Selective Distribution of Saturated Fatty Acids into the Monoglyceride Fraction During Enzymatic Glycerolysis

Gerald P. McNeill*, Dieter Borowitz and **Ralf G. Berger**
Institute for Food Chemistry, Hanover University, 3000 Hanover 91, Germany

Four triglyceride fats and oils (beef tallow, lard, rapeseed oil and soybean oil) were reacted with glycerol while using lipase as the catalyst. For all fats examined, at reaction temperatures above the critical temperature (T_c), the fatty acid compositions of the monoglyceride (MG) and diglyceride (DG) fractions and of the original fat were similar. A relatively low yield of MG was obtained (20–30 wt%). When the reaction was carried out with beef tallow or lard at a temperature below the T_c (40°C), the concentration of saturated fatty acids in the MG fraction was 2 to 4 times greater than that in the DG fraction. Correspondingly, the concentration of unsaturated fatty acids in the DG fraction was more than two times greater than that in the MG fraction. At 5°C, a similar trend was observed for rapeseed oil and soybean oil. Direct analysis of partial glycerides during glycerolysis by high-temperature gas-liquid chromatography showed that below T_c the content of C16 MG increased relatively more than C18 MG. C36 DG and C54 TG were apparently resistant to glycerolysis. Preferential distribution of saturated fatty acids into the MG fraction was accompanied by a high yield of monoglyceride (45–70 wt%) and solidification of the reaction mixture. It is concluded that during glycerolysis below T_c, preferential crystallization occurs for MGs that contain a saturated fatty acid.

KEY WORDS: Fats and oils, glycerolysis, lipase, monoglyceride, selective crystallization.

Considerable interest has been shown in the development of a process for the synthesis of monoglycerides with triacylglycerol lipase (EC 3.1.1.3.) as catalyst. Because enzymes are catalytically active and highly specific at relatively low temperatures, the quality of the monoglyceride produced with lipase should be superior to the chemically synthesized product. This is particularly important because monoglycerides are used as additives in a wide range of food products and cosmetics (1). It has recently been shown that monoglycerides can be enzymatically synthesized by glycerolysis of triglyceride fats and oils without the use of organic solvents in a reaction system with a low water content (2,3). However, to obtain a high yield of monoglyceride, McNeill and co-workers (4) showed that the reaction must be carried out below a critical temperature (T_c). The value of T_c depends on the fat type and is related to the melting point of the fat, *i.e.*, a higher melting point results in a higher value for T_c (5). Using this procedure, yields of monoglyceride of up to 90% on a weight basis can be obtained.

Although a T_c effect could be demonstrated for several different types of fat, no direct evidence was presented for the cause of the phenomenon (5,6). Based on the observation that the reaction mixture always solidified during the course of glycerolysis at temperatures below T_c, the authors of the previous studies suggested that the monoglycerides preferentially crystallize from the reaction mixture, thus shifting the reaction equilibrium further toward synthesis of monoglyceride. The observation that the value of T_c is related to the melting point of the fat, and therefore to the fatty acid composition (5), suggests that only monoglycerides containing saturated fatty acids or monounsaturated fatty acids will crystallize from the reaction mixture.

The aim of the work presented here is to test this hypothesis by analyzing the composition of the reaction mixture in detail during the course of lipase-catalyzed glycerolysis.

EXPERIMENTAL PROCEDURES

Glycerolysis. Water was dissolved in 1.52 g glycerol to give a final moisture content of 3.5%. Lipase powder was dissolved in the glycerol/water solution to give a final lipase concentration of 500 units/g fat, and 7 g fat or oil was added. This results in a mole ratio of glycerol to fat of 2. The reactants were mixed by magnetic stirring (600 rpm) in a tightly stoppered flat-bottomed glass reaction vessel, 7 cm high with an internal diameter of 2.5 cm. The reaction temperature was controlled by keeping the reaction vessel in a glass mantle through which water was continuously circulated from a water bath.

Analysis. The gross composition of the reaction mixture (triglyceride, partial glycerides and free fatty acids) was determined by high-temperature gas-liquid chromatography (GLC) of the trimethylsilyl derivatives. Approximately 10 mg of sample was withdrawn from the reaction mixture and added to 100 μL dry pyridine and 100 μL *bis*(trimethylsilyl) trifluoroacetamide (BSTFA). This silylating reagent was previously shown to be suitable for derivatization of partial glycerides and free fatty acids (7). The mixture was heated for 15 min at 100°C, cooled and diluted with 3 mL hexane. Cold on-column injection of 0.2 μL of sample was performed into a Carlo Erba HRGC gas chromatograph equipped with an on-column injector (Carlo Erba, Milano, Italy). A glass capillary column 10 m long with an internal diameter of 0.3 mm containing SE30 as stationary phase was used. The operating conditions were as follows: initial temperature 80°C, hold for 2 min, heating rate 18°C/min to final temperature of 340°C, hold for 10 min. Hydrogen was used as carrier gas at a flow rate of 20 mL/min with flame ionization detection at 360°C. Response factors were determined with Sigma standards of triolein, diolein and monoolein.

Semi-preparative separation and recovery of the mono- and diglyceride fractions was carried out by thin-layer chromatography (TLC). Approximately 20 mg of the glycerolysis reaction mixture was dissolved in 200 μL of TLC development solvent. Development solvent was chloroform/acetone 96:4 (8). The dissolved sample was applied as a band to a preformed TLC plate of silica gel G, 0.25 mm layer, containing fluorescent indicator, dimensions

*To whom correspondence should be addressed at Institute for Food Chemistry, Hanover University, Wunstorfer Strasse 14, 3000 Hanover 91, Germany.

5 cm × 10 cm. After development and room temperature drying, the bands were visualized under ultraviolet (UV) illumination and scratched off. The mono- and diglyceride fractions were identified by converting the recovered bands to trimethylsilyl derivatives (as described above) followed by high-temperature GLC analysis.

The fatty acid composition of the mono- and diglyceride fractions was determined by capillary GLC. The partial glycerides were converted to glycerol and fatty acid methyl esters by reacting the recovered bands with an excess of 1% sodium methylate in methanol. After extraction of the methyl esters into hexane, separation was achieved on a 30 m × 0.25 mm J&W DB23 column (J&W, Folsom, CA). A 1 μL sample was injected splitless at a column temperature of 80°C (40 s) followed by rapid heating to 150°C. The final temperature of 195°C was reached by means of a temperature program of 3°C/min and was held at that temperature for 5 min. The injector and detector temperatures were 250°C, and a hydrogen carrier gas flow rate of 3 mL/min was used.

Lipase activity was determined by the olive oil/surfactant nonaddition method as previously described (9).

Materials. BSTFA, partial glyceride standards, fatty acid methyl ester standards and preformed TLC plates, 10 cm × 5 cm, 0.25 mm layer containing 254 nm fluorescent indicator were purchased from Sigma Chemical Company, St. Louis, MO. Glycerol (water free) was obtained from Fluka Chemicals, Buchs, Switzerland. Fats and oils were kindly supplied free of charge by the following firms: beef tallow from Robert Unkel Co. Ltd., Wuerzburg, Germany; lard from Caesear and Loretz Co. Ltd., Hilden, Germany; rapeseed oil (zero erucic acid) and soybean oil from Noblee and Thoerl Co. Ltd., Hamburg, Germany. Lipases were kindly donated by the following firms: *Pseudomonas fluorescens* crude (PS) from Amano Pharmaceutical Co. Ltd., Nagoya, Japan; *Chromobacterium viscosum* from Toyo Jozo Co. Ltd., Shizuoka, Japan; *Mucor miehei* (SP 225) and SP398 from Novo Nordisk Biotechnology, Mainz, Germany.

RESULTS

Gross composition of the reaction mixture after glycerolysis. GLC analysis of the reaction mixture, after trimethylsilyl derivatization, resulted in a separation of products on a class basis into free fatty acids (FFA), monoglycerides (MG), diglycerides (DG) and triglycerides (TG), and a further separation within each class according to carbon number. Table 1 shows the composition of the reaction mixture after 24-h reaction time for high-melting fats (beef tallow and lard) and liquid oils (rapeseed oil and soybean oil). The reaction at a temperature above the T_c was compared with the reaction at a temperature below the T_c. For each fat or oil examined, the total MG content was higher at the lower temperature. Closer examination of the carbon number data reveals that the relative proportion of C16 MG to C18 MG was also higher at the lower temperatures. The greatest increase in C16 MG/C18 MG ratio was observed for soybean oil, although the relative increase in total MG was the lowest of the four fats and oils. The concentrations of all molecular species in the DG and TG fractions were lower at the lower temperature for beef tallow and rapeseed oil. In contrast, the content of C36 DG and C54 TG was only slightly reduced for lard, and the C54 TG had slightly increased for soybean oil and the C36 DG was slightly lower.

Effect of temperature on fatty acid composition of the glyceride fractions. Tables 2 and 3 show the fatty acid composition of beef tallow, lard, rapeseed oil and soybean oil in the original fat and in the MG and DG fractions of the reaction mixtures after separation by TLC. After reaction for 24 h at higher temperatures (60°C for the animal fats and 40°C for the vegetable oils), the fatty acid composition of the original fat and both glyceride fractions were almost identical for all fats examined. However, at the lower reaction temperatures (40°C and 5°C, respectively) the fatty acid compositions of the fractions and of the original fat exhibited large differences. For all four fats, the content of the major saturated fatty acids, C18:0

TABLE 1

Effect of Reaction Temperature on the Enzymatic Glycerolysis of Fats and Oils with *Pseudomonas fluorescens* Lipase[a]

	Composition (wt%)												
	FFA		MG			DG				TG			
Fat/oil	C16	C18	C16	C18	C16/C18	C30	C32	C34	C36	C48	C50	C52	C54
Beef tallow													
60°C	1.3	1.8	8.2	15.2	(0.54)	1.1	7.6	19.8	19.9	2.7	7.2	9.6	4.9
40°C	0.8	2.8	28.4	37.7	(0.75)	0.5	3.8	9.1	14.0	0.3	0.9	1.4	0.4
Lard													
60°C	1.3	3.4	7.6	16.3	(0.47)	0.5	6.5	19.6	23.9	1.4	5.1	8.8	5.5
40°C	0.6	3.6	26.6	31.1	(0.86)	0.2	1.4	6.7	18.7	0.2	1.1	3.6	5.2
Rapeseed oil													
40°C	0.2	3.8	1.4	24.5	(0.06)	0.0	0.0	5.5	44.7	0.3	0.0	2.7	14.9
5°C	0.1	4.0	5.1	59.4	(0.09)	0.0	0.1	1.3	22.2	0.8	0.0	0.3	6.0
Soybean oil													
40°C	0.5	3.8	2.6	20.7	(0.13)	0.0	0.6	9.9	38.2	0.0	0.8	6.7	15.8
5°C	0.1	4.4	10.7	30.4	(0.35)	0.0	0.2	2.0	34.9	0.1	0.0	0.8	16.5

[a]Composition of the reaction mixture after 24 h (60°C and 40°C) or 7 d (5°C), determined by high-temperature gas chromatography. FFA, free fatty acid; MG, monoglyceride; DG, diglyceride; TG, triglyceride.

TABLE 2

The Effect of Temperature on the Fatty Acid Composition of the Monoglyceride (MG) and Diglyceride (DG) Fractions After Glycerolysis of Animal Fats with *Pseudomonas fluorescens* Lipase

	Fatty acid composition (wt%)								
Fat/oil	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	Other
Beef tallow									
Original fat	3.1	27.3	2.3	22.8	35.0	2.4	0.3	0.3	6.2
60°C									
MG	3.0	27.3	2.2	21.3	33.2	2.7	0.6	0.2	9.1
DG	3.0	24.9	2.6	18.9	35.4	3.0	0.4	0.2	11.3
40°C									
MG	3.0	38.2	1.1	31.6	17.0	1.2	0.2	0.3	7.3
DG	5.6	15.6	4.2	8.2	49.8	4.1	1.5	0.0	10.0
Lard									
Original fat	1.5	26.4	2.2	17.5	39.0	9.5	0.9	0.3	2.7
60°C									
MG	1.5	24.5	2.1	14.9	41.1	9.8	1.0	0.4	4.7
DG	1.3	21.9	2.4	13.4	44.1	11.6	1.2	0.3	3.8
40°C									
MG	1.7	35.7	1.7	20.6	29.0	6.9	0.8	0.3	3.2
DG	2.3	19.5	3.2	7.0	49.6	12.8	1.4	0.1	3.8

TABLE 3

The Effect of Temperature on the Fatty Acid Composition of the Monoglyceride (MG) and Diglyceride (DG) Fractions After Glycerolysis of Vegetable Oils with *Pseudomonas fluorescens* Lipase

	Fatty acid composition (wt%)								
Oil	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	Other
Rapeseed oil									
Original oil	0.0	5.0	0.0	1.5	54.7	24.4	11.3	0.6	2.6
40°C									
MG	0.0	5.2	0.3	1.5	53.1	24.8	12.1	0.6	2.5
DG	0.0	4.7	0.0	1.3	51.9	25.9	12.9	0.6	2.6
50°C									
MG	0.1	8.4	0.2	2.4	57.9	17.9	8.8	0.8	3.5
DG	0.1	1.4	0.3	0.3	45.8	32.6	16.8	0.2	2.6
Soybean oil									
Original oil	0.0	10.4	0.0	3.6	23.1	55.8	4.5	0.4	2.1
40°C									
MG	0.0	10.8	0.1	3.5	21.9	55.4	4.9	0.4	3.1
DG	0.0	9.2	0.0	3.1	21.5	57.2	6.0	0.4	2.7
5°C									
MG	0.0	26.2	0.0	8.1	18.2	39.4	3.8	0.7	3.5
DG	0.1	2.1	0.0	0.8	23.1	64.5	5.9	0.1	3.5

and C16:0, was higher in the MG fraction and correspondingly lower in the DG fraction. The effect was most obvious for the vegetable oils where the saturated fatty acid content in the MG fraction ranged from six to thirteen times greater than that in the DG fraction. With beef tallow and lard, the excess ranged from two- to fourfold. The opposite was true for the unsaturated fatty acids, which were present in higher concentrations in the DG fraction than in the MG fraction. This effect was greater for tallow and lard with a two- to sixfold excess. The excess in the case of the vegetable oils was less than twofold. Rapeseed oil was an exception where the C18:1 content was higher in the monoglyceride fraction.

Time course of the reaction. Figure 1 shows the change in concentration of the major fatty acids in the MG and DG fractions during the course of beef tallow glycerolysis at 40°C. A short lag period occurred between 0 time and 1 h, where the concentration of most of the fatty acids changed little. After the lag period, most of the changes occurred between 1 and 6 h of the reaction period. In the MG fraction, the content of C16:0 showed an increase up to 4 h reaction time, while the C18:0 content increased linearly up to approximately 6 h. A sharp reduction in the content of C18:1 occurred between 1 and 4 h, with little change thereafter. In the DG fraction, the increase in C18:1 content and the decrease in the C16:0 and C18:0 contents was approximately linear between 1 and 6 h reaction time with little subsequent change. Although little change in the fatty acid composition of the MG and DG fractions occurred after 6 h, the total monoglyceride content in the reaction mixture continued to increase up to 22 h. In Figure 2 the effect of temperature programming

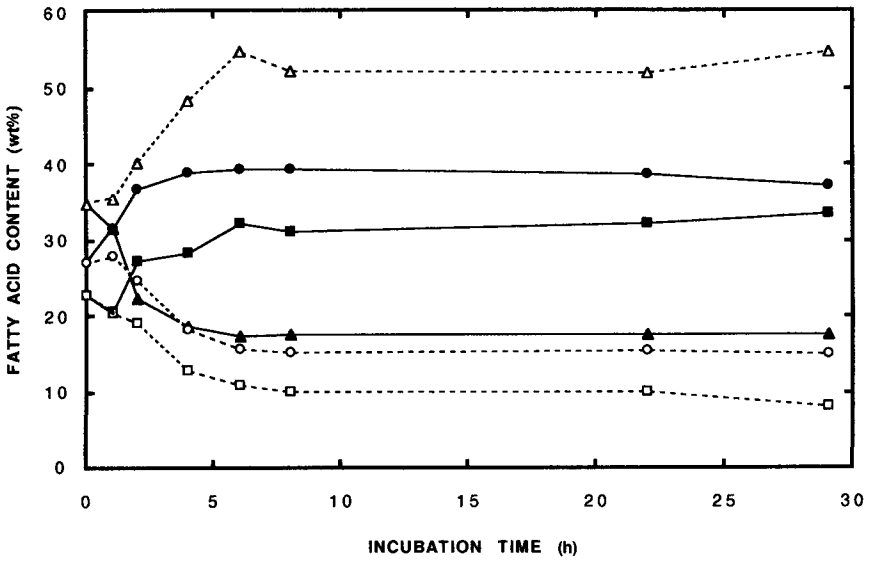

FIG. 1. Change in fatty acid composition of the monoglyceride (MG) and diglyceride (DG) fractions during the course of *P. fluorescens* lipase-catalyzed glycerolysis of beef tallow (40°C): Solid lines = MG, C18:0 (●), C16:0 (■), C18:1 (▲); broken lines = DG, C18:0 (○), C16:0 (□), C18:1 (△).

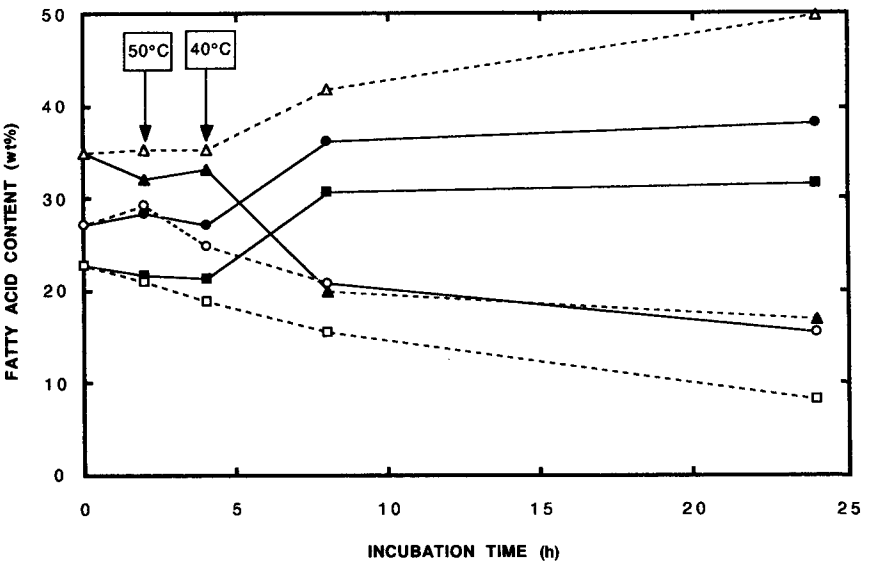

FIG. 2. Effect of altering the temperature during the course of *P. fluorescens* lipase-catalyzed glycerolysis of beef tallow on the fatty acid composition of the monoglyceride (MG) and diglyceride (DG) fractions. Starting temperature is 60°C. Arrows show the time at which the temperature was altered. Solid lines = MG, C18:0 (●), C16:0 (■), C18:1 (▲); broken lines = DG, C18:0 (○), C16:0 (□), C18:1 (△).

on the fatty acid composition of the MG and DG fractions little difference in the fatty acid composition between the fractions occurred, while the total MG content reached approximately 25%. Upon reducing the temperature to 40°C, the monoglyceride content increased further to approximately 60% with an increase in the content of saturated fatty acids and a corresponding decrease in unsaturated fatty acid content in the MG fraction. The corresponding but opposite changes in the fatty acid composition were observed in the DG fraction. In contrast to the data shown in Figure 1, the changes occurred slowly in a 20-h reaction period.

Effect of glycerol content and enzyme type on the fatty acid composition. Table 4 shows that the changes in the fatty acid composition of the MG and DG fractions during glycerolysis of beef tallow with *Pseudomonas fluorescens* lipase at 40°C are independent of the glycerol content over a broad range of concentrations. With *Mucor miehei* and SP398 lipases, the changes in fatty acid composition were qualitatively the same as those observed for

TABLE 4

The Effect of Glycerol Content and Lipase Type on the Fatty Acid Composition of the Monoglyceride (MG) Fraction After Enzymatic Glycerolysis of Beef Tallow for 24 h at 40°C (total MG concentration is also shown)

	Fatty acid composition (wt%)									MG
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	Other	(wt%)
Glycerol/fat mole ratio										
Original fat	3.1	27.3	2.3	22.8	35.0	2.4	0.3	0.3	6.2	
2.2:1	3.0	38.2	1.1	31.6	17.0	1.2	0.2	0.3	7.3	(68.0)
4.5:1	2.8	37.6	1.2	31.2	18.6	1.4	0.3	0.2	6.5	(65.5)
9.1:1	2.9	38.2	1.1	31.5	17.7	1.4	0.2	0.2	6.4	(66.1)
Enzyme type										
Amano PS	3.0	38.2	1.1	31.6	17.0	1.2	0.2	0.3	7.3	(68.0)
SP 225	2.7	34.5	1.6	29.6	24.6	2.0	0.3	0.3	4.1	(43.4)
SP 398	2.4	33.7	1.5	31.8	22.6	1.8	0.3	0.3	5.7	(30.1)

Pseudomonas fluorescens but the magnitude was considerably less, in particular for SP398 lipase. The total MG content was also lower than that obtained with *Pseudomonas fluorescens* lipase.

DISCUSSION

It was previously shown (4–6) that the concentration of monoglyceride in the reaction mixture after enzymatic glycerolysis of triglyceride fats and oils depended strongly on the reaction temperature. Moreover, a higher monoglyceride content was only obtained at lower temperatures, and a sharp transition temperature between the high and low monoglyceride states (called the critical temperature, T_c) was observed for most fats examined. In the work described here, this phenomenon has been confirmed for beef tallow and lard at 40°C and for rapeseed oil and soybean oil at 5°C, by GLC analysis of the reaction mixture. The effect was small for soybean oil, which is also in agreement with previous work (5). However, GLC analysis provides further compositional information that could not be obtained from the Iatroscan analytical method used in the earlier studies.

The ratio of C16/C18 MG shows a clear dependency on the reaction temperature. The observation that the ratio is always higher at the lower temperature shows a preference for synthesis of monoglycerides containing C16 fatty acids. In these fats, the main C16 fatty acid is C16:0; therefore, this monoglyceride is mostly composed of monopalmitin. The C18 monoglycerides are a mixture of monostearin (C18:0) and monoglycerides containing C18 unsaturated fatty acids. Because the absolute amount of C18 monoglycerides was also higher at the lower temperature, it is possible that the monostearin content had increased with no change in the content of other C18 MG species. In this case, the observed increase in the total monoglyceride content is mainly due to synthesis of monopalmitin and monostearin. The changes in the di- and triglyceride fractions support this conclusion. Lard and rapeseed oil have a low content of C18:0 in comparison with beef tallow and soybean oil, respectively. The apparent resistance of lard and rapeseed oil di- and triglycerides, containing only C18 fatty acids (C36 and C54), to conversion to monoglycerides can be explained by assuming they contain mainly unsaturated C18 fatty acids.

Analysis of the fatty acid composition of the MG and DG fractions in the reaction mixture shows clearly that at lower reaction temperatures the monoglycerides contain a higher proportion of saturated fatty acids than the diglycerides. This effect is large: a two- to threefold higher concentration of saturated fatty acids was detected in the monoglyceride fraction. The accumulation of saturated fatty acids in the MG fraction was always accompanied by solidification of the reaction mixture and a large increase in the overall MG concentration. When lipases are used that result in a relatively low yield of MG at the lower temperatures (*Mucor miehei* and SP398), a relatively lower concentration of saturated fatty acids also accumulates in the MG fraction. These observations indicate that the higher concentration of monoglyceride obtained at lower temperatures is due to a disruption of the chemical equilibrium caused by the crystallization of monoglycerides containing saturated fatty acids. Further evidence for the preferential crystallization of monoglycerides is provided by the occurrence of a lag period in the time-course experiments. Crystallization processes are relatively slow and are concentration-dependent. Thus, the lag period can be explained by the need to reach a sufficiently high concentration of MG and a definite period of time to initiate crystallization.

Although the content of saturated fatty acids in soybean is higher than that in rapeseed oil, the final concentration of monoglyceride in the soybean oil reaction mixture after glycerolysis at 5°C was lower. The reverse should be true if further MG synthesis at low temperature is only due to crystallization of monoglyceride containing a saturated fatty acid. The rapeseed oil used in this study had a high content of oleic acid (C18:1, 55%) and was unique among the fats examined because the C18:1 content increased in the MG fraction during glycerolysis at the lower temperature. With both vegetable oils, the content of C18 monoglycerides was higher at the lower reaction temperature, the effect being greater with rapeseed oil. From these observations it may be deduced that at 5°C monoglycerides that contain a fatty acid with a single double bond crystallize from the reaction mixture. This may explain the previous finding (5) that a yield of 90% MG was obtained at 10°C from olive oil (olive oil contains approximately 80% C18:1). It can be postulated that at even lower reaction temperatures, monoglycerides containing two or more double bonds could be induced to preferentially crystallize.

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