Lipase-Catalyzed Acidolysis of Menhaden Oil with CLA: Optimization by Factorial Design

Carlos F. Torres[a], Ernesto Barrios[b], and Charles G. Hill, Jr.[a,*]

[a]Department of Chemical Engineering, University of Wisconsin–Madison, Madison, Wisconsin 53706, and [b]Center for Quality and Productivity Improvement, University of Wisconsin–Madison, Madison, Wisconsin 53705

ABSTRACT: Menhaden oil was interesterified with CLA in a packed-bed reactor containing an immobilized lipase from *Mucor miehei* (L9) as the biocatalyst. Process optimization was studied using a sequence of $2^2 \times 3$ factorial designs involving the mole ratio of reactants, the reaction temperature, and the space-time of the reactor as experimental parameters.Three different responses—percentage of incorporation of CLA, level of n-3 residues remaining, and conversion of CLA—were considered as objective functions. The parameters studied showed opposite effects for incorporation of CLA and the retention level of n-3 residues. A desirability function was constructed to describe a desirable balance of the conflicting response variables. Optimal conditions correspond to a molar ratio of CLA to fish oil of 0.8 to 1, a temperature of 60°C, and a space-time of 5 h.

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KEY WORDS: Acidolysis, conjugated linoleic acid, desirability function, fish oil, *Mucor miehei* lipase, packed-bed reactor, response surface methodology.

CLA is a FA that has attracted the attention of nutritional experts and the food industry because of its potential for incorporation into foods marketed as nutraceuticals. Nutraceuticals are foods (or parts of foods) that provide therapeutic or preventative medicinal values as well as nutritional benefits. The term CLA refers to a mixture of geometrical and positional isomers of linoleic acid ($C_{18:2}$) containing conjugated double bonds. The richest natural sources of CLA are animal fats, especially milk fat and meat from ruminant animals (1). Commercially available CLA is prepared by chemical isomerization, and analysis of these preparations by silver ion HPLC (2) indicates that there are at least 12 different peaks associated with various isomers of CLA. Anticarcinogenic effects (3), decreased risk of atherosclerosis (4), and many other health benefits (5) have been attributed to consumption of CLA. The *cis*-9,*trans*-11 and the *trans*-10,*cis*-12 isomers of CLA are the isomers that are believed to be responsible for the beneficial physiological effects of this substance.

The n-3 PUFA (sometimes referred to as omega-3 FA) found in fish oils also provide health benefits relative to prevention of cardiovascular disease and certain cancers (6).

*To whom correspondence should be addressed at Department of Chemical Engineering, University of Wisconsin–Madison, 1415 Engineering Dr., Madison, WI 53706. E-mail: hill@engr.wisc.edu

One logical approach to facilitating ingestion of both CLA and n-3 FA is to produce fats and oils enriched in these substances *via* enzymatic processes. These fats and oils can be used to fortify foods sold as nutraceuticals, e.g., dairy spreads, frozen desserts, salad dressings, and so on. Incorporation of CLA in fish oils by enzymatic reactions that selectively replace saturated FA and monounsaturated FA residues while leaving the n-3 FA residues relatively untouched permits the production of TAG offering the benefits of both CLA and n-3 FA.

Several reports of enzymatic interesterification of fish oil are available in the literature (7–9). Most approaches to interesterification reactions employ a large molar excess of FA to maximize the level of acyl incorporation in an acidolysis reaction. However, this approach is inappropriate when a highly valuable FA is employed as one of the two basic feedstocks. When a large molar excess of CLA is utilized, the degree of conversion of CLA from a free form to an esterified form is necessarily very low. In order to make more efficient and more economic use of the costly CLA feedstock in a transesterification (acidolysis) reaction, we have conducted a study to determine the reaction conditions that optimize the lipase-catalyzed acidolysis of fish oil with CLA. In this optimization, we have considered the following response variables: (i) the extent of incorporation of CLA as an acylglycerol residue; (ii) the extent to which the starting acylglycerol retains its original n-3 residues, and (iii) the extent of conversion of CLA.

MATERIALS

An immobilized form of a *Mucor miehei* lipase (L9) was obtained from Boëringer-Mannheim (Mannheim, Germany). The biocatalyst has a particle size in the range of 0.2 to 0.8 mm. CLA ($C_{18:2}$) was kindly provided by Natural ASA (Hovdebygda, Norway). Menhaden oil, type II porcine pancreatic lipase, sodium borate, and bile salts were purchased from Sigma (St. Louis, MO). All solvents used were of HPLC grade from Fisher (Chicago, IL). Analysis in our laboratory indicated that the average M.W. of the menhaden oil was 887, a result consistent with that of Haraldsson and Kristinsson (10).

METHODS

Apparatus. The packed-bed reactor consisted of 20 cm of Tygon® tubing (0.32 cm i.d.) containing 550 mg of immobilized enzyme. The Tygon tubing was packed manually with the dry immobilized enzyme. The packing was then fixed in place using

plugs of glass wool. This tubing was submerged in a constant-temperature water bath. Syringe pumps (model 220; KD Scientific, New Hope, PA) were used to supply the CLA and fish oil individually to a Y-shaped connector located at the entrance to the packed-bed reactor. Prior to initiating flow of the CLA and the fish oil to the reactor, nitrogen was passed through the packed bed for 5 min to remove air. Samples were collected manually at the outlet of the reactor.

Operating protocol. Each experiment was initiated by quickly flushing the reactor with a total volume of the mixture of substrates equal to at least twice the void volume of the reactor. After quasi steady-state conditions were achieved, several samples of the effluent stream were taken over a time frame corresponding to at least three reactor space-times. (Under quasi steady-state conditions, the composition of the effluent changes very slowly with time, reflecting deactivation of the enzyme. If the flow conditions approach plug flow, an elapsed time equal to 1.5 space-times should be sufficient to reach a quasi steady-state condition. The reactor space time is the ratio of the void volume of the reactor to the total volumetric flow rate of the two feedstocks.) The experimental data points correspond to the average of three different measurements of the effluent composition at each space-time of interest. The rate at which the immobilized enzyme lost activity was sufficiently low that changes in specific activity with time on stream did not have a significant effect on the average values of the effluent composition.

Determination of the void volume. The void volume (1.90 cm^3/g of catalyst) was calculated using the difference between the weight of the packed-bed reactor (tubing + catalyst), when filled with a FA of known density, and the corresponding weight of the packed-bed reactor in the absence of this fluid. Corrections were made for the regions of the tubing outside the packed bed.

Analytical methods. Samples (0.1 mL) of the menhaden oil were mixed with 1,400 μL of a mixture of chloroform and methanol (volumetric ratio = 2 to 1). Aliquots of the resulting transparent solution (400 μL) were methylated by addition of 1 mL of methanolic NaOH (0.1 N). This mixture was then allowed to stand for 30 min at 60°C. Then 200 μL of water was added. The resulting mixture was extracted with two 1-mL portions of *n*-hexane. The pooled extracts were dried with sodium sulfate and then centrifuged for 2 min at $5{,}035 \times g$. One microliter of sample was injected into a Hewlett-Packard (Avondale, PA) gas chromatograph (Model 5890, Series II) fitted with a 60-m HP Supelcowax 10 column (0.32 mm i.d.). Injector and detector temperatures were 220 and 230°C, respectively. The temperature program was as follows: starting at 100°C and then heating to 180°C at 20°C/min followed by heating from 180 to 220°C at 15°C/min. The final temperature (220°C) was held for 30 min. The carrier gas was helium, and the split ratio employed was 20 to 1. Identification of the various FAME was based on a menhaden oil standard (4-7085) obtained from Supelco (Bellefonte, PA).

Purification of TAG from the reaction mixture. Separation and recovery of the TAG were accomplished *via* solid-phase extraction on silica gel columns (10 g, grade 60, 70–230 mesh) (Aldrich, Milwaukee, WI) (11). The columns were conditioned by washing with 25 mL hexane, taking care to prevent them from becoming dry. The sample (10 mL containing TAG at a concentration of *ca.* 100 mg/mL as determined by stoichiometric calculations) was applied to a column and then eluted under vacuum (5 mm Hg) with solvent mixtures of increasing polarity: first, 30 mL hexane/diethyl ether (200:3, vol/vol), and second, 100 mL hexane/diethyl ether (96:4, vol/vol). The eluate from the second elution (containing the TAG) was collected and evaporated for subsequent analysis of the distribution of FA residues along the glycerol backbone.

Positional distribution of FA residues in TAG. Modified versions of the methods of Luddy *et al.* (12) and Williams *et al.* (13) were employed to release FA from the *sn*-1,3 positions of acylglycerols. A known weight of TAG and an appropriate (*ca.* 20–50 mg) weight of porcine pancreatic lipase were added to a 60-mL stoppered flask. Next, 0.65 mL Tris buffer (1 M, sodium salt, pH 8.0), 0.35 mL sodium borate (0.19 M), 0.1 mL $CaCl_2$ (22%, w/w), and 0.25 mL bile salts (0.1%, w/w) were added. The resulting mixture (pH = 7.91) was maintained at 40°C for 1 min without shaking and then shaken at 300 rpm at 40°C for 7 min. The reaction was stopped by addition of 1 mL acetic acid (0.1 M). The mixture was extracted three times with 1 mL of chloroform/methanol (2:1, vol/vol). The pooled organic phases were passed through a 0.45-μm syringe filter and then methylated with 0.1 M methanolic NaOH as described in the Analytical Methods section. This protocol provides information concerning the distribution of FA residues at the s*n*-2 position. The distribution of FA residues at the *sn*-1,3 positions was then calculated by subtracting the amount of FA residue at the *sn*-2 position from the total quantity of this FA present in the corresponding unhydrolyzed acylglycerols as determined by GC.

Statistical experimental design. A sequence of factorial experiments was planned for this study. The advantages of a two-level factorial design are well known (14). Hence, two levels were employed for the molar ratio of reactants and the temperature. However, because of the expected nonlinear dependence of the three response variables (extents of incorporation of CLA, retention of n-3 residues, and conversion of CLA) on the reactor space-time, three levels were employed for this independent variable. Consequently, $2^2 \times 3$ factorial designs were carried out in a fully randomized order. In addition, for each of the three space-times of interest, runs at the center of the molar ratio × temperature combination were performed at the beginning or end of the experiment in order to check for the reproducibility of the experiments and to assess the curvature of the three responses. Response surface methodology (15) was employed to ascertain how to improve the process. The entries in Tables 1–4 define the experimental design: the order for each experiment, the parameter levels employed, and the corresponding responses. R language software was used in the analysis of the data and preparation of the associated plots (16).

RESULTS AND DISCUSSION

Preliminary experiments (data not shown), as well as theoretical considerations, indicated a nonlinear dependence (curva-

TABLE 1
Experimental Design 1: Parameter Settings and Responses[a]

Run		Factor settings			Coded factor settings				Responses			
No.	Order	MR	Temp	St	*M*	*T*	t_l	t_q	CLA	n-3	Conv	Des
1	15	1	25	1	–1	–1	–1.225	0.707	15.67	25.37	47.01	0.03
2	4	2	25	1	1	–1	–1.225	0.707	18.36	25.39	27.54	0.00
3	7	1	55	1	–1	1	–1.225	0.707	19.65	24.88	58.92	0.41
4	10	2	55	1	1	1	–1.225	0.707	28.99	20.98	43.48	0.26
5	13	1	25	3	–1	–1	0.000	–1.414	18.15	24.44	54.45	0.32
6	5	2	25	3	1	–1	0.000	–1.414	26.84	23.26	40.26	0.27
7	8	1	55	3	–1	1	0.000	–1.414	20.91	23.39	62.7	0.44
8	11	2	55	3	1	1	0.000	–1.414	31.13	20.41	46.69	0.27
9	14	1	25	5	–1	–1	1.225	0.707	20.63	23.5	61.86	0.43
10	6	2	25	5	1	–1	1.225	0.707	30.83	22.45	46.24	0.33
11	9	1	55	5	–1	1	1.225	0.707	23.32	23.21	69.93	0.54
12	12	2	55	5	1	1	1.225	0.707	34.02	19.78	51.03	0.10
13	2	1.5	40	1	0	0	–1.225	0.707	23.28	24.42	46.54	0.33
14	3	1.5	40	3	0	0	0.000	–1.414	24.45	23.51	48.91	0.35
15	1	1.5	40	5	0	0	1.225	0.707	25.63	22.6	51.25	0.36

[a]Factors: MR, mole ratio of CLA to menhaden oil; Temp, temperature (°C), and St (h), space-time. Coded factor settings for the MR, temperature, and the linear and quadratic trends are denoted by *M, T,* t_l, and t_q, respectively. Responses are desirability function (Des), extent of incorporation of CLA (CLA), retention of n-3 residues (n-3), and conversion of CLA (Conv).

TABLE 2
Experimental Design 1: Coefficients of Fitted Models Corresponding to the Coded Factors[a]

Response	Mean	*M*	*T*	t_l	*M*T*	T^*t_l	SD	R^2
CLA	24.04	4.32	2.30	2.67			2.06	.92
n-3	23.09	–1.04	–0.98	–0.78	–0.68		0.44	.96
Conv	50.84	–8.30	4.62	5.32			2.36	.97
Des	0.284	–.078[a]	.052[b]	.070[a]	–.049[b]	–.077[a]	.866	.87

[a]All coefficients have $P < 0.001$, except for those bearing superscripts a and b, for which the P is < 0.025 and < 0.085, respectively. For a description of factors and other abbreviations see Table 1.

TABLE 3
Experimental Design 2: Parameter Settings and Responses[a]

Run		Factor settings			Coded factor settings				Responses			
No.	Order	MR	Temp	St	*M*	*T*	t_l	t_q	CLA	n-3	Conv	Des
1	11	0.8	30	1	–1	–1	–1.225	0.707	15.12	27.74	56.77	0.04
2	14	1.6	30	1	1	–1	–1.225	0.707	19.21	23.5	36.06	0.02
3	6	0.8	60	1	–1	1	–1.225	0.707	15.7	26.69	58.93	0.22
4	10	1.6	60	1	1	1	–1.225	0.707	26.38	24.17	49.51	0.30
5	8	0.8	30	3	–1	–1	0	–1.414	17.82	26.02	66.89	0.43
6	4	1.6	30	3	1	–1	0	–1.414	24.01	25.14	45.06	0.26
7	3	0.8	60	3	–1	1	0	–1.414	18.71	25.76	70.23	0.49
8	13	1.6	60	3	1	1	0	–1.414	27.44	24.05	51.5	0.33
9	5	0.8	30	5	–1	–1	1.225	0.707	17.02	26.36	63.87	0.36
10	7	1.6	30	5	1	–1	1.225	0.707	23.43	25.51	43.97	0.25
11	9	0.8	60	5	–1	1	1.225	0.707	20.86	26.27	78.29	0.63
12	12	1.6	60	5	1	1	1.225	0.707	29.87	23.32	56.06	0.23
13	2	1.2	45	1	0	0	1.225	0.707	19.75	26.78	49.42	0.30
14	1	1.2	45	5	0	0	–1.225	0.707	24.26	24.04	60.72	0.41
15	16	1.2	45	1	0	0	1.225	0.707	18.71	26.62	46.83	0.25
16	15	1.2	45	5	0	0	–1.225	0.707	23.71	24.91	59.33	0.42

[a]See Table 1 for a description of factors and abbreviations.

ture) of each of the different response variables on the space-time parameter. These initial studies also showed a high negative correlation between the responses for incorporation of CLA and retention of n-3 residues (–0.86). However, conversion of CLA did not exhibit a significant correlation to either incorporation of CLA or retention of n-3 residues.

To check for the expected curvature, experiments involving at least three different levels of the space-time parameter were necessary. Consequently, a $2^2 \times 3$ factorial design was employed at the beginning of this optimization study. Factor (parameter) settings and observed responses for the first set of experiments are shown in Tables 1 and 2. Responses are also displayed in a

TABLE 4
Experimental Design 2: Coefficients of Fitted Models Corresponding to the Coded Factors[a]

Response	Mean	*M*	*T*	t_l	t_q	M^*t_l	SD	R^2
CLA	21.29	3.76	1.86	1.51			1.64	.92
n-3	25.37	–1.10					0.79	.70
Conv	56.42	–9.40	4.32	4.18			3.82	.93
Des	0.297	–.065[b]	.070[a]	.091	–0.57[b]	–.058[b]	0.880	.88

[a]All coefficients have $P < 0.001$, except for those bearing superscripts a and b, for which the P is < 0.025 and < 0.05, respectively. For a description of factors and abbreviations see Table 1.

design layout arrangement in Figure 1. For purposes of analysis it was convenient to decompose the two DF associated with the space-time factor into constituent linear (t_l) and quadratic (t_q) trends by means of orthogonal polynomials (see Orthogonal Polynominals section, p. 464). The corresponding coded factor levels are also shown in Table 1. Thus, four experimental factor main effects and the corresponding interactions were considered for analysis. Moreover, for purposes of optimization the three different responses (extent of incorporation of CLA, retention of n-3 residues, and conversion of CLA) were combined

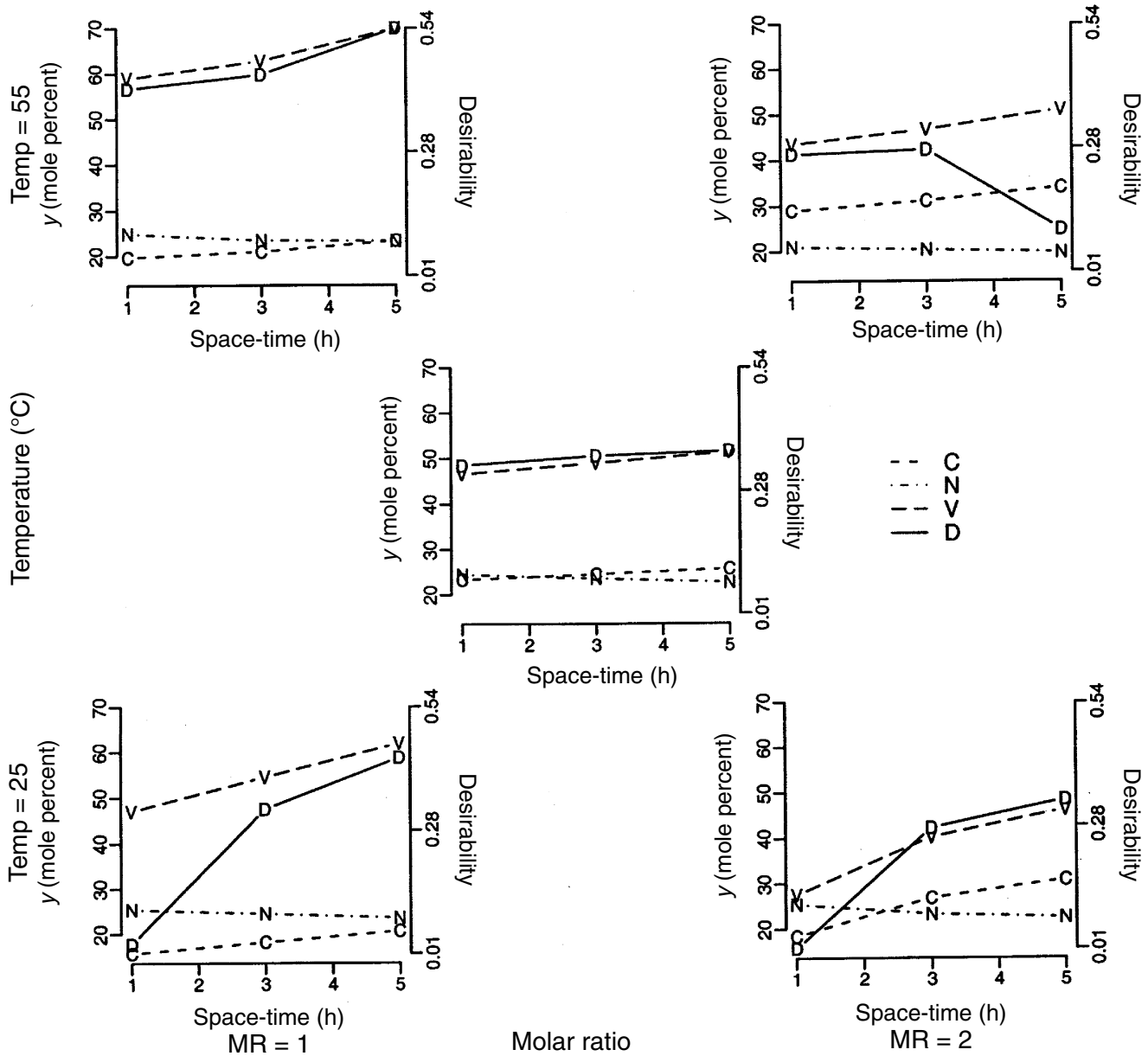

FIG. 1. Experimental design 1: Responses and factor levels. *y* represents the mole percentage of one of these optimization responses depending on the plot in question: C = extent of incorporation of CLA, N = retention of n-3 residues, and V = conversion of CLA. D = desirability function.

into a desirability function according to Derringer and Suich (17). Each response is first transformed to a desirability value, $d = [(y - y_{min})/(y_{max} - y_{min})]^{r}$, and the geometric mean of the three desirability values is employed for simultaneous optimization (Eq. 1),

$$\text{Des} = (d_1\, d_2\, d_3)^{1/3} \qquad [1]$$

The coefficients employed for the construction of the desirability function (Des) are shown in the Desirability Functions section (p. 465).

Normal plots of the effects are shown in Figure 2 for the three responses (14). Examination of the normal plots indicates that in all cases the extent of incorporation of CLA and the degree of conversion of CLA have opposite signs. This result implies that greater incorporation of CLA is obtained when using a high mole ratio of CLA to fish oil but that use of such ratios involves a concomitant lower level of conversion of CLA. Because of the interaction between the mole ratio of CLA to fish oil and the temperature, when the operating temperature increases from 25 to 55°C, a greater decrease in the level of n-3 residues can be observed at a mole ratio of CLA to fish oil of 2 than at a mole ratio of 1. Factor effects that are of opposite signs for different response variables imply that moving the factor setting in a direction that increases one response variable will lead to a decrease in the other response variable. To achieve an appropriate balance between conflicting responses, the use of a desirability function is in order.

In the first experimental design, the highest value of the desirability function employed corresponds to process conditions in which the mole ratio of CLA to menhaden oil is 1, the temperature is 55°C, and the space-time is 5 h. In the present situation the desirability function has no direct physical interpretation and may be regarded as a somewhat arbitrary, but nonetheless useful, construct that seeks a meaningful com-

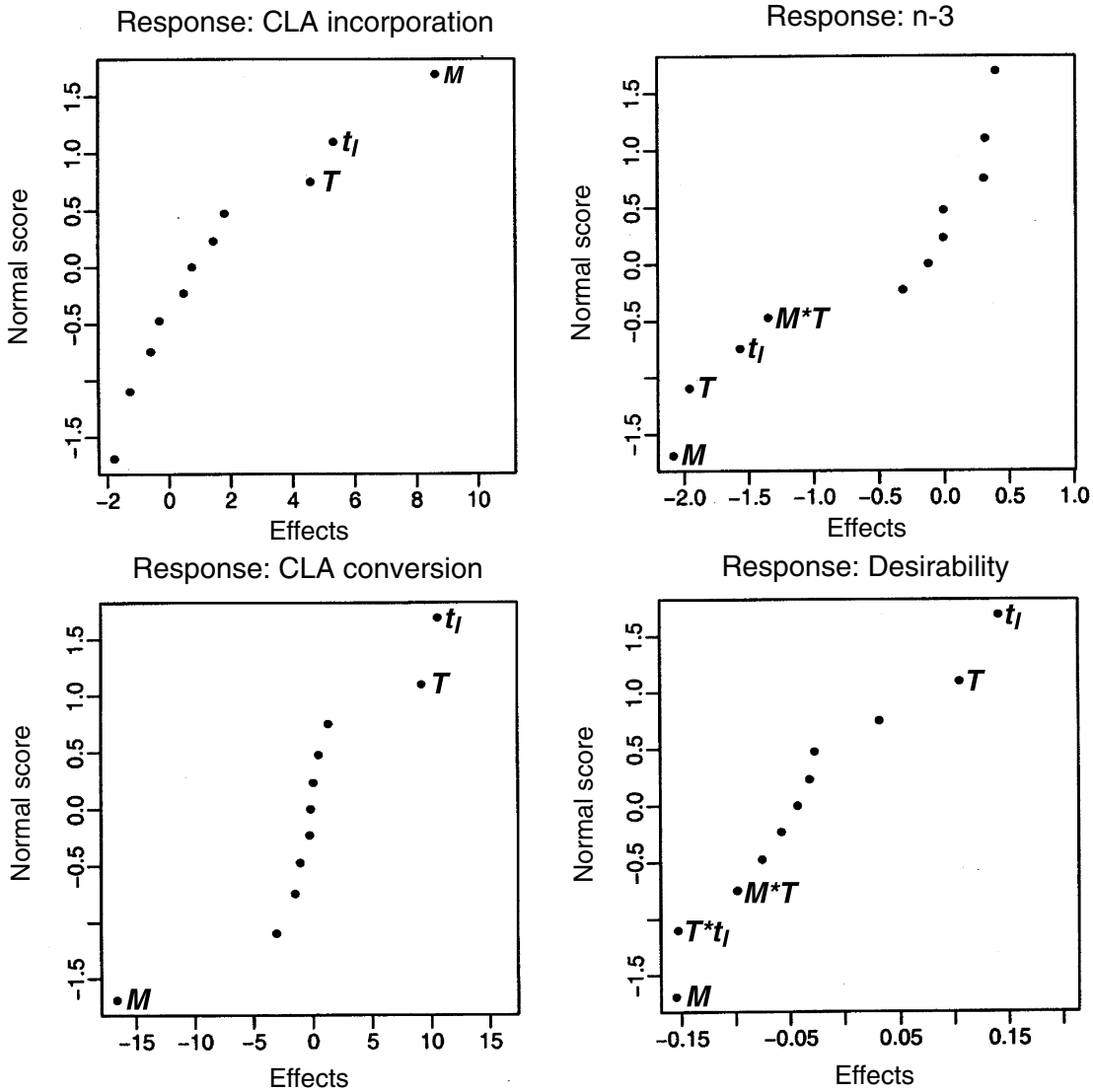

FIG. 2. Normal plots of effects for experimental design 1. M^*T and T^*t_l denote interactions for the mole ratio and temperature, and the temperature and the linear time trend, respectively.

promise among the various objective functions. The desirability function provides an overall measure of the combined responses and is used to simultaneously adjust all three responses into regions in which the overall results are improved. In this first stage of experimentation, the best response of the desirability function corresponds to a mole ratio of CLA to menhaden oil of 1 and an operating temperature of 55°C. This fact is apparent from inspection of Figure 1, in which the upper left-hand plot exhibits consistently higher levels of the desirability function than the other experimental conditions.

Empirical linear models were built for each response. The estimated coefficients are shown in Table 2. For example, for the first experimental design the fitted model for the extent of incorporation of CLA was

$$\text{CLA} = 24.04 + 4.32 \times M + 2.30 \times T + 2.67 \times t_l \quad [2]$$

with an estimated SD = 2.06 and a multiple correlation coefficient $R^2 = 0.92$. Similarly, for the desirability function

$$\text{Des} = 0.284 - 0.078 \times M + 0.052 \times T + 0.070 \times t_l - 0.049 \times M \times T - 0.077 \times T \times t_l \quad [3]$$

with SD = 0.866 and $R^2 = 0.87$. Three main effects (mole ratio of CLA to menhaden oil, temperature, and the linear component of the space-time factor) are significant for all the responses at the 99% significance level ($P < 0.01$). For example, the coefficient for the (coded) mole ratio in the model for CLA is 4.32. On average, the CLA concentration then increases by 8.64 units when the coded level of the mole ratio changes from –1 to +1, that is, when the mole ratio of CLA to menhaden oil is 2 rather than 1. Moreover, the interaction between the mole ratio and the temperature is relevant for the retention of n-3 residues ($P < 0.01$). This result implies that when both the temperature and the mole ratio are increased, a higher percentage of n-3 residues is released. That is, on average at 25°C, the effect of changing the mole ratio from 1 to 2 reduces the coded level of n-3 residues remaining by 0.7 units, whereas at 55°C, the corresponding average reduction is 3.4 units.

A second experimental design was employed to improve the global response of the process. Different aspects were considered to determine the optimal parameter values for this experimental design. Again, three levels of the space-time factor were considered to ascertain whether the response exhibits curvature; the same values of the space-time were used in this design, namely, 1, 3, and 5 h. Thus, only the mole ratio and temperature were considered in the determination of the steepest ascent path (15). This approach was selected on the basis of the response of the desirability function in the first experimental design. The settings calculated using the steepest ascent path were 1.25 for the mole ratio of CLA to fish oil and 45.18°C for the temperature. For practical reasons, we chose to employ a mole ratio of 1.2 and a temperature of 45°C as the center of the second experimental design. Factor settings and the responses observed for this second $2^2 \times 3$ factorial design are summarized in Tables 3 and 4 and displayed in Figure 3. Experiments corresponding to additional center points for estimation of the experimental error, reproducibility, and checking for curvature were conducted at the beginning and at the end of the series of experiments constituting the experimental design.

Analysis of the data for this second design again led to a negative correlation between the responses for the extent of incorporation of CLA and the extent of retention of n-3 residues (80%). The response indicating the extent of conversion of CLA showed the highest correlation with the desirability function (77%) and thus had the greatest influence on the desirability function. Hence, the role assigned to the percentage conversion of CLA in the desirability function is the dominant factor determining the experimental conditions that provide the best response.

The normal plots shown in Figure 4 indicate those factors (effects) likely to have a significant influence on the observed responses. At the 99% confidence level, all three main effects (mole ratio, temperature, and the linear component of the space-time factor) are important for two of the response functions. For the extent of retention of n-3 residues, only the mole ratio of reactants has a significant effect at the 99% confidence level ($P < 0.01$). The parameters for the final fitted model for each response are presented in Table 4.

Analysis of the data for the response corresponding to the extent of retention of n-3 residues indicates that only the mole ratio of CLA to fish oil is a major factor. Interpretation of the model for the retention of n-3 residues indicates that in the region of parameter space corresponding to the second experimental design, the negative effects of temperature and space-time on the retention of n-3 residues have been minimized. The correlation coefficient associated with the working model for this response is smaller ($R^2 = 0.7$), but the error associated with this model is just 1.8 times the experimental error. Hence, the final model can be considered as satisfactory.

Response trends for this second set of experiments (Fig. 4) were similar to those for the first design. In this case, the best global results were obtained at a mole ratio of 0.8, a temperature of 60°C, and a space-time of 5 h. Attempts to obtain further improvements using mole ratios of CLA to fish oil below 0.8 were not considered because of the low levels of incorporation of CLA obtained under these conditions.

Finally, the effect of space-time was studied in more detail at these best factor settings to better define the composition of the effluent stream corresponding to the approach to equilibrium for the various reactions. The space-time course of the acidolysis reaction at 60°C for a mole ratio of CLA to menhaden oil of 0.8 is displayed in Figure 5. To facilitate interpretation of the data, both the extent of incorporation of CLA into fish oil acylglycerols and the total concentration of n-3 FA are plotted. Extents of release of several individual saturated and monounsaturated major FA are also indicated. Visual inspection of Figure 5 indicates that the n-3 FA were virtually unaffected by the acidolysis reaction. Under the experimental conditions employed, only very small quantities of

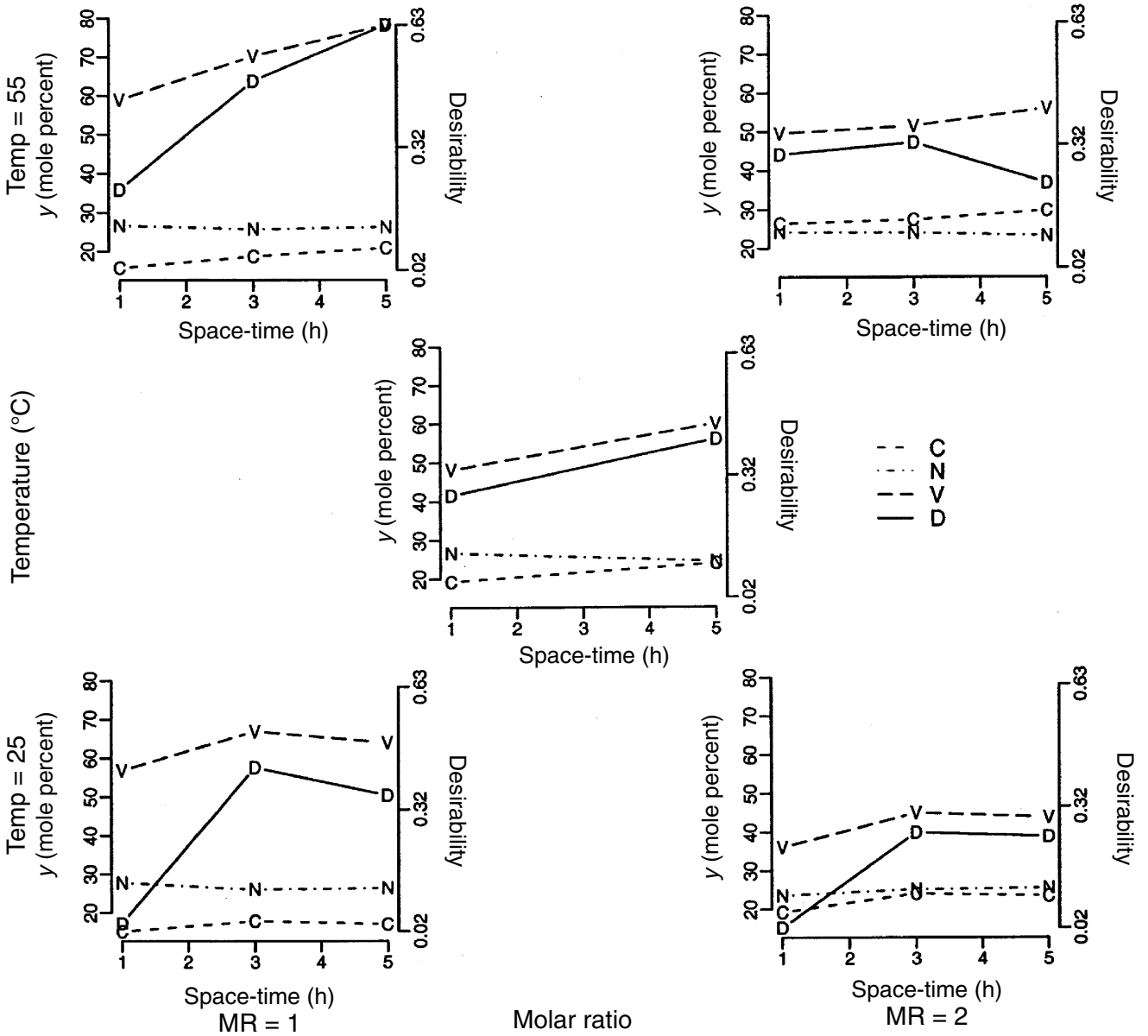

FIG. 3. Experimental design 2: responses and factor levels. *y* represents the mole percentage of one of these optimization responses depending on the plot in question. For abbreviations see Figure 1.

n-3 residues were released. Hence, incorporation of CLA residues into the acylglycerols of menhaden oil occurred primarily at the expense of other less desirable major FA residues, namely, C_{14}, C_{16}, $C_{16:1}$, C_{18}, and $C_{18:1}$. At the longest space-time (5 h), *ca.* 10% of the product acylglycerols were DAG. No MAG were observed in the product mixture. Moreover, the extent of incorporation of CLA obtained at a space-time of 5 h was approximately 21%. This value corresponds to *ca.* 80% consumption of the CLA fed to the reactor. Since the purity of the CLA employed was close to 90%, the results reported here reflect a marked improvement in the efficiency of utilization of CLA compared to the results obtained using a larger molar excess of CLA relative to menhaden oil.

The positional distributions of the various FA residues in the native fish oil and in the interesterified TAG product are presented in Table 5. The relative positions of the n-3 residues change only to a small degree in the interesterified product relative to the original menhaden oil. As expected for an *sn*-1,3 selective enzyme, the losses of n-3 residues occurred mainly at the *sn*-1,3 positions. The relationship between the extent of the retention of n-3 residues at the *sn*-1,3 sites and incorporation of the replacement FA mediated by *Mucor miehei* lipase has been described by Xu *et al.* (9). It should be noted that in the product, the predominant FA at the *sn*-2 position were n-3 residues (36%). It has been reported that FA at the *sn*-2 position are readily absorbed, regardless of the type of FA residue present at this position (18). We also observed migration of some FA residues from *sn*-1,3 to *sn*-2 sites on the glycerol backbone. For example, it should be noted that 18% of the *sn*-2 sites were occupied by CLA

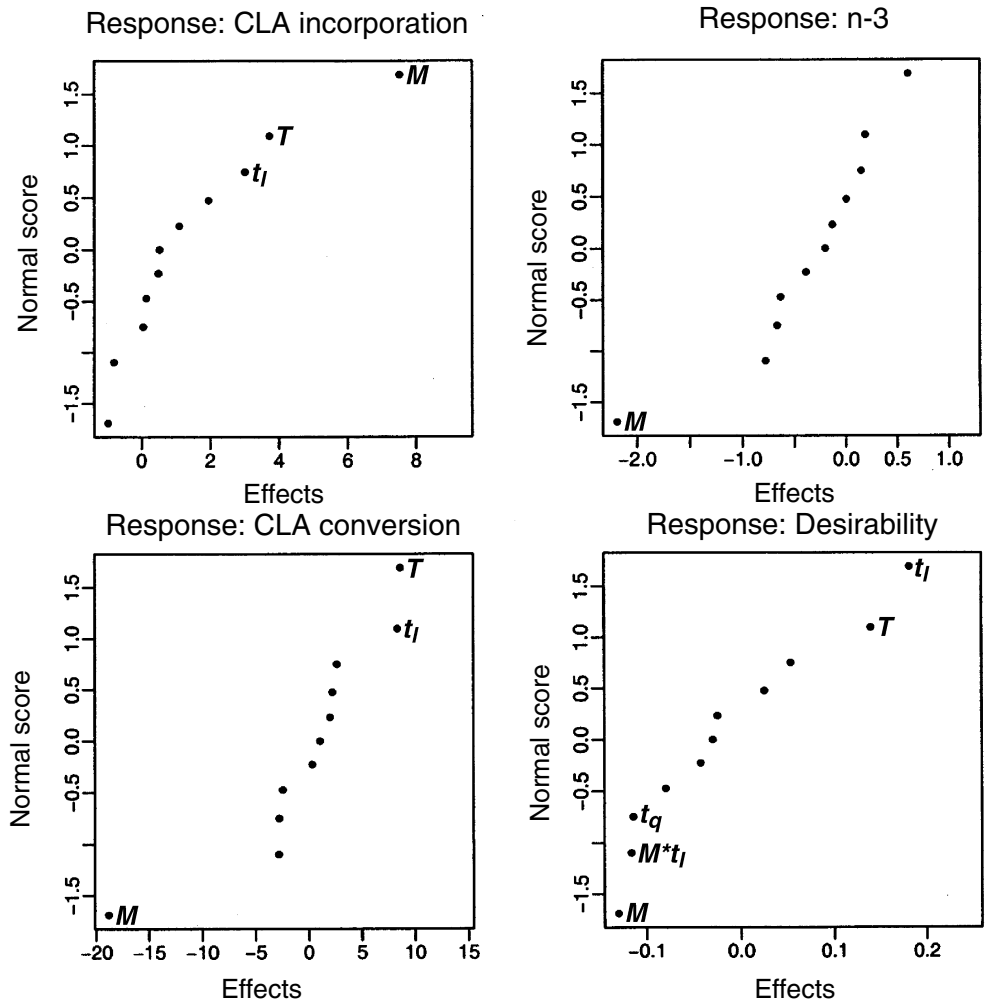

FIG. 4. Normal plots of effects for experiment design 2. M^*t_l denotes the interaction of the mole ratio and the linear trend.

residues. It has previously been reported that reaction time, water content, lipase loading, temperature, acyl donor type, and lipase type can influence acyl migration (19,20) of the indicated type. The results obtained in the present work indicate that both the 1,3-regioselectivity of the lipase employed and the FA selectivity that the *M. miehei* lipase has previously demonstrated relative to DHA (21) play important roles in determining the final distribution of FA residues along the glycerol backbone. Both facts explain the preservation of n-3 residues with regard to their positional distribution and their respective percentage in the final interesterified product.

APPENDICES

(i) Orthogonal polynomials. The experimental designs employed in this study considered orthogonal factors with either two or three levels of these factors. Associated with the space-time factor are three levels and two DF. These DF can be decomposed into linear and quadratic trend effects, each with one DF. This approach is possible with all quantitative factors and with equidistant levels. In the present study, orthogonal polynomials were used to decompose the space-time factor into two factors, linear and quadratic time trends, whose effects are easier to interpret (22). The linear and quadratic components are determined by the following two orthogonal polynomials:

$$t_{linear} = (-1, 0, +1)/\sqrt{2} \quad [4]$$

and

$$t_{quadratic} = (+1, -0, +1)/\sqrt{2} \quad [5]$$

The new trend factors are orthogonal to one another and preserve the orthogonality relations of the space-time to the mole ratio and temperature factors, thereby avoiding confounding the effects of these factors. Further scaling of the trend factors was necessary so that the corresponding effect estimates have the same SE as the other terms considered in the models. The factor decomposition was accomplished by coding the space-time levels as indicated in Table 6. This re-scaling not only provides a fair representation of their effects in the

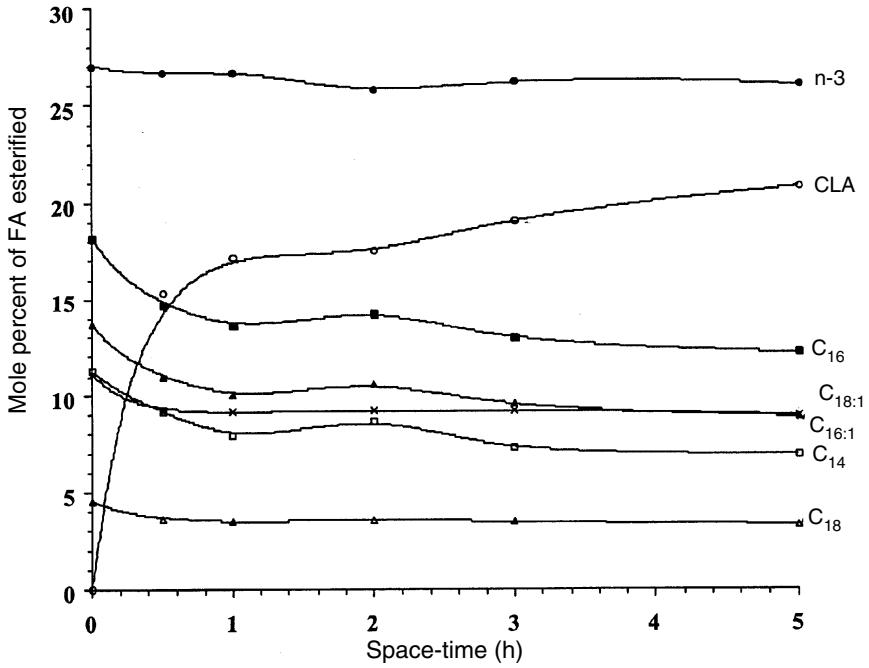

FIG. 5. Dependence of the effluent composition on reactor space-time for the acidolysis of menhaden oil with CLA. Conditions: Mole ratio of CLA to menhaden oil = 0.8:1; 60°C; (□), C_{14}; (■), C_{16}; (▲), $C_{16:1}$; (△), C_{18}; (×), $C_{18:1}$; (●), n-3; and (○), CLA.

normal plots of Figures 2 and 4 but also renders the model coefficients in Tables 2 and 4 comparable.

(ii) Desirability functions. Desirability functions were introduced by Harrington (23) and extended by Derringer and Suich (17) to deal with the need for simultaneous optimization of several responses. If one is concerned with k different response variables, each of the individual responses y_i can be transformed into a corresponding measure of the desirability d_i. The geometric mean can then be used as a joint measure of the desired response. This mean is defined here as the desirability function Des:

$$\text{Des} = (d_1 d_2 d_3 \ldots d_k)^{1/k} \quad [6]$$

This function is assumed to provide an overall assessment of the various responses and is used as the objective function for optimization. There are various types of transformations that may be used for individual responses. In our case, we developed a modification of Derringer and Suich's one-sided transformation. That is, for a response variable, y, the corresponding desirable value, d, is given by

$$d = \left(\frac{y - y_{\min}}{y_{\max} - y_{\min}} \right)^r \quad [7]$$

where $y_{\max}$ and $y_{\min}$ are the maximum and minimum values that one can expect to obtain, Because r is greater than 0, d must lie between zero and one. Desirability functions for different values of r are shown in Figure 6. Note that the exponent r determines the shape and trend of the desirability function. The desirability function, d, is concave, open upward for $r > 1$ and downward for $r < 1$. If $r = 1$, d corresponds to a linear rescaling of y with slope $1/R_y$, where $R_y = y_{\max} - y_{\min}$ is the potential range of the response variable. For $r \neq 1$, the *indifference point*, at which d has the same slope as the curve for $r = 1$, is given by

$$y_0 = y_{\min} + R_y r^{-1/(r-1)} \quad [8]$$

In practice, experience will suggest appropriate values of $y_{\max}$ and $y_{\min}$. However, it will be more difficult to envision practical working values of r. Instead, it would be easier to assign work-

TABLE 5
Positional Distribution of FA Residues in TAG of the Native Menhaden Oil and the Interesterified Product

	Native menhaden oil			Interestified product		
	% of total	% of *sn*-1,3	% of *sn*-2	% of total	% of *sn*-1,3	% of *sn*-2
C_{14}	11.3	11.7	9.9	6.9	9.1	6.0
C_{16}	18.2	19.2	15.1	12.2	14.9	11.3
$C_{16:1}$	13.7	14.2	12.1	8.9	10.9	7.9
C_{18}	4.6	5.0	3.3	3.3	3.4	3.8
$C_{18:1}$	11.1	12.9	5.6	9.0	10.1	7.4
CLA	0.0	0.0	0.0	20.9	22.8	18.0
n-3	27.0	23.7	37.3	26.1	19.7	36.0

TABLE 6
Decomposition of Space-Time Factor into Linear and Quadratic Trends *via* Orthogonal Polynominals

Space-time	Linear trend	Quadratic trend
1 h	–1.225	+0.707
3 h	0.00	–1.414
5 h	+1.225	+0.707

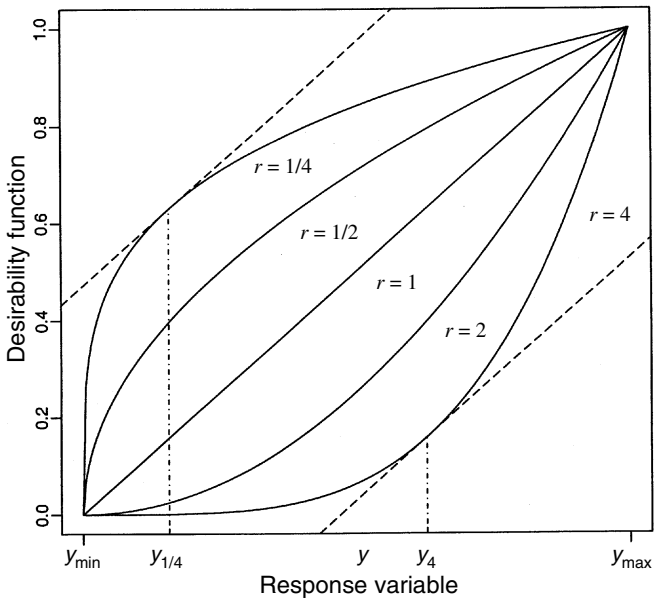

FIG. 6. Desirability transformations for various values of the exponent *r*. Indifference points for $r = 1/4$ and $r = 4$ are indicated by $y_{1/4}$ and y_4, respectively.

ing values to the indifference point, y_0, and to compute the corresponding value of *r* by solving Equation 8. After initial values of the parameters y_{min}, y_{max}, and *r* are established, they can subsequently be fine-tuned to reflect the appropriate desirability function for the optimization task of interest. Parameter values used for each of the transformed responses in the present paper are summarized in Table 7. Finally, the individual desirability values d_i, are combined using Equation 6 to represent the compromises involved in overall optimization of the process. The computed value of the desirability factor for each experimental run is reported in the last column of Tables 1 and 3.

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TABLE 7
Coefficients Used in Equation 7 for Computation of the Desirability Function Des

	Response				
	Original	Transformed	y_{min}	y_{max}	*r*
CLA	y_1	d_1	15.12	26.66 (53.328)[a]	1.1
n-3	y_2	d_2	23.32	28.00	0.3
Conv	y_3	d_3	36.00	100.00	1.1

[a]The value of y_{max} depends on the mole ratio of CLA to menhaden oil employed.
Conv, conversion of CLA.

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