wt % (of the enzyme mass) distillated water was added to compensate for water loss from the enzyme carrier, EnzL ≈ 7 wt % (of the oil mass); the enzymes were reused, and 4% extra was added in batches 2−5 to compensate for loss; stirring = 400−500 rpm. Oil and glycerol were equilibrated to 65 °C before the enzymes were added. Reactions were followed by vacuum filtration at 1.5−2 bar to separate the enzymes from the reaction mixture.

To purify DAG from the reaction mixture, batch deodorization and SPD were applied. Deodorization was conducted to remove excess glycerol and FFA in a 40 L conventional batch deodorizer. The equipment is described elsewhere (*26*). The vacuum was adjusted to less than 500 Pa, and the temperature was 190 °C. The total

Table 3. Outline of Distillation Steps and Conditions Used to Purify DAG Oil from the Deodorized Reaction Mixture by SPD

distillation step and oil used	purpose	feed rate (L/h)	evaporator temp (°C)	heat exchange temp (°C)
1. distillation: deodorized oil	removal of MAG	2.6	175	80
2. distillation: residue from 1. distillation	separation of DAG and TAG	1.9	250/258[a]	100
3. distillation: residue from 2. distillation	separation of DAG and TAG	1.9	260/265[a]	100

[a] The two temperatures refer to distillation of DAG oil from sunflower oil and from rapeseed oil, respectively.

deodorization time was 4 h. Afterward, the deodorized oils were purified further by SPD. Xu et al. have described the SPD system used (*26*). The nonvarying conditions were as follows: evaporator and degasser vacuum, 0.1 Pa; condenser temperature, 60 °C; and roller speed, 400 rpm. The varying distillation conditions are listed in **Table 3**.

High-Performance Liquid Chromatography (HPLC) Analysis of Acylglycerol Composition. Analysis was performed on equipment described by Mu et al. using evaporative light scattering detection (*27*). The separation of TAG, 1,3- and 1,2-DAG, and MAG was performed on a Hypersil silica column (l = 10 cm, i.d. = 2.1 cm, particle size = 5 μm) from Thermo Hypersil-Keystone (Bellafonte, PA). A binary solvent system of heptane (A) and heptane/ethyl acetate/2-propanol/acetic acid (B) = 80:10:10:1 (v/v/v/v) was used. The content of solvent B in the mobile phase was increased from 2 to 35% over 10 min at a flow of 0.50 mL/min. The content of solvent B was then increased further to 98% for 1 min and was maintained for 6 min before reverting to 2%, all at a flow rate of 1 mL/min.

The reaction samples were dissolved in chloroform (approximately 0.25 mg/mL), and 10 μL aliquots were injected for the HPLC analysis with double determinations. Calibration curves were established to quantify the amount of the different acylglycerols in the samples. The contents of TAG, 1,3-DAG, 1,2-DAG, and MAG are given as wt % of the total acylglycerol content in the lipid sample.

Gas Chromatography (GC) Analysis of FA Composition. The FA compositions of rapeseed oil, sunflower oil, and the two DAG oils from rapeseed and sunflower oil, respectively, were determined by GC after transmethylation with KOH in methanol to form fatty acid methyl esters (FAME).

FAMEs were analyzed on an Hewlett-Packard 6890 gas chromatograph with a capillary column (SP2380, 60 m, i.d. 0.25 mm, 0.2 μm film, Supelco, Bellefonte, PA). The injector was 260 °C and used in the split mode with a split ratio of 11:1. The initial oven temperature was 70 °C followed by temperature programming: 15 °C/min until 160 °C followed by 1.5 °C/min until 200 °C, which was maintained for 15 min, and finally, the temperature was raised to 225 °C and maintained for 10 min. The temperature of the detector (flame ionization) was 270 °C. Samples were run in duplicate, and results are presented as wt % FAME.

Determination of FFA Content. The amount of FFA in rapeseed oil, sunflower oil, and the two DAG oils from rapeseed and sunflower oil, respectively, was determined according to AOCS official method (*28*). The amount of FFA was calculated as wt % oleic acid. Analyses were made in duplicate.

Determination of Tocopherol Content. Tocopherols were determined by normal phase HPLC with fluorescence detection as described by AOCS Official Method (*29*). The amount of α-tocopherol equivalents (α-TE) was calculated on the basis of the vitamin E activity of α-, β-, γ-, and δ-tocopherols, which is 1.0, 0.5, 0.1, and 0.03, respectively, for the four tocopherols (*30*).

Statistical Analysis. The data were analyzed by means of RSM with Modde 7.0. Second-order coefficients were generated by regression analysis with backward elimination. Responses were fitted with the factors by multiple regression. The fit of the model was evaluated by the coefficients of determination (R^2 and Q^2) and analysis of variance (ANOVA). The insignificant coefficients were eliminated after examining the coefficients, and the model was finally refined.

RESULTS AND DISCUSSION

Shift of Glycerolysis Equilibrium. In a previous work (*21*), we found that in batch glycerolysis with Novozym 435 a DAG yield of 60 wt % could be achieved after 7 h where equilibrium was obtained. Glycerol addition in a fed-batch manner did not cause any shift in the obtained acylglycerol equilibrium toward higher DAG content and neither did absorbing glycerol on silica gel. In the present work, we examined two other approaches, which are likely to shift the glycerolysis equilibrium. These approaches are addition of MAG and lowering of the temperature. The methodologies were tested after the equilibrium level of DAG was obtained in the glycerolysis reaction. From **Table 1**, it can be seen that 1 h after MAG addition the same amount of DAG is present in the reaction demonstrating that the additional MAG (9 wt % of substrate) was not able to shift the equilibrium toward higher DAG content. The use of commercial MAGs in transesterification with rapeseed oil has been examined using Lipozyme RM IM (*22*). The acylglycerol composition after 6 h was practically similar to the composition observed for glycerolysis in this work so the use of more expensive MAG preparations instead of glycerol is not advantageous.

For the temperature experiment, the reaction mixture became pastelike and almost solid after approximately 24 h at 5 °C but no increase in DAG yield was observed (**Table 1**). Lowering the temperature has been used extensively as an approach to shift reactions toward higher MAG yield. MAG has the highest melting point (35 °C for monoolein and 12 °C for monolinolein) of the acylglycerols (MAG, DAG, and TAG), and this physical property can be exploited to precipitate MAG from the reaction mixture hereby shifting the reaction toward higher MAG yield (*31*−*34*).

To increase MAG in the reaction mixture can be advantageous in a DAG production if TAG is simultaneously decreased, since MAG and DAG are more easily separated than DAG and TAG. So, a reaction product low in TAG and high in DAG and MAG is valuable because MAGs can be separated and sold as, for example, food emulsifiers or reused in the production of DAG. The remaining oil after MAG separation will have a high DAG and low TAG content and thereby be a nutritional valuable oil where removal of TAG is unnecessary. Unfortunately, we only observed a decrease in TAG content of 6 wt % during the 3 days at 5 °C (**Table 1**). Coteron et al. (*20*) also investigated the effect of lowering the temperature of the glycerolysis mixture (olive oil, Novozym 435) to 10 °C after 4 h. They found no effect on any of the acylglycerols for the 50 h that they followed the reaction. These findings are in good correlation with this work. To the contrary, McNeill et al. (*32*) found that the MAG content in the glycerolysis of rapeseed oil (nonimmobilized *Pseudomonas fluorescens*) doubled in 3 days when lowering the temperature from 30 to 5 °C. Comparing these previous findings with the results from this work, the lack of MAG increase could be due to the used lipase, Novozym 435. This immobilized lipase might be susceptible to blockage of the carrier (macroporous acrylic resin) when the reaction mixture becomes more viscous and pastelike, hereby hindering contact between reaction mixture and lipases.

In conclusion, none of the two approaches to shift the acylglycerol equilibrium succeeded. The DAG yield remained

Table 4. Scaled and Centered Regression Coefficients and Significance (*P*) Values for Total DAG Response in wt %

	total DAG	
variables [a]	coefficients	*P*
intercept	56.393	2.089×10^{-19}
R_{time}	9.118	5.445×10^{-11}
EnzL	4.782	1.441×10^{-7}
R_{temp}	11.008	5.017×10^{-12}
WatC	–2.081	6.715×10^{-4}
SubR	4.576	2.399×10^{-7}
$R_{time} \times R_{time}$	–4.259	3.242×10^{-3}
EnzL × EnzL	–9.039	3.702×10^{-6}
$R_{temp} \times R_{temp}$	–6.944	5.527×10^{-5}
$R_{time} \times$ WatC	–2.923	5.471×10^{-5}
$R_{time} \times$ SubR	–3.171	2.444×10^{-5}
EnzL × R_{temp}	–1.605	6.633×10^{-3}
EnzL × WatC	–2.066	1.136×10^{-3}
EnzL × SubR	4.905	2.124×10^{-7}
$R_{temp} \times$ WatC	–2.123	9.201×10^{-4}
$R_{temp} \times$ SubR	–2.414	3.166×10^{-4}

[a] R_{time} = reaction time (h), EnzL = enzyme load (wt % of oil mass), R_{temp} = reaction temperature (°C), WatC = water content (wt % of glycerol mass), and SubR = substrate molar ratio (oil/glycerol). The size of the coefficient represents the change in response when a factor varies from average to high level, while the other factors are kept at their averages. The coefficients with confidence intervals including zero are insignificant and have been removed from the model (backward elimination).

at approximately 60 wt %. This indicates, on the other hand, that the glycerolysis of rapeseed oil catalyzed by Novozym 435 is a very stable reaction yielding approximately 60 wt % DAG under much varying reaction conditions. Because it seemed very difficult or impossible to shift the acylglycerol equilibrium, we chose to focus on optimizing the system so the most economical process settings yielding a high amount of DAG could be determined.

Optimization and Up Scaling of Process. The factors to be optimized were chosen based on experiences both from former work (*21*) and from literature (*11*, *35*). The factor, which influences the overall cost of a given production most, is the amount of enzymes used. Moreover, reaction time and reaction temperature are also production parameters influencing the cost. The water content can be difficult to control in a production and a high water content often results in a high amount of FFA in the final reaction mixture; hence, it is desirable to have it as low as possible. Regarding the substrate molar ratio, low glycerol content is preferred due to the high viscosity of the component. Appropriate levels of the five factors were identified (**Table 2**).

Model Evaluation. The best-fitting model was determined by multiple regressions with backward elimination, whereby insignificant factors and interactions are removed from the model. The model coefficients and *P* values for the response value (DAG yield) are given in **Table 4**. All *P* values were less than 0.01. The model evaluation was very satisfactory having coefficients of determination $R^2 = 0.993$ and $Q^2 = 0.950$ and the ANOVA showing no lack of fit for the model. This evaluation showed that the model was acceptable and could be used for optimizing the glycerolysis system with respect to DAG yield. The predicted results being very close to the observed with a maximum error of 3.64 (**Table 2**) also confirmed the applicability of the model.

Main Effects of Parameters. The optimization results were well-evaluated from the main effects plot where the major influence of each parameter on DAG yield is shown (**Figure 1**). The only single factor having a negative effect on DAG yield was water content; hence, it is not necessary to add extra water to the reaction mixture to ensure lipase activity. The macropores acrylic carrier used for Novozym 435 contained 1−2 wt % water. This amount of water appears to be enough to achieve catalytic active lipases.

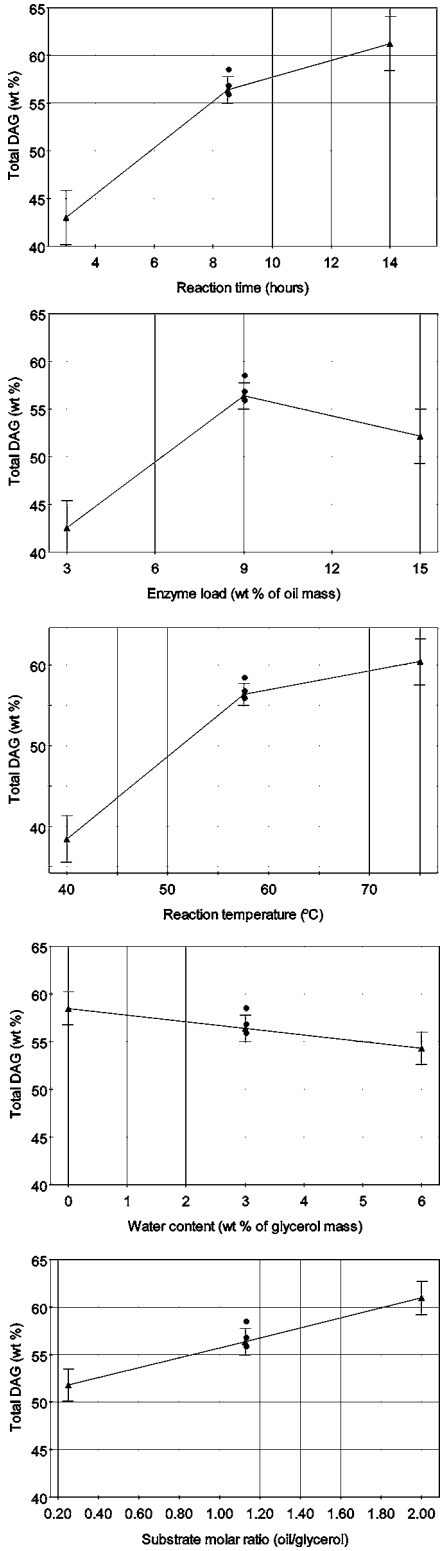

Figure 1. Main effects of factors on total DAG yield with 95% confidence intervals. The effect of each factor when it is varied from a low to a high level and all other factors that are kept at their averages are displayed.

The parameter, which influenced the DAG yield most, was reaction temperature. The optimal temperature for maximum activity of Novozym 435 is 70−80 °C although it is recom-

mended by the producer to use temperatures in the range of 40−60 °C for optimum productivity due to thermal inactivation. The model showed that a temperature of 75 °C gave the highest DAG yield, which was in correspondence with the optimum activity of Novozym 435. The effect of temperature on DAG yield was however not linear. There was a large increase in DAG yield from 40 to 57.5 °C whereas there was only a slight increase from 57.5 to 75 °C of approximately 4 wt % DAG.

The reaction time also had a positive nonlinear effect on DAG yield with a large increase from 3 to 8.5 h and a smaller increase in DAG yield (5 wt % DAG) from 8.5 to 14 h.

In this work, the levels examined for substrate molar ratio were chosen to be from a molar ratio of 0.25 (oil/glycerol) where glycerol would be in molar excess to the stoichiometric molar ratio of 2. The molar stoichiometry of DAG synthesis by glycerolysis is shown in eq 1.

$$2\ \text{TAG} + 1\ \text{glycerol} \rightarrow 3\ \text{DAG} \qquad (1)$$

The effect of substrate molar ratio on DAG yield was positive, which suggests that excess glycerol was not favorable. In previous reports using an immobilized *Candida antarctica* lipase B (Novozym 435 and Chirazym L-2) for glycerolysis, it was found that a large excess of glycerol did not alter the glycerolysis acylglycerol composition (*19*, *20*). These reports, however, were not aiming for DAG but for the mixture of MAG and DAG or for high MAG yield, respectively, and the substrate molar ratios examined were in the range of 0.17−0.55 (oil/glycerol). The present work reveals that even lower glycerol content than previous examined can be applied when aiming for DAG synthesis. The possibility to use a low amount of glycerol, only approximately 5 wt % of the oil mass (equals a molar ratio of 2), is very beneficial for a large-scale production, since potential problems caused by the high viscosity of glycerol can be avoided.

The effect of enzyme load on DAG yield increased from low to center level and then decreased to high level of enzyme load. The decrease in DAG yield when large amounts of lipases are employed can be explained by a poorer mixing of the reaction and thereby occurrence of mass transfer limitations. This result is in accordance with previous reports showing that an increase in lipase load above a certain amount (approximately 10 wt %) did not increase the yield but might increase the reaction rate (*11*, *15*, *19*). Although these reports used different lipases and strategies, all reactions were solvent-free. Solvent-free systems are preferred when products are aimed for the food industry. To achieve proper mixing and hereby a satisfactory mass transfer is of major importance to gain high yields in these often highly viscous reaction systems. Therefore, it is economically advantageous to determine the optimal amount of immobilized lipases needed to achieve the highest yield.

Interaction between Parameters. The largest parameter interaction was observed from the regression coefficients (**Table 4**) to be the positive interaction between enzyme load and substrate ratio. To evaluate the interaction between enzyme load and substrate ratio, contour plots were constructed for high and center levels of temperature and reaction time, since these factors had the most significant effects on DAG yield (**Figure 2**). From the contour plots, it is observed that the optimal amount of lipases was approximately 12 wt % for all combinations of center and high reaction temperature and time. Moreover, the stoichiometric molar ratio of 2 (oil/glycerol) for DAG synthesis was favored as also revealed by the main effects plot. However, if a low amount of enzyme is used, the contour plots suggest that excess glycerol and a high temperature are advantageous.

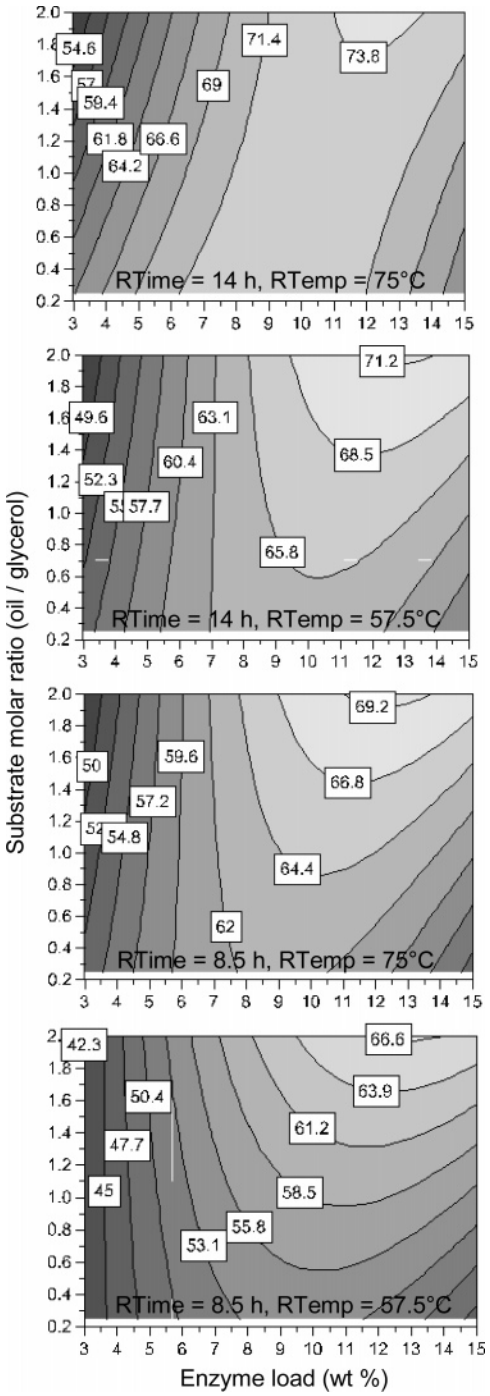

Figure 2. Contour plots of the interaction of EnzL and SubR for the combinations of high and center levels of R_{time} and R_{temp} all with WatC = 0. The contour levels equal the predicted total DAG yield at the given settings.

The high temperature lowers the viscosity and increases the solubility of glycerol in the oil phase, which is beneficial especially when added in higher amounts. Another observation was that if a shorter reaction time was more important than to lower lipase load the model suggests that a high substrate ratio and high amount of lipases were applied. A set of approximate optimal reaction conditions can be predicted from the contour plots in **Figure 2**. We have chosen the following conditions to be optimal: R_{time} = 14 h, EnzL = 10 wt %, R_{temp} = 75 °C, WatC = 0 wt %, and SubR = 2. The reason for the enzyme load only being 10 wt % and not 12 wt % (optimal from **Figure 2**) is due to cost considerations.

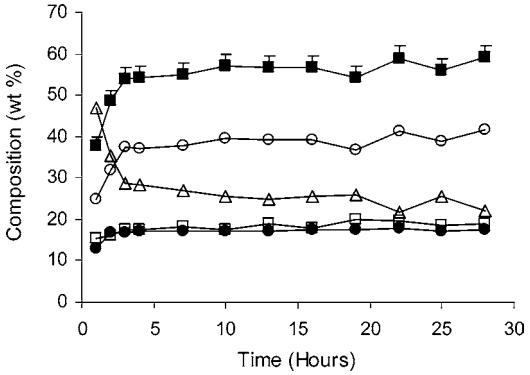

Figure 3. Time course for the predicted optimal reaction. △, TAG; ○, 1,3-DAG; ●, 1,2-DAG; □, MAG; and ■, total DAG. For total DAG, the error is approximately 5% of the measured value.

Table 5. Pilot Plant Productions and the Total DAG Yield from Each Production

batch no.	oil	oil mass (kg)[a]	DAG yield (wt %)
1	sunflower	15	60
2	sunflower	17	58
3	sunflower	17	54
4	rapeseed	20	50
5	rapeseed	20	50

The lipases have been filtered and reused for all productions. Production conditions were as follows: $R_{time} \approx 18$ h, EnzL ≈ 7 wt %, $R_{temp} = 65$ °C, WatC = 0%, and SubR = 0.9. [a] Mass of oil used in the reaction. Abbreviations: R_{time} = reaction time (h), EnzL = enzyme load (wt % of oil mass), R_{temp} = reaction temperature (°C), WatC = water content (wt % of glycerol mass), and SubR = substrate molar ratio (oil/glycerol).

Model Verification and Process Scale-up. The model predicted a DAG yield of approximately 72 wt % for the optimal conditions chosen above. To verify the model, the optimal reaction was run and samples were collected in intervals from 1 to 28 h to construct a time course. **Figure 3** shows that the yield never increases to above 60 wt % during 28 h of reaction. On this background, it can be concluded that a reaction time of approximately 4−5 h is optimal since DAG yield does not increase significantly thereafter due to the obtained equilibrium. The inability to obtain the predicted 72 wt % DAG yield is ascribed to the lack of the model to take a molecular equilibrium into account. However, the model provides good insight in how process settings can be chosen to, e.g., minimize production costs. For example, regarding enzyme load, it can be seen from **Figure 2** that 60 wt % DAG should be achievable with only 5 wt % lipases (instead of 10 wt % as used in **Figure 3**) when all other factors are at the optimal level.

Five pilot plant batch productions were run both to test if direct up scaling was possible and to examine the potential for purification of DAG oil by means of batch deodorization and SPD. **Table 5** reveals that the up scaling was practically possible with the five batches yielding DAG in the rage of 50−60 wt %. The lipases were reused in the five pilot plant batch reactions. They were filtered from the reaction mixture and kept in some of the remaining glycerol in the refrigerator until the next batch. It is known that lipases lose some activity especially in batch reactions where mechanic stirring is applied. From **Table 5**, a decrease of 10 wt % DAG is observed from the first to the last pilot plant production. The mechanic stirring might have crushed some of the immobilized lipase particles, which explains the lower yield. Another possibility is loss of lipases during filtration, since some trouble caused by the excess glycerol in the reaction mixture was experienced. Extra 4% lipases were added to each production to compensate for the given loss, but this may not have been enough. To hinder or minimize these troubles in the future, less glycerol (molar ratio of 2, oil/glycerol) will be recommended for glycerolysis. The reason to use more glycerol than optimal predicted was that the contour plots in **Figure 2** show that a higher glycerol content is favored when using a lower lipase load (7 wt %).

Table 6. Purified Yield for the Two Different DAG Oils and Characteristics of the Two DAG Oils and the Oils that They Were Made from, Rapeseed Oil and Sunflower Oil

	rapeseed oil	DAG oil (rapeseed)	sunflower oil	DAG oil (sunflower)
amount of purified oil (kg)		≈20		≈21
FFA (%)[a]	0.04	0.05	0.04	0.05
α-TE (mg/kg)[b]	419	331	606	526
acylglycerol composition (wt %)				
TAG	100	ND	100	ND
1,3-DAG	ND[c]	58	ND	57
1,2-DAG	ND	35	ND	35
MAG	ND	7	ND	8
FA composition (wt % FAME)				
16:0	4.8	5.1	5.9	6.0
18:0	1.7	2.0	3.9	3.9
18:1 n-9	57.1	54.7	25.6	25.8
18:1 n-7	3.2	3.1	0.9	0.9
18:2 n-6	21.1	24.1	61.3	61.0
18:3 n-3	9.1	8.4	0.9	0.9
20:0	0.5	0.5	0.3	0.3
20:1 n-9	1.2	1.2	0.3	0.3
22:0	0.4	0.4	0.7	0.6
other	0.9	0.5	0.2	0.3

[a] $n = 2$. [b] α-TE = α-tocopherol equivalents, $n = 2$. [c] ND = not detected.

The overall conclusion regarding the optimal conditions when taking the results from **Figures 2** and **3** and **Table 5** into consideration is the following: No extra water is needed in the reaction; only a low content of glycerol is necessary (molar ratio of 2), an approximate temperature of 60−65 °C is fine, only 4−5 h of reaction time is needed, and finally 5 wt % lipases is enough to achieve an acceptable DAG yield. All in all, these conditions are satisfactory because they enable easy and low cost batch production of DAG. Moreover, it is possible to scale-up the glycerolysis process to a 20 kg production in a pilot plant batch reactor and achieve a similar DAG yield as in lab scale. However, the reusability of the lipases needs further examination to minimize loss of catalytic activity.

Product Purification and Characterization. Two DAG oils were purified, DAG oil produced from rapeseed oil [DAG oil (rapeseed)] and from sunflower oil [DAG oil (sunflower)]. Purification of the two DAG oils was carried out in several steps. First, excess glycerol and some FFA were removed by batch deodorization, which accounted for less than 1 wt %. Hereafter, the products were further purified in three steps using SPD (**Table 3**). The loss of DAG oil from reaction mixtures during purification was 31 and 7% for DAG oil (sunflower) and DAG oil (rapeseed), respectively. The high loss during purification of DAG oil (sunflower) might be due to some operational problems experienced during the first two SPD steps. Both of the purified DAG oils had a white color, but they were not totally clear at refrigerator temperature (5 °C). The purity of the two purified DAG oils is listed in **Table 6**. The DAG content in the two oil was very satisfactory being 92−93 wt %, and almost no FFAs were detected, which is excellent due to the well-known bad flavor of this component. In previous work where DAG oil from butterfat was purified in a similar manner,

a purity of 86 wt % with a DAG loss during purification of 23% was achieved (*9*). In the present work, both loss and purity of final DAG oils were more acceptable.

The loss of tocopherols during production and purification was 21 and 13% for DAG oil (rapeseed) and DAG oil (sunflower), respectively. This is less than previously found for structured lipids where the same purification method was applied (*26*). The explanation might be that DAG oil was colleted as the distillate in the two last SPD steps, which is contrary to the structured lipids that were collected as the residue. The tocopherols are likely to evaporate under the given purification conditions and will therefore end up in the distillate.

The FA profiles for the final two DAG oils were very similar to the two starting oils, rapeseed and sunflower oil. This indicates that Novozym 435 does not have specificity toward any specific FAs. Moreover, it is an advantage to have similar FA profiles for studies where the DAG oils are to be compared with the oils from which they are made.

ACKNOWLEDGMENT

We thank Nina Kjeldsen, Bert Nielsen, Preben Bøje Hansen, and Malene Leidecker for technical assistance.

LITERATURE CITED

(1) D'Alonzo, R. P.; Kozarek, W. J.; Wade, R. L. Glyceride composition of processed fats and oils as determined by glass capillary gas chromatography. *J. Am. Oil Chem. Soc.* **1982**, *59*, 292−295.

(2) Tada, N. Physiological actions of diacylglycerol outcome. *Curr. Opin. Clin. Nutr. Metab. Care* **2004**, *7*, 145−149.

(3) De Guzman, D. Kao's fat-reducing DAG oil scheduled for US introduction. *Chem. Market Rep.* **2001**, *259*, 8.

(4) Meng, X. H.; Zou, D. Y.; Shi, Z. P.; Duan, Z. Y.; Mao, Z. G. Dietary diacylglycerol prevents high-fat diet-induced lipid accumulation in rat liver and abdominal adipose tissue. *Lipids* **2004**, *39*, 37−41.

(5) Maki, K. C.; Davidson, M. H.; Tsushima, R.; Matsuo, N.; Tokimitsu, I.; Umporowicz, D. M.; Dicklin, M. R.; Foster, G. S.; Ingram, K. A.; Anderson, B. D.; Frost, S. D.; Bell, M. Consumption of diacylglycerol oil as part of a reduced-energy diet enhances loss of body weight and fat in comparison with consumption of a triacylglycerol control oil. *Am. J. Clin. Nutr.* **2002**, *76*, 1230−1236.

(6) Taguchi, H.; Watanabe, H.; Onizawa, K.; Nagao, T.; Gotoh, N.; Yasukawa, T.; Tsushima, R.; Shimasaki, H.; Itakura, H. Double-blind controlled study on the effects of dietary diacylglycerol on postprandial serum and chylomicron triacylglycerol responses in healthy humans. *J. Am. Coll. Nutr.* **2000**, *19*, 789−796.

(7) Murase, T.; Aoki, M.; Wakisaka, T.; Hase, T.; Tokimitsu, I. Anti-obesity effect of dietary diacylglycerol in C57BL/6J mice: Dietary diacylglycerol stimulates intestinal lipid metabolism. *J. Lipid Res.* **2002**, *43*, 1312−1319.

(8) Taguchi, H.; Nagao, T.; Watanabe, H.; Onizawa, K.; Matsuo, N.; Tokimitsu, I.; Itakura, H. Energy value and digestibility of dietary oil containing mainly 1,3-diacylglycerol are similar to those of triacylglycerol. *Lipids* **2001**, *36*, 379−382.

(9) Yang, T.; Zhang, H.; Mu, H.; Sinclair, A. J.; Xu, X. Diacylglycerols from butterfat: Production by glycerolysis and short-path distillation and analysis of physical properties. *J. Am. Oil Chem. Soc.* **2004**, *81*, 979−987.

(10) Sonntag, N. O. V. Glycerolysis of fats and methyl esters—Status, review and critique. *J. Am. Oil Chem. Soc.* **1982**, *59*, 795A−802A.

(11) Noureddini, H.; Harmeier, S. E. Enzymatic glycerolysis of soybean oil. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1359−1365.

(12) McNeil, G. P.; Yamane, T. Further improvement in the yield of monoglycerides during enzymatic glycerolysis of fats and oils. *J. Am. Oil Chem. Soc.* **1991**, *68*, 6−10.

(13) Watanabe, Y.; Shimada, Y.; Yamauchi-Sato, Y.; Kasai, M.; Yamamoto, T.; Tsutsumi, K.; Tominaga, Y.; Sugihara, A. Synthesis of MAG of CLA with *Penicillium camembertii* lipase. *J. Am. Oil Chem. Soc.* **2002**, *79*, 891−896.

(14) Berger, M.; Laumen, K.; Schneider, M. P. Enzymatic esterification of glycerol I. Lipase-catalyzed synthesis of regioisomerically pure 1,3-sn-diacylglycerols. *J. Am. Oil Chem. Soc.* **1992**, *69*, 955−960.

(15) Watanabe, T.; Shimizu, M.; Sugiura, M.; Sato, M.; Kohori, J.; Yamada, N.; Nakanishi, K. Optimization of reaction conditions for the production of DAG using immobilized 1,3-regiospecific lipase Lipozyme RM IM. *J. Am. Oil Chem. Soc.* **2003**, *80*, 1201−1207.

(16) Lo, S. K.; Baharin, B. S.; Tan, C. P.; Lai, O. M. Lipase-catalysed production and chemical composition of diacylglycerols from soybean oil deodoriser distillate. *Eur. J. Lipid Sci. Technol.* **2004**, *106*, 218−224.

(17) Watanabe, T.; Sugiura, M.; Sato, M.; Yamada, N.; Nakanishi, K. Diacylglycerol production in a packed bed bioreactor. *Process Biochem.* **2005**, *40*, 637−643.

(18) Liao, H.-F.; Tsai, W.-C.; Chang, S.-W.; Shieh, C.-J. Application of solvent engineering to optimize lipase-catalyzed 1,3-diacylglycerols by mixture response surface methodology. *Biotechnol. Lett.* **2003**, *25*, 1857−1861.

(19) Torres, C.; Lin, B.; Hill, C. G., Jr. Lipase-catalyzed glycerolysis of an oil rich in eicosapentaenoic acid residues. *Biotechnol. Lett.* **2002**, *24*, 667−673.

(20) Coteron, A.; Martinez, M.; Aracil, J. Reactions of olive oil and glycerol over immobilized lipases. *J. Am. Oil Chem. Soc.* **1998**, *75*, 657−660.

(21) Kristensen, J. B.; Xu, X.; Mu, H. Diacylglycerol synthesis by enzymatic glycerolysis: Screening of commercial available lipases. *J. Am. Oil Chem. Soc.* **2005**, *82*, 329−334.

(22) Weber, N.; Mukherjee, K. D. Solvent-free lipase-catalyzed preparation of diacylglycerols. *J. Agric. Food Chem.* **2004**, *52*, 5347−5353.

(23) Sugiura, M.; Shimizu, M.; Yamada, N.; Yamada, Y. Process for producing diglycerides. KAO CORP (JP) Ed. Patent WO9909119, 1999.

(24) Xu, X. Response surface methodology for experimental optimization. *Lipid Technol.* **2002**, 17−19.

(25) Montgomery, D. C. Response surface methods and other approaches to process optimization. In *Design and Analysis of Experiments*; Robey, C., Ed.; John Wiley and Sons: New York, 1997.

(26) Xu, X.; Jacobsen, C.; Nielsen, N. S.; Heinrich, M. T.; Zhou, D. Purification and deodorization of structured lipids by short path distillation. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 745−755.

(27) Mu, H.; Kalo, P.; Xu, X.; Høy, C.-E. Chromatographic methods in the monitoring of lipase-catalyzed interesterification. *Eur. J. Lipid Sci. Technol.* **2000**, *102*, 202−211.

(28) Official Method Ca 5a-40 Free Fatty Acids. In *AOCS Official Methods and Recommanded Practices of the American Oil Chemists' Society*; AOCS Press: Champaign, IL, 1998.

(29) AOCS Official Method Ce 8-89 Determination of tocopherols and tocotrienols in vegetable oils and fats by HPLC. In *Official Methods and Recommanded Practices of the American Oil Chemists' Society*; AOCS Press: Champaign, IL, 1998.

(30) Padley, F. B.; Gunstone, F. D.; Harwood, J. L. Occurrence and characteristics of oils and fats. In *The Lipid Handbook*; Gunstone, F. D., Harwood, J. L., Padley, F. P., Eds.; University Press: Cambridge, United Kingdom, 1994.

(31) Yamane, T.; Kang, S. T.; Kawahara, K.; Koizumi, Y. High-yield diacylglycerol formation by solid-phase enzymatic glycerolysis of hydrogenated beef tallow. *J. Am. Oil Chem. Soc.* **1994**, *71*, 339−342.

(32) McNeil, G. P.; Shimizu, S.; Yamane, T. High-yield enzymatic glycerolysis of fats and oils. *J. Am. Oil Chem. Soc*. **1991**, *68*, 1−5.

(33) Rosu, R.; Uozaki, Y.; Iwasaki, Y.; Yamane, T. Repeated use of immobilized lipase for monoacylglycerol production by solid-phase glycerolysis of olive oil. *J. Am. Oil Chem. Soc.* **2004**, *74*, 445−450.

(34) Arcos, J. A.; Otero, C. Enzyme, medium, and reaction engineering to design a low-cost, selective production method for mono- and dioleoylglycerols. *J. Am. Oil Chem. Soc.* **1996**, *73*, 673−682.

(35) Ferreira-Dias, S.; Correia, A. C.; Baptista, F. O.; da Fonseca, M. M. R. Contribution of response surface design to the development of glycerolysis systems catalyzed by commercial immobilized lipases. *J. Mol. Catal. B: Enzym.* **2001**, *11*, 699−711.

Received for review April 6, 2005. Revised manuscript received June 14, 2005. Accepted June 15, 2005. The Danish Dairy Research Foundation and the Directorate for Food, Fisheries, and Agri Business in Denmark are thanked for financial support.

JF0507745