Enzymatic Methylation of Canola Oil Deodorizer Distillate

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Methylation of canola oil deodorizer distillate catalyzed by a nonspecific lipase was investigated. The conversion of fatty acids to methyl esters has been optimized by using a statistical design. Up to 96.5% conversion of fatty acids to their methyl esters has been achieved without the aid of vacuum or any water-removing agent. The effects of temperature, ratio of the reactants (methanol: fatty acids in the deodorizer distillate) and enzyme concentration on the equilibrium conversion were studied. The temperature and ratio of the reactants showed a significant effect on the conversion of fatty acids to methyl esters and they exhibited a strong interactive effect. Enzyme concentration in the range of 2.7% to 4.3% did not show a significant effect on the equilibrium conversion of fatty acids. Greater than 95% conversion of fatty acids to methyl esters was achieved at temperatures around 50°C and at a ratio of the reactants between 1.8 and 2.0. The inhibitory effect of hydrophilic methanol on the enzyme activity was largely reduced by working at the lower temperature range (around 50°C).

KEY WORDS: By-product utilization, canola oil, deodorizer distillate, esterification, fatty acid, lipase, methylation, oil processing, sterols, tocopherols.

Deodorizer distillate is a valuable by-product obtained during the deodorization of vegetable oils. During processing, 0.3-0.5% of the deodorizer feedstock ends up as the distillate. It is a complex mixture of free fatty acids, sterols and their esters, tocopheryl esters, and mono-, di- and triacylglycerols. Tocopherols and sterols find extensive application in cosmetic and pharmaceutical industries, and the deodorizer distillate is the principal raw material source for the manufacture of natural vitamin E. The price of these distillates is based on the tocopherol content, which is normally between 2 to 15% of the distillate (1).

Preparation of high-purity concentrates of sterols and tocopherols involves a series of physical and chemical treatment steps, designed to separate the free fatty acids from the distillate in the initial step, followed by separation of sterols to yield a rich tocopherol concentrate. A number of processes have been established for the manufacture of tocopherols from the deodorizer distillate and some are commercially practiced. Some of these processes involve urea adduct formation, liquid-liquid extraction with polar and nonpolar solvent pairs, supercritical fluid extraction, double distillation, alkali saponification and molecular distillation (2-6). None of these processes have been completely satisfactory. For example, tocopherol molecules are sensitive at the usual temperatures employed and are extremely labile under alkaline conditions (7). Molecular distillation is the commonly employed process, wherein the tocopherol molecules are distilled under high vacuum at relatively low temperatures. However, this is an energy-intensive process and also requires the use of special equipment for generating a constant source of high vacuum. Modification of the free fatty acids can be envisaged as a way to facilitate the separation of sterols and tocopherols from the deodorizer distillate.

The boiling points of the fatty acids can be lowered by producing their methyl esters with the enzyme lipase as the catalyst. Lipases are enzymes that belong to the category of hydrolases, and they catalyze the hydrolysis of triacylglycerols under normal conditions. However, under limiting conditions of water, they catalyze the reverse reaction, namely esterification (8). High ester yield requires removal of water of reaction formed. Lipases have been widely employed for esterification of fatty acids with alcohols. However, with short-chain alcohols ($<C_4$), low ester yields (17% to 62% for methyl ester synthesis) have been obtained (9-11). It is believed that short-chain alcohols inhibit the reaction by inactivating the enzyme. A number of factors play a critical role in determining the yield of the esterification reaction, and they include enzyme concentration, moisture content of the enzyme, time and temperature of the reaction and the ratio of the reactants (12). It is difficult to arrive at the optimum conditions for maximum ester yield by trial and error because the various factors exhibit interaction effects.

Response surface methodology (RSM) is one technique that offers a solution to multivariable optimization problems. A two-level fractional design has been utilized as an initial step to find the path of steepest ascent (PSA) close to the region of optimum response, followed by complete description of the response surface near the optimum by using a central composite design (CCD) (13,14).

In this research, lipase-catalyzed methyl esterification of the fatty acids in canola oil deodorizer distillate has been studied as a preliminary step to the recovery of tocopherols and sterols. RSM has been employed to optimize the ester yield. The effect of temperature, ratio of the reactants and enzyme concentration on the equilibrium conversion have been studied along with their interaction effects. The use of molecular sieves as water-trapping agents has also been studied.

MATERIALS AND METHODS

Substrate and enzyme. Canola oil deodorizer distillate was supplied by CSP Foods Ltd., Saskatoon, Saskatchewan, Canada. It was a brownish yellow solid (at 25°C) with the following characteristics: acid value 157.7(15), saponification value 199.6(16), iodine value 68.6(17), unsaponifiable matter 14.8%(18).

The lipase Randozyme SP-382, immobilized on a bead-shaped acrylic resin, was a gift of Novo Industri A/S, Copenhagen, Denmark. The final moisture content of the enzyme was adjusted by adding a specific amount of water to a fixed amount of the enzyme, and the mixture was equilibrated for 24 hr before use. The moisture content of the enzyme was determined in the Karl Fischer-Automat (Metrohm AG, Herisau, Switzerland). Methanol used for the methylation reaction was of Omnisolv grade (BDH Chemicals, Toronto, Canada) and contained 0.05% water.

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Chemicals. Unless otherwise specified, all chemicals were of reagent grade. Karl Fischer reagent (comp-5; 1 mL equivalent to 5 μg of water) was purchased from BDH Chemicals. Molecular sieve (type-5A) was purchased from Fisher Scientific Co., Fairlawn, NJ.

Pretreatment of the deodorizer distillate. The deodorizer distillate sample was dried at 80°C for 2 hr under 30 mm Hg vacuum. It was then stored in air-tight containers at −18°C prior to use.

Esterification reaction. The reactions were carried out in a magnetically stirred 100- or 250-mL jacketed Wheaton flask (Celstir, Canlab, Burnaby, Canada) depending on the amount of the reactants (free fatty acids in the deodorizer distillate and methanol). The required temperature of the reaction was obtained by using a circulating water bath (Haake, Berlin, Germany), which contained a water-ethylene glycol mixture. Specific amounts of deodorizer distillate and molecular sieve were placed in the flask and the headspace was flushed with nitrogen. The enzyme and methanol were added after attainment of the desired temperature. Samples were drawn at specific intervals for analysis.

Acid value. The degree of conversion of free fatty acids to methyl esters was determined by measurement of acid value (AV) (Equation 1).

$$\% \text{ conversion} = \{\text{Initial AV of deodorizer distillate} - \text{AV of the reaction mixture at time 't'}\} \times 100/\{\text{Initial AV of the deodorizer distillate}\} \quad [1]$$

Five mL of the reaction mixture was pipetted into a tube containing 4 mL hexane and 2 mL water. After gentle swirling, the hexane layer was transferred to a tube containing 1-2 g of anhydrous sodium sulphate, shaken well, and allowed to settle. The clear hexane layer was transferred to another tube and the solvent was evaporated under a stream of nitrogen; AV was then determined on the sample (15).

Experimental procedure. A statistical design was adopted to optimize the reaction conditions for conversion of free fatty acids to methyl esters in the deodorizer distillate. Moisture content of the enzyme (X_1, wt% of the enzyme), temperature of the reaction (X_2, °C), enzyme concentration (X_3, wt% of the reactants), time (X_4, hr), molar ratio of the reactants (X_5, methanol:free fatty acids in the deodorizer distillate) and % molecular sieve (X_6, wt% of the reactants) were selected as the variables to maximize the response (conversion of free fatty acids to methyl esters in the deodorizer distillate).

It was found that 3 variables, moisture content of the enzyme, time and % molecular sieve did not exhibit any profound effect on the response (preliminary experiments not presented here). A CCD (orthogonal and rotatable) with 3 variables, temperature (X_2), enzyme concentration (X_3) and molar ratio of the reactants (X_5) was used to elucidate the surface response near the optimum. It included 8 factorial points, 9 center point replicates and 6 axial points (13,14). The levels of the variables and the complete design are presented in Table 1 and Table 2.

RESULTS AND DISCUSSION

The SAS RSREG (Statistical Analysis System-RSREG, 1985) procedure was used to fit a second order polynomial equation to the results in Table 2. The fitted equation is given by (Equation 2):

$$Y = -152.6 + 7.1X_2 - 17.9X_3 + 270.4X_5 + 0.3X_2X_3 - 3.5X_2X_5 + 10.5X_3X_5 - 0.02X_2^2 - 2.3X_3^2 - 32.3X_5^2 \quad [2]$$

The ANOVA (analysis of variance) for response Y (percent conversion) of the CCD is presented in Table 3. From

TABLE 1

Coded and Actual Levels of Three Variables (moisture content of enzyme (X_1) = 3% (wt% of enzyme), time (X_4) = 2.5 hr, and % molecular sieve (X_6) = 3% (wt% of the reactants)

Variable	Coded level of variable[a]				
	−1.68	−1	0	1	1.68
Temperature (X_2, °C)	51.6	55.0	60.0	65.0	68.4
Enzyme concentration (X_3, wt% of the reactants)	2.7	3.0	3.5	4.0	4.3
Ratio of reactants (X_5, methanol:free fatty acids)	1.3	1.4	1.6	1.8	1.9

[a]Coded level = 2 (actual value − mean of high and low level of the variable)/(difference between high and low level of the variable). High level and low level are the actual values of the variable for the coded level 1 and −1.

TABLE 2

Coded Level Combinations for a Three-Variable Central Composite Orthogonal and Rotatable Design (CCD) (X_2:temperature, °C; X_3: enzyme concentration, wt% of the reactants, dry basis; X_5: molar ratio of the reactants (methanol:free fatty acids in the deodorizer distillate))

Test run no.[a]	Coded level of variable			Percent conversion[b]
	X_2	X_3	X_5	
1	−1	−1	−1	91.5
2	1	−1	−1	91.2
3	−1	1	−1	92.3
4	1	1	−1	92.1
5	−1	−1	1	92.9
6	1	−1	1	75.5
7	−1	1	1	94.8
8	1	1	1	83.8
9	0	0	0	94.0
10	0	0	0	91.0
11	0	0	0	92.5
12	0	0	0	91.5
13	0	0	0	93.5
14	0	0	0	93.8
15	0	0	0	91.8
16	0	0	0	92.7
17	0	0	0	92.0
18	1.68	0	0	89.8
19	−1.68	0	0	93.9
20	0	1.68	0	93.7
21	0	−1.68	0	90.4
22	0	0	1.68	90.7
23	0	0	−1.68	89.1

[a]Test runs were performed in a random order.

[b]Percent conversion (average of two determinations) = {Initial AV of deodorizer distillate − AV of the reaction mixture at time 't'} × 100/{Initial AV of the deodorizer distillate}.

TABLE 3

Analysis of Variance, Showing the Effect of the Variables as a Linear Term, Quadratic Term and Interactions (cross product) on the Response Y (percent conversion of fatty acids to methyl esters) of the Central Composite Design (% variability explained (r^2) = 79.09%)

Source	DF[a]	Sum of squares	Mean square	F ratio
Model	9	287.56	31.95	5.46[b]
Linear	3	136.37		
Quadratic	3	39.05		
Cross product	3	112.14		
Residual	13	76.01	5.85	
Lack of fit	5	67.05	13.41	11.97[c]
Pure error	8	8.96	1.12	
Total	22	363.57		

[a]Degrees of freedom.
[b]Significant at 1% level.
[c]Significant at 5% level.

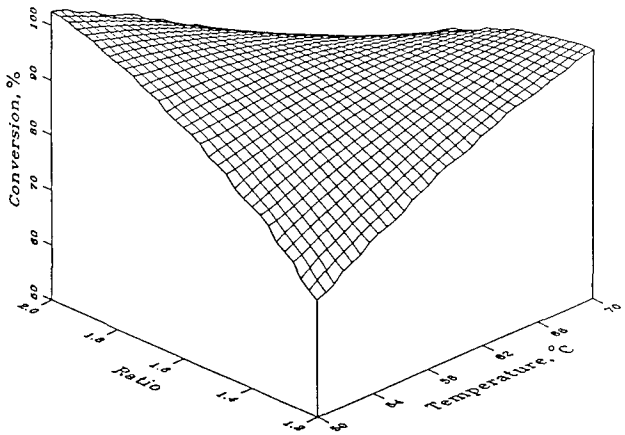

FIG. 1. Three-dimensional response, showing the effect of temperature and ratio with enzyme concentration held constant at 4.5%, on the percent conversion of fatty acids to methyl ester.

TABLE 4

Analysis of Variance, Showing the Significance of the Effect of the Three Variables on the Response Y (% conversion of fatty acids to methyl ester)

Variable	DF[a]	Sum of squares	Mean square	F ratio
Temperature (X_2)	4	202.88	50.72	8.67[b]
Enzyme concentration (X_3)	4	41.80	10.45	1.79
Ratio (X_5)	4	155.65	38.91	6.66[b]

[a]Degrees of freedom. [b]Significant at 1% level.

the table it is seen that, although the fitted model is significant, it also shows statistically significant lack of fit. Such situations often arise if the model does indeed fit the data well and if the estimated variance due to experimental uncertainty is relatively small (19).

ANOVA for the effect of the 3 variables on the response is given in Table 4. Temperature (X_2) and the ratio of the reactants (X_5) were found to have significant effects on the conversion of free fatty acids. Grids from the data were generated to plot 3-D graphs and contour maps by using the SAS program. The 3-D graphs and contour maps were plotted by the Surfer program (20). The solution of the model is a saddle point. The 3-D graph (for enzyme concentration = 4.5% — Fig. 1) illustrates that point clearly.

Contour maps were plotted by keeping enzyme concentration (X_3) constant and varying temperature (X_2) and ratio (X_5) at the same time. The effect of temperature and the ratio of the reactants are clearly exhibited in the contour maps (Fig. 2a–2f, Fig. 3a–3b). The effect of temperature and molar ratio of the reactants on the response are discussed separately.

Temperature. Temperature has been shown (10,21) to have a considerable effect on esterification reactions in previous studies. The temperature effect may also be related to the enzyme source, type of substrate and water content of the reaction medium. The enzyme used in this study has a recommended operating temperature range of 60–80°C (22). From the contour maps (Fig. 2a–2f) it is evident that for a particular enzyme concentration, a change in temperature (within the range 50–70°C) had a marked effect on the percent conversion of the free fatty acids to methyl esters.

Ratio of the reactants had an interactive effect with temperature in deciding the equilibrium conversion. The influence of temperature for a fixed enzyme concentration is maximum for the extremes of the ratio (around 1.2 and 2.0) and was minimal near a ratio of 1.6. The optimum region clearly existed in the lower temperature region <55°C and at a ratio of >1.8 (Fig. 2a–2f). With increase in enzyme concentration above 2.5%, improvement in conversion was attainable at 50°C and a ratio of 1.8–2.0. This reached a pleateau after 3.7% enzyme concentration. Higher conversions were also possible by working at higher temperatures and lower ratios. However, availability of methanol for the reaction would be slightly limited due to its low boiling point, and in addition the higher temperature would increase the stress on the enzyme activity. Lower operational temperatures would be ideally suited for an industrial operation and it would also favor enhanced stability of the enzyme (23). However, reactions at lower temperatures (<45°C) would be limited by the melting point of the deodorizer distillate. A suitable solvent could be used to solubilize the deodorizer distillate, and the esterification reaction could be carried out in a nonaqueous medium at lower temperatures.

A 3-D graph and contour map (Figs. 3a and 3b) were plotted for a constant ratio of 1.96 (maximum conversion was effected at this ratio in trial experiments) by changing temperature and enzyme concentration. A temperature change from 50°C to 70°C at enzyme concentration of 4.5% resulted in a 25% drop in percent conversion of fatty acids to methyl esters. The same temperature change at an enzyme concentration of 2.5% resulted in a 40% drop in percent conversion. The negative effect of the increase in temperature at a particular ratio was lessened to some extent by an increase in enzyme concentration.

Ratio of the reactants. A molar excess of methanol with respect to the amount of free fatty acids in the deodorizer

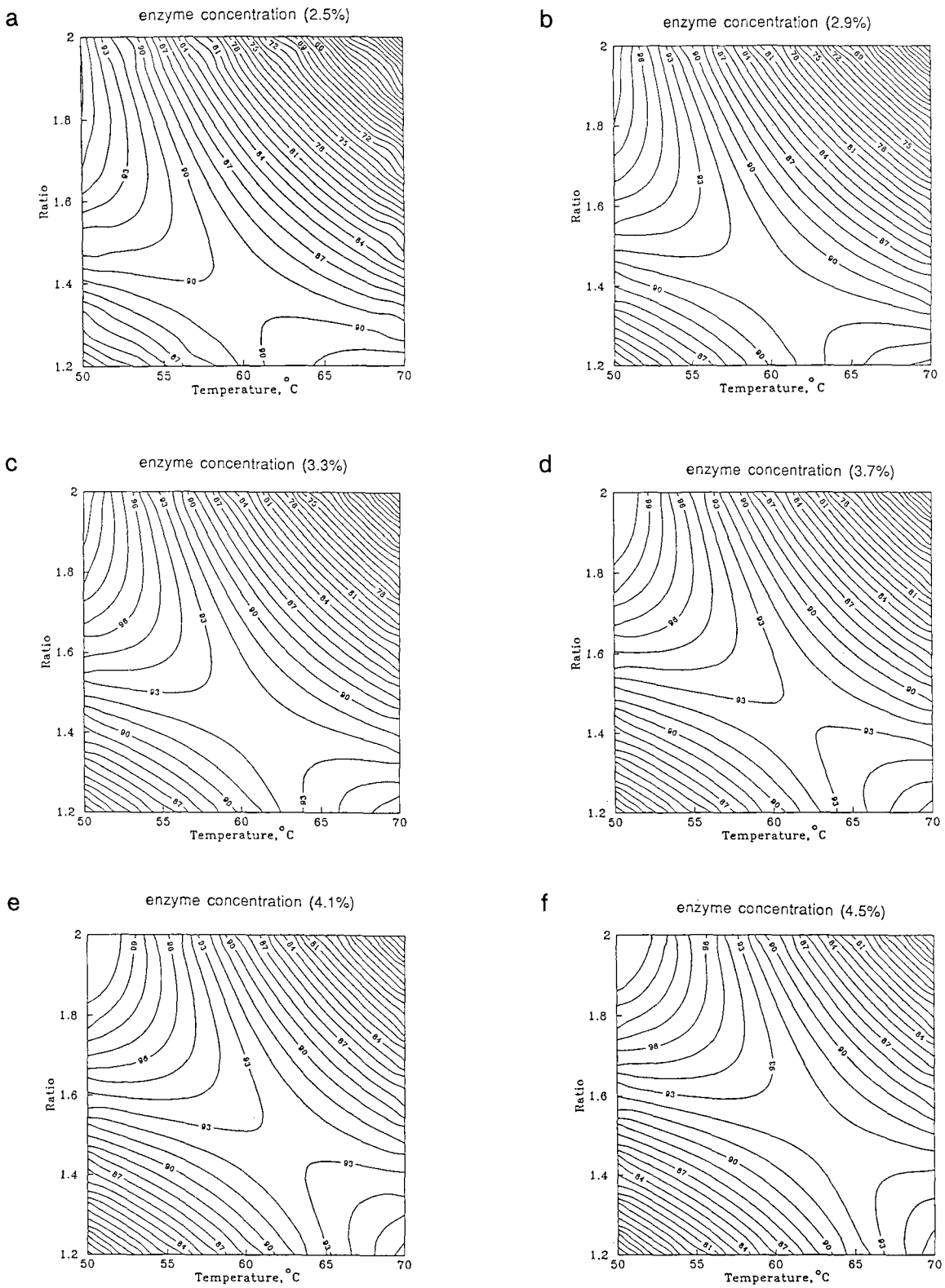

FIG. 2a–2f. Two-dimensional contour plots of the percent conversion of fatty acids to methyl ester, showing the effect of temperature and ratio (methanol:free fatty acids in the deodorizer distillate) with enzyme concentration.

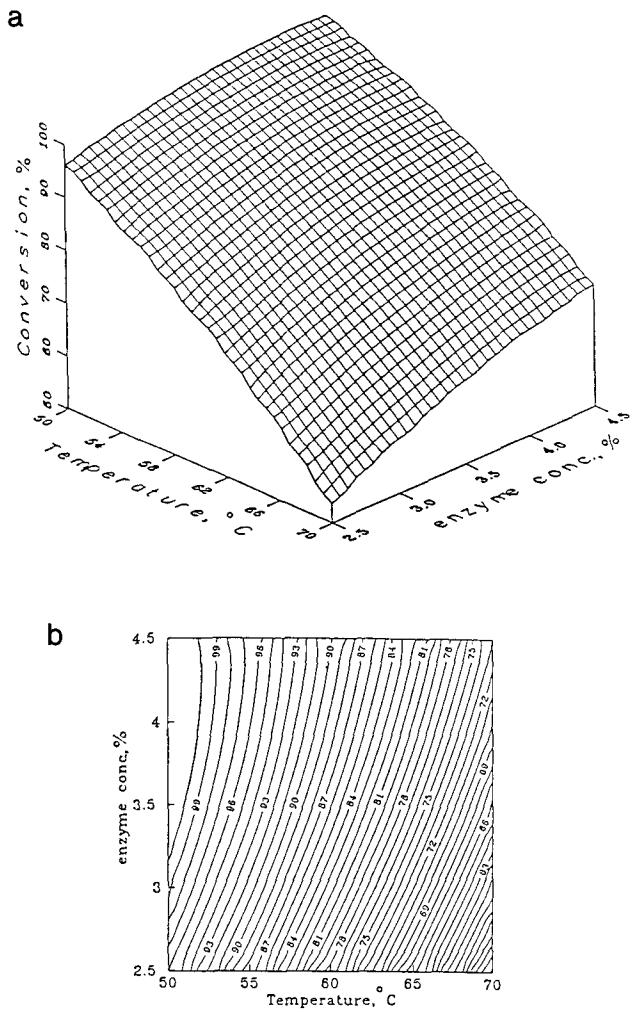

FIG. 3. The effect of temperature and enzyme concentration (wt% of the reactants), with ratio (methanol:free fatty acids in the deodorizer distillate) held constant at 1.96, on the percent conversion of fatty acids to methyl ester in the deodorizer distillate. (3a: three-dimensional response graph, 3b: two-dimensional contour plot.)

distillate was found to be necessary to attain optimum yield. As mentioned earlier, yields of 16% to 62% of methylation of the fatty acids catalyzed by lipases have been achieved in previous studies. About 71% yield of methyl oleate has been reported for the lipase-catalyzed reaction of methanol and oleic acid at a pressure of 0.032 bar (10). It has been proposed that hydrophilic solvents stripped the water surrounding the enzyme, thereby lowering its activity (21,24).

The effect of the ratio of reactants in conjunction with other parameters that affect the reaction equilibrium had not been studied previously. In this study, it was found that the ratio of the reactants had a significant effect on the equilibrium conversion and showed a strong interaction effect along with temperature. The contour maps (Figs. 2a–2f) show that, for a fixed enzyme concentration, change in the ratio from 1.2 to 2.0 produces maximum change in the response (percent conversion) at temperature extremes (around 50°C and 70°C), whereas minimum change in response was observed at temperatures close to 60°C.

The combined effect of the ratio and temperature produced interesting results. High conversions (>90%) were obtained at combinations of high temperature (70°C) and low ratio (1.2) and for combinations of low temperature (50°C) and high ratio (2.0), for enzyme concentrations from 2.5%–4.5%. It should be noted that the initial moisture content of the enzyme was being held constant at 3%. It is well known that some amount of moisture is essential to keep the enzyme in the active state (24). In all the test runs of the CCD, 75% of the equilibrium conversion was reached within 1 hr after initiation of the reaction. The combined effect of temperature and ratio of the reactants seems to play a vital role in the reaction kinetics and in determining the equilibrium conversion of the fatty acids to methyl esters.

The lower conversion found at higher temperatures and ratios (70°C and 2.0, respectively) may be due to the inactivation effect of high temperature and high concentration of hydrophilic methanol. However, at lower ratio (1.2) and at 70°C, methanol was consumed rapidly as the reaction progresses, leaving little methanol in the reaction mixture to participate in the enzyme inhibition process. At 50°C (lower end of the temperature range), a lower ratio (1.2) resulted in slightly lower conversion (~80%) than at 70°C for the same ratio (~90%). High conversions (~95%) were obtained at 50°C and a ratio of 2.0. The exact reason for this behavior is not known. Following the reasoning applied for higher temperature, a higher ratio should have inhibited the esterification reaction, resulting in lower conversions, but this was not the case. The lower temperature seems to have largely reduced the inhibition effect of methanol on the enzyme. Further studies are needed along this line to explain the interactive effect on the enzyme chemistry.

The role of molecular sieves in absorbing water in the reaction mixture is well known, and in many studies it has been used to advantage. In this study, preliminary trial experiments showed that the use of molecular sieves in the reaction resulted in an increased percent conversion. Conversions of up to 96.5% were obtained by working around the optimum conditions (experiments based on conditions predicted by Ridge analysis on the CCD). Experiments also performed at optimum conditions, but without added molecular sieve, resulted in similar high conversions (~96.5%). This suggests that the presence of water in the reaction mixture is favored when one of the reactants is hydrophilic.

Continuing studies on the role of water under optimum conditions are now in progress in our laboratory. Research to define the chemical nature of the sterol and tocopherol fractions in the deodorizer distillate, along with studies on the technology required to separate these valuable fractions, is currently being conducted.

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[Received May 14, 1991; accepted October 31, 1991]