Saponification of Oils Rich in Polyunsaturated Fatty Acids: Optimization of Conditions by Response Surface Methodology

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A methodological approach was used to optimize experimental conditions leading to complete saponification of cod liver oil without loss of polyunsaturated fatty acids. Each experiment, using conditions imposed by a Doehlert design, was analyzed for the percentages of fatty acids with different chain lengths and degrees of unsaturation: $Y1=\%C22{:}6\omega3$, $Y2=\%C20{:}5\omega3$, $Y3=\%C18{:}4\omega3$, $Y4=\%C18{:}1\omega9+\omega7$, $Y5=\%C16{:}0$. The difference between values obtained in each experiment and values obtained by transesterification allowed the extent of fatty acid degradation to be quantified. The experimental results were expressed by response surfaces allowing the choice of saponification conditions for each type of fatty acid.

An optimum zone for saponification was observed which was larger when carried out under nitrogen. Nitrogen did not eliminate degradation, but degradation was less marked under nitrogen than under air. The fatty acid having the greatest degree of unsaturation in this study, C22:6ω3, was the first to be degraded. The extension of the Doehlert design to four factors showed that under air, alkali concentration and reaction time were the major factors governing loss of polyunsaturated fatty acids.

Fatty acid compositions of oils can be studied either after hydrolysis and methylation or after transesterification. Various experimental conditions have been used for saponification (1-6). Fatty acid compositions of common vegetable oils generally are not modified during saponification, whereas marine oils, rich in long chain polyunsaturated fatty acids (PUFA), may be partially isomerized or oxidized under certain hydrolytic conditions (7-9). This is not observed with transesterification. Nevertheless, saponification is necessary when both fatty acids and unsaponifiable matter of oil need to be analyzed or when fatty acid compositions of oils rich in unsaponifiable matter, such as shea butter (10) and certain marine animals, are analyzed (11).

The objective of the present investigation was to optimize conditions leading to complete saponification without loss of long chain PUFA. Four parameters were studied: reaction time, alkali concentration, alkali volume and bath temperature. In previous work, the first two parameters were used at different levels but without systematic study of their influence on the loss of fatty acids. A methodological approach like that used in the present study provides an extremely efficient means of determining the influence of the various parameters while requiring a minimum of experiments (12). The choice of the methodology depends on the relationship between experimental responses and the parameters being studied. Experimental responses were the percentages of five fatty acids chosen on the basis of their differences in chain length and degree of unsaturation.

Personal experience and literature indicate that the percentages are not proportional to variations in saponification. We have thus postulated a second degree equation leading to a Doehlert uniform lattice (13) as the most appropriate experimental design. Two designs were carried out, each with two factors (reaction time and alkali concentration), to determine the influence of the factors under air and nitrogen. The experimental design under air was then extended to four factors to show the influence of alkali volume and bath temperature.

EXPERIMENTAL PROCEDURE

Saponification. Commercial cod liver oil (∼100 mg) was saponified under air and under nitrogen with KOH/ethanol in a round-bottomed flask (10 ml). Reaction time, alkali concentration, alkali volume and bath temperature were varied. Water (10 ml) was added to the reaction mixture, which was then acidified with hydrochloric acid (2M). Fatty acids were extracted with diethyl ether (3 × 10 ml) after the addition of nonadecanoic acid as internal standard (one ml of a 5 g/l solution in CH_2Cl_2). Unsaponifiable matter was not extracted in order to minimize the loss of fatty acids. The degree of saponification was qualitatively measured by thin layer chromatography (TLC) (Si 60F Merck, hexane/ether, 8/2, v:v).

Esterification of fatty acids. Fatty acids were esterified by the Metcalfe method (14). BF3-10% in methanol (Fluka, Büchs, Switzerland) (one ml) was added to the fatty acids (20 mg) in a test tube and heated to boiling. Heating was maintained for a few seconds until the reaction mixture became cloudy. Water (six ml) was added and the methyl esters were extracted with hexane (3 × 2 ml).

Analysis of fatty acid composition by GLC. An Intersmat IGC gas chromatograph (Delsi, France), equipped with a flame ionization detector (FID) and fused silica column (51 m, 0.32 mm i.d., 0.22 μm df) coated with CP Wax 57 CB (Chrompack) was used. Temperatures were 190 C for the column and 260 C for injector and detector ovens. Inlet pressure of hydrogen, used as carrier gas, was 0.9 bar, split 60 ml/min. Peak areas were calculated by electronic integrator (Enica 10, Delsi, France). The quantitative analysis was accomplished with an internal standard (nonadecanoic acid, Fluka). Weight response factors were not taken into account.

Experimental methodology. The Doehlert design (13) has peculiar properties. A set of points uniformly distributed in space following a rhombic lattice allows the exploration of the whole region. The number of factors can be increased during the study. Lattice extension can be accommodated in any direction. It is also possible to explore a neighboring surface by reusing the shared points of the lattice adjacent to the new area to

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be explored. The number of factors fixes the experimental design and thus the number of experiments and the values taken by the variables (the coded variables X take levels between -1 and +1). The design construction requires the choice of an experimental domain be defined by its center and its variation step for each factor. The position of each variable was chosen according to experimental constraints, the order of variables being unimportant.

The four parameters studied were examined in the range of commonly employed values: reaction times of 0–2 hr; alkali concentrations, 0–2M; alkali volumes, 0–10 ml, and bath temperatures, 65–95 C (such that saponifications were carried out at or below the boiling point of the reaction medium). An initial experimental design with two factors (X1 = reaction time, X2 = alkali concentration) was used for two series of seven experiments under air or nitrogen; subsequently, 14 experiments were added to extend the experimental design to four factors (X3 = alkali volume, X4 = bath temperature) under air (Table 1).

The second order equation postulated was:

$$\begin{aligned} Y = {} & b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_1{X_1}^2 \\ & + b_22{X_2}^2 + b_33{X_3}^2 + b_4{X_4}^2 + b_12X_1X_2 \\ & + b_13X_1X_3 + b_14X_1X_4 + b_23X_2X_3 \\ & + b_24X_2X_4 + b_34X_3X_4 \end{aligned}$$

TABLE 1

Doehlert Design: Real Variables[a]

Experiments [b]	U1[c] (min)	U2[c] (M)	U3[c] (ml)	U4[c] (C)
1	65[d]	1.000[d]	5.0[d]	80[d]
2	125	1.000	5.0	80
3	5	1.000	5.0	80
4	95	1.866	5.0	80
5	35	0.134	5.0	80
6	95	0.134	5.0	80
7	35	1.866	5.0	80
8	95	1.289	8.0	80
9	35	0.711	2.0	80
10	95	0.711	2.0	80
11	65	1.577	2.0	80
12	35	1.289	8.0	80
13	65	0.423	8.0	80
14	95	1.289	5.75	95
15	35	0.711	4.25	65
16	95	0.711	4.25	65
17	65	1.577	4.25	65
18	65	1.000	7.25	65
19	35	1.289	5.75	95
20	65	0.423	5.75	95
21	65	1.000	2.75	95

[a] Calculated from coded variables X (13).

[b] Experiments from 1 to 7, design with 2 factors; from 1 to 21, design with 4 factors.

[c] U1 = *reaction time*; U2 = *alkali concentration*; U3 = alkali volume; U4 = bath temperature. The bath temperatures of 80, 95, 65 C correspond to 76, 78, 63 C, respectively, inside the reactional medium.

[d] Value of the variable at the experimental domain center. The variation step of each variable is: ΔU1 = 60 min; ΔU2 = 1M; ΔU3 = 3.676 ml; ΔU4 = 18.97C.

The polynomial coefficients b were calculated by the least squares method. The results were given by the response surfaces, calculated by Nemrod software (15), in order to facilitate their interpretation. The response surfaces were drawn up to 5% of the maximum experimental values obtained under nitrogen.

RESULTS AND DISCUSSION

TLC indicated that saponification reaction was complete for all experiments (the disappearance of triglycerides and polar lipids).

The 5 response values Y1 = %C22:6ω3, Y2 = %C20:5ω3, Y3 = %C18:4ω3, Y4 = %C18:1ω9+ω7, Y5 = %C16:0, are given in Table 2. The maximum value of each response was similar to that obtained by transesterification methods (2,16) on cod liver oil and in good agreement with literature (17). This showed that, in the explored experimental domain, certain conditions led to a total hydrolysis without loss of fatty acids.

The percentages of C18:1ω9+ω7 (Y4) and C16:0 (Y5) varied slightly. This was expected for fatty acids which are saturated or monounsaturated. The percentage variations of C22:6ω3 (Y1), C20:5ω3 (Y2) and C18:4ω3 (Y3) indicated degradation for some conditions. The greater the degree of unsaturation, the greater is the extent of degradation. Degradation was less severe under nitrogen than under air; nevertheless, some degradation of PUFA occurred during saponification under inert gas.

The examination of residues partition and statistical tests were carried out on the basis of the results of the designs with two factors (X1 = reaction time, X2 = alkali concentration) under nitrogen and air. They indicated that the model postulated for the responses fit well with the results measured in the definite experimental space. The equations allowed the different response surfaces to be drawn. Figure 1 gives response surfaces for %C22:6ω3 (Y1). Response surfaces varied in the same range for %C20:5ω3 (Y2) and %C18:4ω3 (Y3) and only very slightly for %C18:1ω9 + ω7 (Y4) and %C16:0 (Y5). Under both nitrogen and air, the b2 coefficients were higher than the b1 coefficients for the responses Y1, Y2 and Y3. Thus, the percentages of PUFA were more dependent on alkali concentration (X2) than reaction time (X1). This was confirmed by examining the response surfaces. Figure 1 shows that a displacement (-1 to +1) on the alkali concentration axis induced a greater variation of the fatty acid percentages (9 to 8%, N2; 9 to 6.5%, air) than a displacement (-1 to +1) on the reaction time axis (9 to 8.4%, N2; 9 to 7.2%, air). The influences of these parameters were less under nitrogen. The optimum domain of the five responses was obtained by superimposition of the maximum values (Figs. 2 and 3). Under nitrogen, Figure 2 shows a large surface limited by the %C22:6ω3. The optimum can be obtained with conditions ranging from short reaction times and medium alkali concentrations up to long reaction times and low alkali concentrations. The optimum zone of each response increased from Y1 to Y5 when unsaturation and chain length decreased. Under air, the optimum domain was slightly reduced and still limited by the %C22:6ω3 (Fig. 3). But Y1,Y2,Y3 limits were more closely spaced. For Y4 and Y5, a limit was noted with short reaction times and low alkali

TABLE 2

Saponification Results[a] Under Air and Under Nitrogen (wt %)

Experiments	Y1[b] Air	Y1[b] N2	Y2[b] Air	Y2[b] N2	Y3[b] Air	Y3[b] N2	Y4[b] Air	Y4[b] N2	Y5[b] Air	Y5[b] N2
1	8.83	(8.97)	6.78	(7.03)	1.89	(1.97)	20.41	(20.13)	8.50	(8.47)
2	7.13	(8.42)	5.81	(6.75)	1.70	(1.91)	20.16	(20.02)	8.45	(8.35)
3	8.95	(9.26)	6.86	(7.28)	1.90	(2.02)	19.33	(20.44)	8.05	(8.67)
4	6.12	(7.89)	5.20	(6.44)	1.53	(1.84)	20.20	(20.02)	8.24	(8.27)
5	9.05	(9.45)	6.78	(7.32)	1.83	(2.02)	19.74	(20.45)	8.31	(8.50)
6	9.43	(9.28)	7.03	(7.27)	1.94	(2.01)	20.57	(20.41)	8.67	(8.67)
7	7.36	(8.49)	5.84	(6.98)	1.64	(1.97)	20.08	(20.43)	8.67	(8.60)
8	7.02		6.16		1.85		20.83		8.68	
9	8.98		7.10		1.94		19.95		8.32	
10	8.38		6.89		1.99		20.41		8.49	
11	8.13		6.68		1.89		20.39		8.37	
12	8.93		7.03		1.95		20.42		8.40	
13	9.10		7.28		2.04		20.62		8.51	
14	6.79		6.03		1.83		20.67		8.59	
15	9.40		7.40		2.07		20.34		8.59	
16	9.30		7.38		2.07		21.02		9.00	
17	9.28		7.26		1.98		20.55		8.40	
18	9.44		7.20		1.93		19.89		8.16	
19	8.36		6.89		1.98		20.56		8.45	
20	9.97		7.47		2.03		20.73		8.45	
21	7.95		6.56		1.89		19.92		8.26	

[a]Mean of 2 experiments.

[b]Y1 = %C22:6ω3, Y2 = %C20:5ω3, Y3 = %C18:4ω3, Y4 = %C18:1ω9+ω7, Y5 = %C16:0.

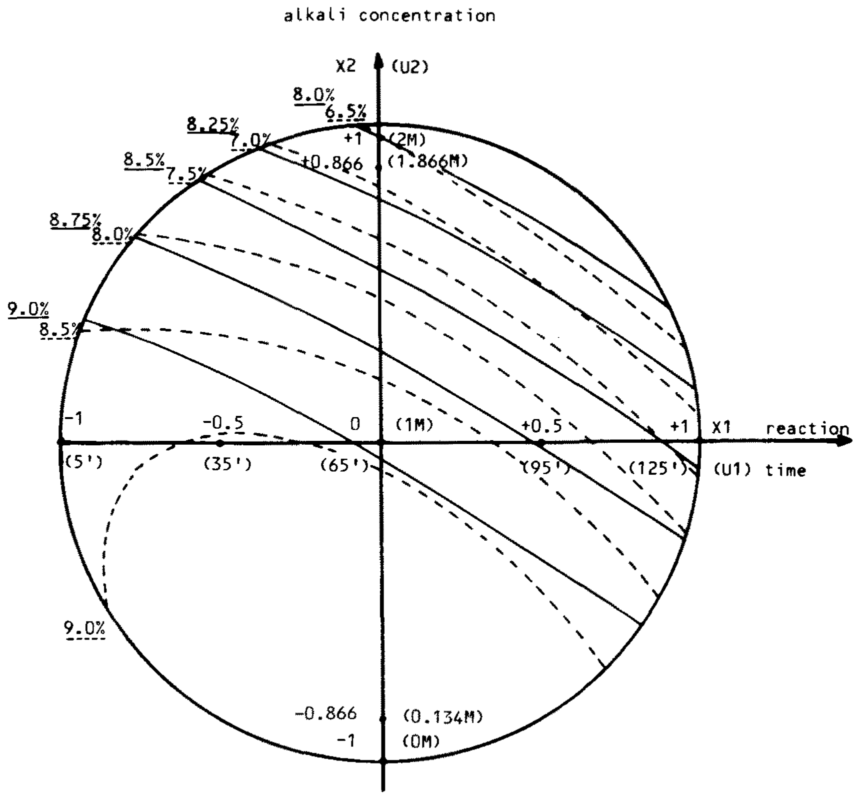

FIG. 1. Response surfaces of %C22:6ω3 under nitrogen and under air. Y1 = f(X1,X2); X3 = 0 (U3=5 ml); X4 = 0 (U4=80 C); ——, nitrogen; — — — —, air.

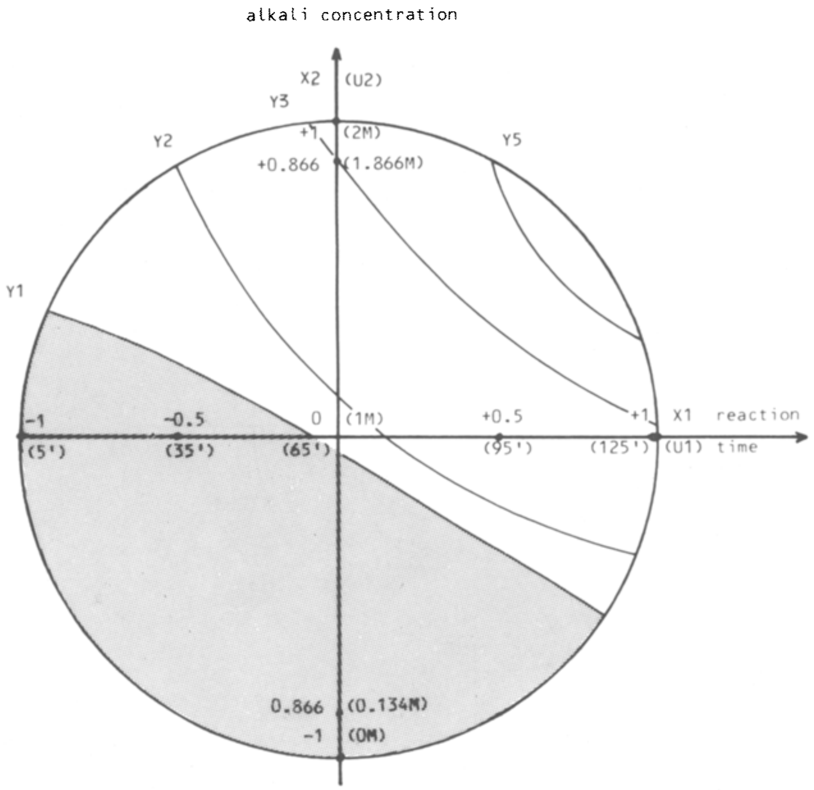

FIG. 2. Optimum domain under nitrogen (dark zone). X3 = 0 (U3=5 ml); X4 = 0 (U4=80 C).

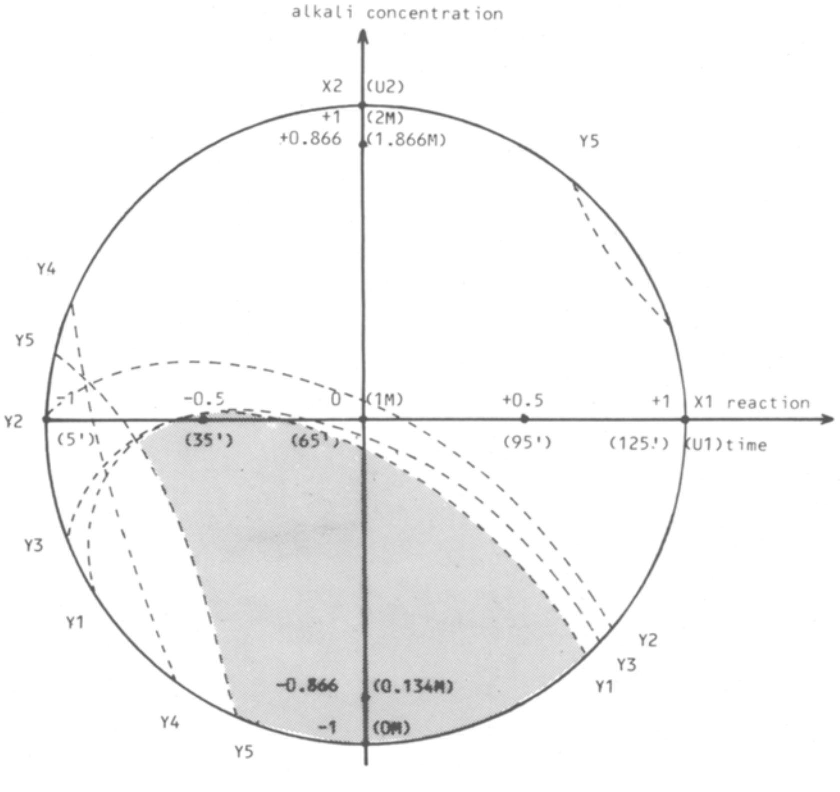

FIG. 3. Optimum domain under air (dark zone). X3 = 0 (U3=5 ml); X4 = 0 (U4=80 C).

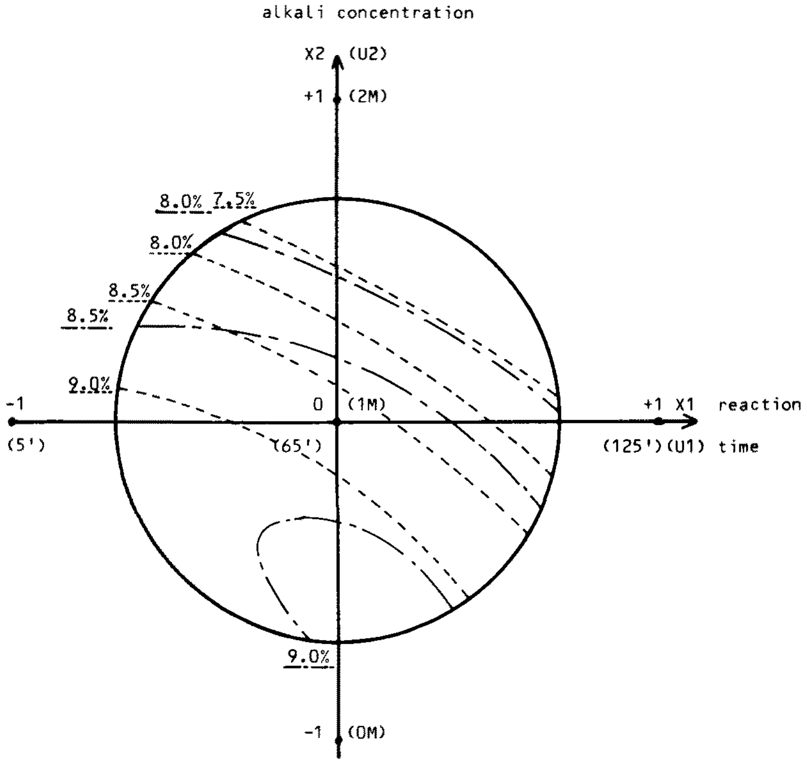

FIG. 4. Response surfaces of %C22:6ω3 under air. Y1 = f(X1,X2); X4 = 0 (U4=80 C); X3 = -0.75 (U3=2.25 ml), — — —; X3 = +0.75 (U3=7.75 ml) - - - -.

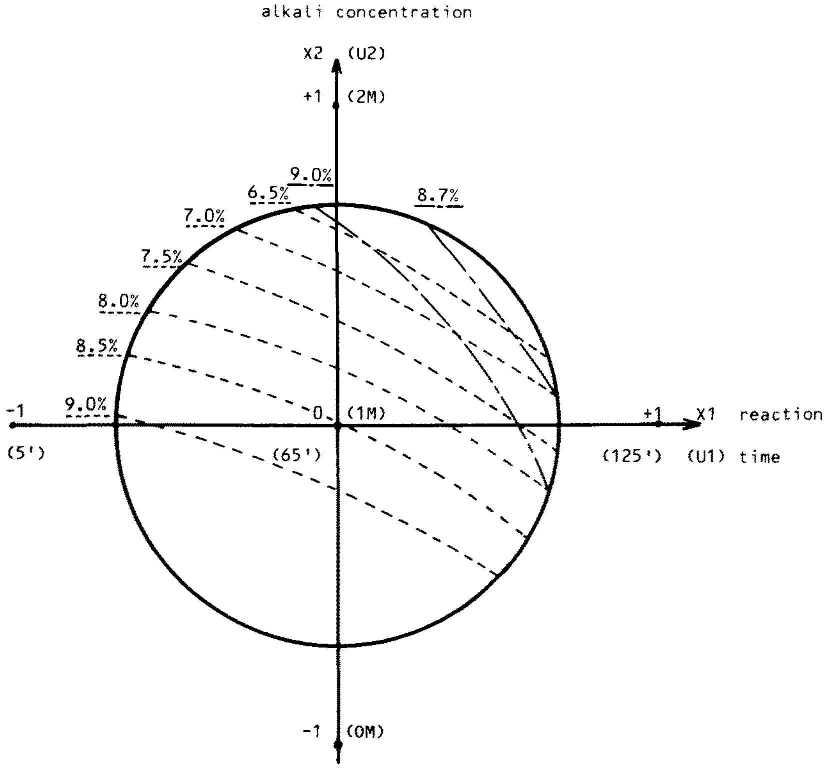

FIG. 5. Response surfaces of %C22:6ω3 under air. Y1 = f(X1,X2); X3 = 0 (U3=5 ml); X4 = -0.75 (U4=65.7 C) — — —; X4 = +0.75 (U4=94.2 C) - - - -.

concentrations. The optimum zone of each response was larger under nitrogen than under air. Analysis of responses [calculated by Nemrod (13)] showed high correlation within the couples Y1/Y2, Y2/Y3, Y4/Y5, medium correlation in the couple Y1/Y3 and no correlation in the other couples.

Two other factors were studied in order to obtain a better understanding of the reaction: alkali volume (X3) and bath temperature (X4). To obtain maximum response variations due to factors X3 and X4, a study involving four factors was conducted under air because greater degradation occurred than under nitrogen. Experimental results are given in Table 2. The postulated model fit well with the experimental results. These values showed that X2 (alkali concentration) and X1 (reaction time) had similar influence to that shown in the experiment with two factors, and X2 was the predominant factor for the responses Y1, Y2 and Y3. X3 (alkali volume) was the least significant of the parameters for the responses Y1, Y2 and Y3. X4 (bath temperature) acted on responses Y1 and Y2 but had no significant effect on the other responses. The examination of response surfaces confirmed these results. The optimum zone was still limited by %C22:6ω3. Figures are given for only the Y1 response as this showed the greatest variation and thus the strongest influence of each parameter. Figure 4 gives the response surfaces Y1 = f(X1,X2) for X4=0 (80 C) and X3 at 2 levels - and +0.75 (2.25 and 7.75 ml). A clear reduction of the optimum surface was noted at 2.25 ml while for a volume of 7.75 ml no difference was found by comparison with five ml (Fig. 1).

Figure 5 gives the response surfaces Y1 = f(X1,X2), for X3 = 0 (5 ml) and X4 at 2 levels - and +0.75 (65.7 and 94.2 C). At 65.7 C (corresponding to 63 C in the reaction medium) the surface was optimum in almost all the domain except for long reaction times and high alkali concentrations where slight degradation appeared. At 94.2 C (medium at 78 C), the domain was limited to low alkali concentrations and short reaction times with much degradation in the rest of the domain (9 to 6.5%); these results were similar to those observed at 80 C (medium at 76 C) (Fig. 1). In order to show the combined influences of the alkali volume and bath temper-

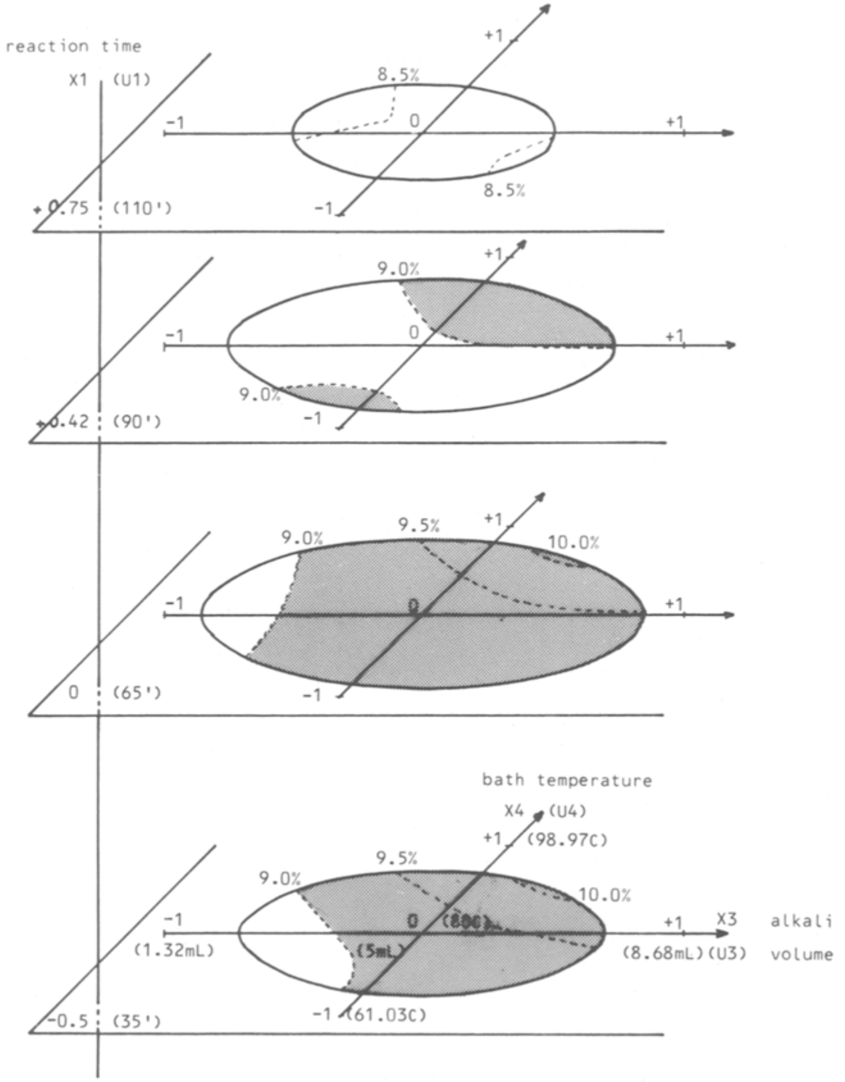

FIG. 6. Response surfaces of %C22:6ω3 under air (optimum in dark zones). Y1 = f(X3,X4); X2 = -0.5 (U2=0.5M); X1 = -0.5; 0; +0.42 +0.75 (U1=35, 65, 90, 110 min).

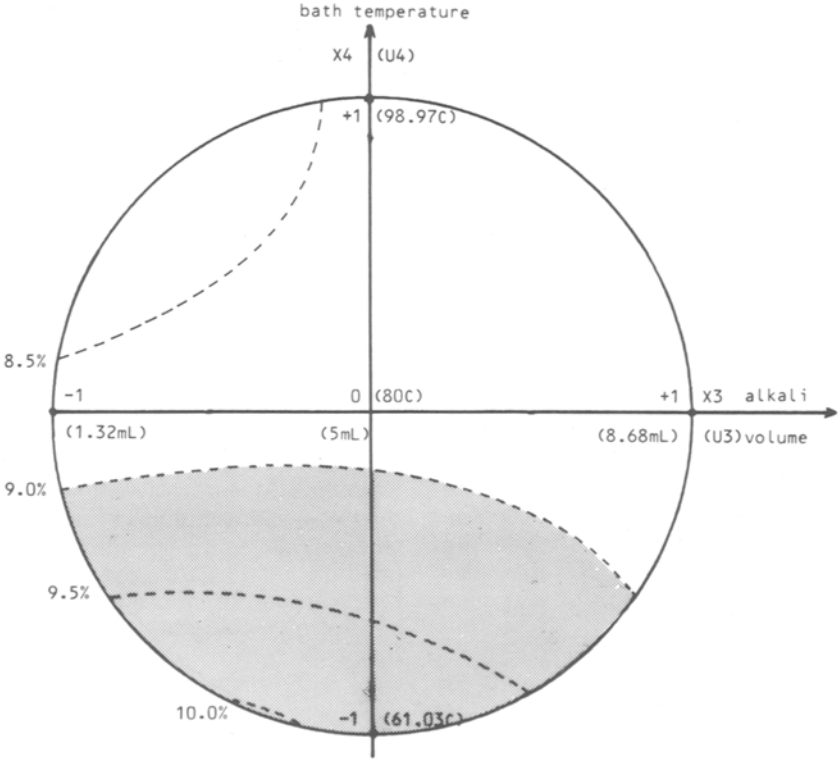

FIG. 7. Response surfaces of %C22:6ω3 under air (optimum in dark zone). Y1 = f(X3,X4); X1 = -0.08 (U1=60 min); X2 = 0 (U2=1M).

ature parameters, the response surfaces Y1 = f(X3,X4) were drawn for different conditions of reaction time and alkali concentration taken in the optimum domain of the design with two factors (Figs. 6 and 7). Figure 6 gives the Y1 response surfaces for X2 = -0.5 (0.5 M) and X1 = -0.5, 0, 0.42 and 0.75 (35, 65, 90 and 110 min, respectively). The response surfaces indicated that up to 65 min, optimum zones were obtained with volumes greater than three ml at all temperatures. At 90 min the surface was reduced, and at 110 min it disappeared. Figure 7 gives the Y1 response for X2 = 0 (1M) and X1 = -0.08 (60 min); these conditions were evaluated because they have been widely reported in the literature (1,6). Y1 was not at an optimum in the whole surface but only for bath temperatures below 80 C combined with all levels of alkali volume.

This work gave a range of conditions leading to saponification without degradation of PUFA. This allows one to select suitable conditions for saponification (alkali concentration and volume, reaction time and bath temperature) in the presence of air or nitrogen. Other marine oils rich in unsaponifiable matter, hydrocarbons, diacylglyceryl ethers also were saponified with experimental conditions selected from the optimum domain (U1 = 65 min, U2 = 0.225 M, U3 = 5 ml, U4 = 80 C). The percentages of fatty acids obtained were similar to those found by transesterification (16), confirming the validity of the chosen experimental conditions.

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