Chapter 2

MATERIALS AND METHODS

Materials

1. Lipases

Lipase PS (*Pseudomonas* sp.), lipase AK (*Pseudomonas fluorescens*), lipase AY (*Candida rugosa*), lipase D (*Rhizopus delemar*), lipase M (*Mucor javanicus*), lipase F (*Rhizopus oryzae*) were gifts from Amano Pharmaceutical Co. Ltd., Nagoya, Japan. Lipase OF (*Candida rugosa*) and lipase PL (*Alcaligenes* sp.) were gifts from Meito Sangyo Co. Ltd., Japan. Lipase LP (*Chromobacterium viscosum*) was a gift from Asahi Chemical Industry Co. Ltd., Japan.

2. Supports

The supports were celite 545 (200 μm) from Wako Pure Chemical Industries, Ltd., silica gel 60 (200 μm) from Merck Co. Ltd., $CaCO_3$ (Softon 3200) from Shiraishi Calcium Co. Ltd. The polypropylene powder EP-100 : Accurel (<200 and 200-400 μm) was a gift from Akzo Nobel (Obernburg, Germany). The activated charcoal was purchased from Fluka Chemical Co. Ltd.

3. Raw materials

Palm olein was purchased from Morakot Industry Co. Ltd., Thailand. Glycerol was purchased from Carlo Erba Reagenti Co. Ltd., Italy.

4. Chemicals

Company	Chemicals
Carlo Erba Reagenti Co. Ltd., Italy	Glycerol
	Sodium carbonate
	Sodium dihydrogen phosphate
	Sodium hydroxide
	Sodium phosphate
	Sodium sulphate anhydrous
	Sodium-potassium tartrate
Fluka Chemie GmbH, Germany	Albumin serum
	Cupric acetate
	Folin-Ciocalteu's phenol reagent
	Potassium hydroxide
	Potassium iodide
	Pyridine
J.T. Baker Inc., USA	Hydrocloric acid
Labscan Asia Co. Ltd., Thailand	Acetone
	Benzene
	Chloroform
	Ethanol
	Hexane
	Isooctane
	Methanol
Merck KGaA, Germany	Acetic acid
	Phenolphthalein
Nacalai Tesque Inc., Japan	1,2-Dioleoyl-rac-glycerol
	1,3-Diolein
	Capric acid
	Caprylic acid
	Lauric acid

Company	Chemicals
Nacalai Tesque Inc., Japan	Linoleic acid
	Myristic acid
	Oleic acid
	Palmitic acid
	Stearic acid
	Triolein
	Monopalmitin
Sigma Chemical Co., USA	Copper sulfate pentahydrate

5. Instruments

Instruments	Model	Company
Evaporator	SB-651	Tokyo Rikakikai Co. Ltd., Japan
Gas chromatography	XL	Perkin-Elmer Corporation, USA
Hot air oven	UM200	Memmert Ltd, Germany
IATROSCAN	MK5	Iatron Laboratories Inc., Japan
Incubator	MIR-153	Sanyo Electric Co. Ltd., Japan
Magetic stirrer	RCT, RO 5	Ika Werke GmbH, Germany
Microcentrifuge	8080	Centurion Scientific Ltd., UK
Peristatic pump	EP-1	Bio-Rad Laboratories Ltd., USA
PH meter	420A	Orion Research Inc., USA
Spectrophotometer	U-2000	Hitachi Ltd., Japan
Suction pump	A-3S	Tokyo Rikakikai Co. Ltd., Japan
Water bath	W350	Memmert GmbH & Co. KG, Germany

Methods

A. Analytical methods

1. Acid value was measured by the method of AOAC (1999) (Appendix 1).
2. Iodine value was determined by the method of AOAC (1999) (Appendix 2).
3. Soluble protein was determined by the method of Lowry *et al*. (1951) (Appendix 3).
4. Sponification value was determined by the method of AOAC (1999) (Appendix 4).
5. Hydrolytic activity of lipase (Lee and Rhee , 1993)

Hydrolytic activity of lipase was assayed by the modified cupric acetate method. At first, 5 % (w/v) aqueous solution of cupric acetate was prepared and filtered, the pH being adjusted to 6.1 using pyridine. For the lipase reaction in two-phase system, 0.2 mL of enzyme solution (2.0 mg for immobilized enzyme), 1.0 mL of 0.1 M phosphate buffer (pH 7.0), and 1.5 mL of 10 % palm olein in isooctane was incubated at 500 rpm and 30 °C for 30 min. The enzyme reaction was stopped by adding 0.3 mL of 6 M HCl.

The upper isooctane layer of 1.0 mL was taken out and mixed with 0.4 mL cupric acetate solution. Free fatty acids dissolved in isooctane were determined by measuring the absorbance of isooctane solution at 715 nm against the control, which contained no free fatty acid. Lipase activity was determined by measuring the amount of fatty acids from the standard curves of palmitic acid (Appendix 5). One unit of enzyme activity was difined as the enzyme necessary to release 1 μmol of palmitic acid per minute at the specified condition.

6. Fatty acid compositions (Shimada *et al*., 1994)

The fatty acid compositions of triglycerides were determined by converting all fatty acids of triglycerides into the corresponding fatty acid methyl esters followed by gas chromatography (GC) analysis (McNeill *et al*., 1996). After evaporation of excess solvent *in vacuo*, 10 μL of the oily residue were methylated with 0.5 % NaOH in methanol (500 μL) and then incubated for 10 min at 60 °C. The methyl esters were extracted with hexane (400 μL) for 1 min. The hexane layer was washed with 200 μL distilled water and dried over anhydrous sodium sulfate. Analysis was carried out with a PERKIN-ELMER AutoSystem XL Gas Chromatograph (Perkin-Elmer Corporation Norwalk, CT, USA) on a Optima 5 column (Optima-5-0.25 μm 25 m x 0.25 mm ID, Macherey-Nagel, Germany) (Shimada *et al*., 1994) and split ratio 50:1. Helium was used as the carrier gas with 1.65 mL/min flow rate. The temperature program used was 150 °C (40 °C/min, 0.5 min), 170 °C (5 °C/min), 195 °C (10 °C/min) and 215 °C (9.5 min). Injector and detector temperatures were 250 °C. Response factors were determined using a standard mixture of fatty acid methyl esters (Appendix 6).

7. Oil compositions (Rosu *et al*., 1997)

The course of glycerolysis was monitored by intermittent sampling (150 mg) and dissolved in 0.3 mL chloroform/methanol (1:1). The enzyme was separated by centrifugation at 10,000 rpm for 5 min. The removal of glycerol from the supernatant solution was achieved by extraction with water (0.1 mL). For an effective extraction of partial glycerides (soluble in water to some extent), two subsequent extractions of the water layer with 0.3 mL chloroform each were performed. The total chloroform extract (approximately 0.9 mL) was concentrated to dryness in a N_2 stream. Chloroform (0.2 mL) was added to the concentrate, and the solution was analyzed by a thin-layer chromatography/flame ionization detector (TLC/FID) (Iatroscan MK5, Iatron

Laboratories Inc., Japan) with Chromarod S III quartz rods. One to 3 μL chloroform extract was applied to the rods, followed by development in benzene/chloroform/acetic acid (50:20:0.7) for 10 cm. The rods were dried and scanned under the following conditions: hydrogen flow rate, 160 mL/min, air flow rate, 2.0 L/min, 30 s/scan. TAG, 1,3-DAG, 1,2(2,3)-DAG, MAG and FFA were effectively separated. Peak areas were calculated with the chromatography data system ChromStar. All results throughout this article were expressed as percentage of peak areas of the reaction mixture's components on a glycerol-free basis. They were regarded as weight percentages, and may vary slightly from the actual weight percent (Appendix 7).

B. Experiments

The experiments were designed in Completely Randomized Design (CRD). Analysis of Varian (ANOVA) and Duncan's Multiple Range Test (DMRT) were used for data analysis. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc.).

1. Commercial lipase for MAG production

The hydrolytic and specific activity of nine commercial lipases were determined using the modified cupric acetate method (Lee and Rhee, 1993).

2. Physical and chemical properties of palm olein

Physical and chemical properties of palm olein were determined. The parameters studied were sponification value, iodine value, acid value and fatty acid composition.

3. Selection of commercial lipases for MAG production

Selection of commercial lipases for MAG production was performed in batch system. The reaction mixture consisted of palm olein (15 g), glycerol contained 4% (w/w) water (4.46 g) and lipase (500 U). The glycerol to palm olein molar ratio was 2.7:1 (Borncheruer and Yamane, 1994). The reaction

mixture was mixed by magnetic stirrer at 300 rpm and the temperature was controlled at 30 °C. The reaction mixture was sampled for 6 h and analyzed using TLC/FID analyzer. The lipase which gave the highest % MAG in the reaction mixture will be chosen for further study.

4. Selection of support for immobilized lipase

Lipase (section 3) 500 U was dissolved in 5 mL 0.1 M phosphate buffer pH 7.0. Several supports (0.5 g): Accurel EP100 (<200 and 200-400 μm), $CaCO_3$, celite, silica gel and activated charcoal were added in the solution and the mixture was stirred using a magnetic stirrer for 60 min at 100 rpm and 30 °C. Hydrophobic support material was prewet with ethanol before addition to the lipase solution. Afterward, 5 mL of 0.1 M phosphate buffer pH 7.0 was added and the suspension was filtered through a Buchner funnel. The immobilized enzyme was washed on the filter paper with another 5 mL of 0.1 M phosphate buffer pH 7.0 to remove unadsorbed soluble enzyme and dried in a vacuum desiccator overnight (Rosu *et al*., 1997). The hydrolytic activity of unadsorbed soluble enzyme and immobilized enzyme were determined. The immobilized yield was calculated using the following formula:

$$\text{Immobilized yield} = \frac{\text{Total immobilized enzyme activity (U)}}{\text{Total initial soluble enzyme activity (U)}} \times 100$$

5. Optimal condition for enzyme immobilization

The parameters that effect on immobilized lipase were investigated.

5.1 Effect of lipase concentration

Lipase (section 3) was immobilized as described above with suitable support (section 4) and varying concentration of lipase at 5, 10, 50, 100 and 150 U/mL. The suitable concentration of lipase for immobilized lipase was chosen for further study. The immobilized activity and immobilized yield were calculated.

5.2 Effect of temperature

Lipase (section 3) was immobilized as described above with suitable support (section 4), suitable concentration of lipase (section 5.1) and varying temperature for immobilization at 4, 25, 30 °C. The suitable temperature for immobilized lipase was chosen for further study. The immobilized activity and immobilized yield were calculated.

5.3 Effect of time

Lipase (section 3) was immobilized as described above with suitable support (section 4), suitable concentration of lipase (section 5.1), suitable temperature (section 5.2) and varying the immobilization time for 5, 10, 15, 20, 30, 60, 120 and 240 min. The suitable immobilization time for immobilized lipase was chosen for further study. The immobilized activity and immobilized yield were calculated.

6. Properties of immobilized lipase

6.1 Optimum temperature

The effect of temperature on the hydrolytic activity of immobilized lipase (section 5) was assayed at various temperature of 30, 35, 40, 45, 50, 55, 60 and 65 °C. Immobilized lipase activities were expressed as relative activity.

6.2 Thermostability

Thermostability of immobilized lipase (section 5) was studied by incubating immobilized lipase for 5, 10, 15, 20 and 25 h at 45 °C. The remaining activity were determined and expressed as relative activity.

7. Selection of reactor types for MAG production

MAG production was performed in several reactors.

7.1 Continuous MAG production in CSTR

The immobilized lipase (1.5 g) was placed in a jacketed cylindrical vessel (4.5 cm ID, 6.0 cm height) and agitated by magnetic stirrer at 300 rpm. The substrate included palm olein, glycerol and water. The molar ratio of glycerol to palm olein was 2.7:1 and glycerol containing 4 % (w/w) distilled water. The substrate was introduced on the top with the flow rate of 0.02 mL/min. The product was filtered by polyvinylidene fluoride (PVDF) membrane (pore size, 0.45 μm) and removed near the bottom. The reaction temperature was controlled at 45 °C (Figure 6). The composition of oil phase was analyzed using TLC/FID analyzer.

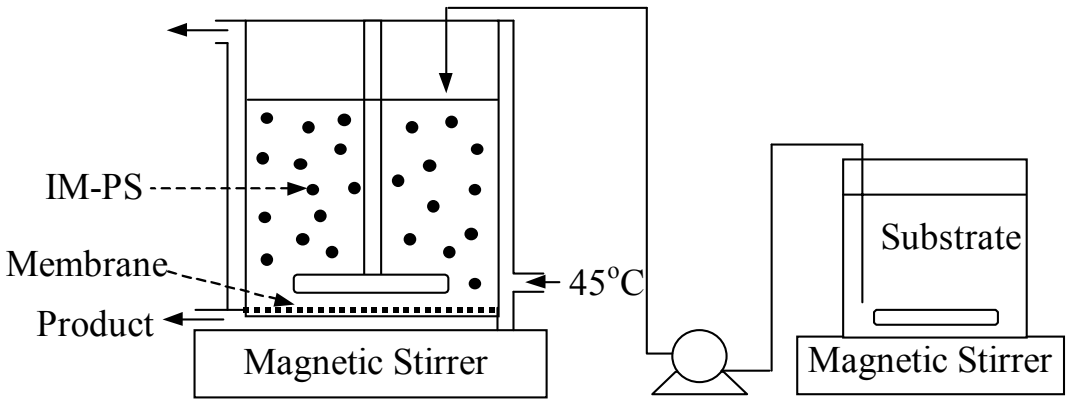

Figure 6 Schematic diagram for continuous glycerolysis of palm olein by IM-PS in CSTR

7.2 Continuous MAG production in PBR

The immobilized lipase (1.5 g) was packed in a jacketed column (0.68 cm ID, 25 cm long). The substrate included palm olein, glycerol and water. The molar ratio of glycerol to palm olein was 2.7:1 and glycerol containing 4 % (w/w) distilled water. The substrate was introduced downward the column with the flow rate of 0.02 mL/min. The reaction temperature was controlled at 45 °C. The product was removed at the bottom of the column (Figure 7). The composition of oil phase was analyzed using TLC/FID analyzer.

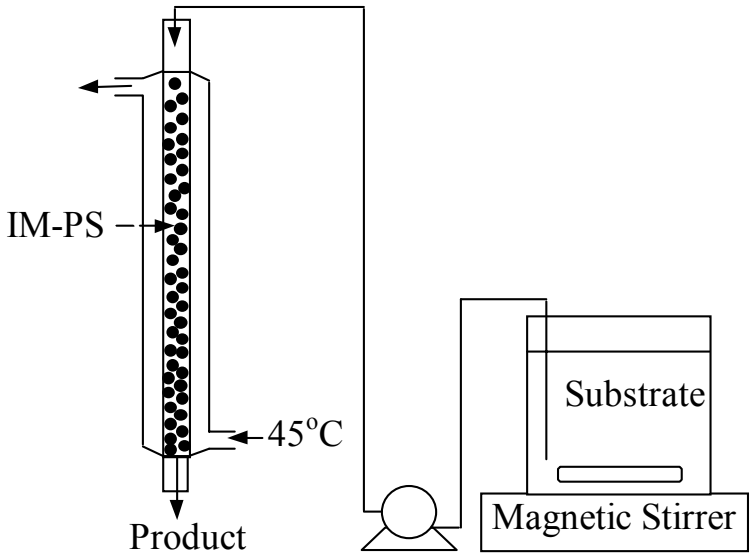

Figure 7 Schematic diagram for continuous glycerolysis of palm olein by IM-PS in PBR

The reactor which gave the highest % MAG in the reaction mixture was chosen for further study.

8. Optimization of MAG production in solvent system

8.1 Effect of solvent type

The initial condition for MAG production in solvent system included 250 U immobilized lipase, 20 ml of 30 %(w/v) palm olein in organic solvent (acetone, hexane and isooctane), 1.78 g glycerol and 0.1 g water. The reaction mixture was stirred with magnetic stirrer at 300 rpm and temperature was controlled at 45 °C for 24 h. The composition of oil phase was analyzed using TLC/FID analyzer. The suitable solvent for MAG production was chosen for further study.

8.2 Stability of immobilized lipase in organic solvents

Stability of immobilized lipase in a suitable solvent (section 8.1) was tested at 4 and 45 °C for 24 h.

8.3 Effect of water content in glycerol

MAG production was performed at initial condition with suitable solvent (section 8.1). The water content in glycerol was varied at 4, 6, 8, 10 and 12 %. The composition of oil phase was analyzed using TLC/FID analyzer. The suitable water content in glycerol for MAG production was chosen for further study.

8.4 Effect of the molar ratio of glycerol to palm olein

MAG production was performed in initial condition above with suitable solvent (section 8.1) and water content in glycerol (section 8.3). The molar ratio of glycerol to palm olein was varied at 1.5:1, 2:1, 2.7:1, 3:1 and 3.5:1. The composition of oil phase was analyzed using TLC/FID analyzer. The suitable molar ratio of glycerol to palm olein for MAG production was chosen for further study.

8.5 Effect of palm olein cencentration in solvent

MAG production was performed in initial condition as described above with suitable solvent (section 8.1), suitable water content in glycerol (section 8.3), suitable molar ratio of glycerol to palm olein (section 8.4). The palm olein concentration was varied at 5, 10, 20, 30, 40, 50, 60 and 70 % in solvent. The composition of oil phase was analyzed using TLC/FID analyzer. The suitable palm olein concentration for MAG production was chosen for further study.

8.6 Effect of amount of immobilized lipase

MAG production was performed in initial condition with suitable solvent (section 8.1), suitable water content in glycerol (section 8.3), suitable molar ratio of glycerol to palm olein (section 8.4), suitable palm olein concentration in solvents (section 8.5). The amount of immobilized lipase was varied at 10, 20, 30, 40, 50 and 60 % in oil. The composition of oil phase was analyzed using TLC/FID analyzer. The suitable amount of immobilized lipase for MAG production was chosen for further study.

8.7 Effect of temperature

MAG production was performed in initial condition with suitable solvent (section 8.1), suitable water content in glycerol (section 8.3), suitable molar ratio of glycerol to palm olein (section 8.4), suitable palm olein concentration in solvents (section 8.5), suitable amount of immobilized lipase (section 8.6). The temperature of the reaction was varied at 25, 35, 45 and 55 °C. The composition of oil phase was analyzed using TLC/FID analyzer. The suitable temperature for MAG production was chosen for further study.

9. MAG production under optimum condition

MAG production was performed at optimal condition (section 8.7). The reaction mixture was sampled at time interval for 24 h to analyze for the composition of oil phase using TLC/FID analyzer.

10. Kinetics of glycerolysis of palm olein with glycerol by soluble and immobilized lipase

The condition of glycerolysis was set as optimized condition that was chosen previously. The Michaelis-Menten constants of soluble and immobilized lipase were determined by varying the concentration of the single substrate (palm olein) in the range of 30 to 60 mM in the presence of a fixed saturating concentration of the other . The kinetic parameters of soluble and immobilized lipase were determined using the Lineweaver-Burk plot of initial velocity against varied substrate concentration.

11. Continuous MAG production in solvent system

11.1 Continuous MAG production in CSTR

The immobilized lipase (1.5 g) was placed in a jacketed cylindrical vessel (4.5 cm ID, 6.0 cm height) and agitated by magnetic stirrer at 300 rpm. The suitable composition of substrate: palm olein, glycerol, water and organic solvent (section 8.6) was introduced near the top with the flow rate of 0.02 mL/min and the product was removed near the bottom. The reaction temperature was controlled at suitable temperature (section 8.7). The composition of oil phase was analyzed using TLC/FID analyzer.

11.2 Continuous MAG production in PBR

The immobilized lipase (1.5 g) was packed in a jacketed column (0.68 cm ID, 25 cm long). The suitable composition of substrate: palm olein, glycerol, water and organic solvent (section 8.6) was introduced on the top of the column with the flow rate of 0.02 mL/min. The temperature was controlled at suitable temperature (section 8.7). The product was removed at the bottom of the column. The composition of oil phase was analyzed using TLC/FID analyzer.

The reactor which gave the highest % MAG in the reaction mixture was chosen for further study.

12. Optimization of continuous MAG production in solvent system

Several effects on MAG production in the suitable reactor (section 11) were investigated.

12.1 Effect of molar ratio of glycerol to palm olein

Continuous MAG production in solvent system was performed in the suitable reactor (section 11). The molar ratio of glycerol to palm olein was varied at 0:1, 4:1, 8:1, 12:1 and 16:1. The composition of oil phase in product was analyzed using TLC/FID analyzer. The suitable molar ratio of glycerol to palm olein was chosen for further study.

12.2 Effect of water content in glycerol

Continuous MAG production in solvent system was performed in the suitable reactor (section 11) with suitable molar ratio of glycerol to palm olein (section 12.1). The water content in glycerol was varied at 5, 10, 15, and 20 % (w/w). The composition of oil phase in product was analyzed using TLC/FID analyzer. The suitable water content in glycerol was chosen for further study

12.3 Effect of substrate flow rate

Continuous MAG production in solvent system was performed in the suitable reactor (section 11) with suitable molar ratio of glycerol to palm olein (section 12.1), suitable water content in glycerol (section 12.2). The substrate flow rate was varied at 0.01, 0.02, 0.04 and 0.06 mL/min. The composition of oil phase in product was analyzed using TLC/FID analyzer. The suitable substrate flow rate was chosen for further study

12.4 Effect of temperature

Continuous MAG production in solvent system was performed in the suitable reactor (section 11) with suitable molar ratio of glycerol to palm olein (section 12.1), suitable water content in glycerol (section 12.2), suitable substrate flow rate (section 12.3). The temperature of reaction was varied at 35,

45 and 55 °C. The composition of oil phase in product was analyzed using TLC/FID analyzer. The suitable temperature was chosen for further study

12.5 Continuous MAG production under optimum condition

Continuous MAG production in solvent system was performed in the suitable reactor (section 11) with suitable molar ratio of glycerol to palm olein (section 12.1), suitable water content in glycerol (section 12.2), suitable substrate flow rate (section 12.3) and suitable temperature (section 12.4). The composition of oil phase in product was analyzed using TLC/FID analyzer.

13. Time course of continuous MAG production in solvent system

Time course of continuous MAG production in solvent system in the suitable reactor (section 11) was investigated using optimal condition (section 12). The products were sampled and analyzed using TLC/FID analyzer.

14. Large scale of continuous MAG production in solvent system

The semi-fundamental method in combination with different methods (e.g., dimensional analysis/regime analysis, rules of thumb, scale-down approach/regime analysis, trial and error) were used to scale up reactor and MAG production processes.

Scaling up of the suitable reactor for continuous MAG production (section 11) to 10 times was performed under the optimal condition (section 12) for MAG production processes.

15. Recovery of MAG

15.1 Crystallinzation at low temperature

After continuous MAG production in PBR, residual glycerol was removed from the product mixture by separation with separating funnel and used for further study.

15.1.1 Effect of temperature on MAG crystallization

MAG product was recovered from product mixture using temperature control at 10, 5, 0, -5, -10 and –20 °C for 8 h. Afterward, the mixture was filtered under reduced pressure to remove uncrystallized oil. The MAG product was dried in desiccator for 8 h. The composition of product mixture and MAG product were determined by TLC/FID analyzer. The purity and yield of MAG product were calculated using the following formula. The temperature that gave the highest yield of MAG product was chosen for MAG crystallization in further study.

$$\text{The purity of MAG (\%)} = \text{\% of MAG in MAG product}$$

$$\text{The yield of MAG (\%)} = \frac{\text{Weight of MAG product (g)} \times 100}{\text{Weight of product mixture (g)}}$$

15.1.2 Effect of time on MAG crystallization

MAG product was recovered from product mixture using suitable temperature (section 15.1.1) and varying time for crystallization at 2, 4, 8, 12, 18 and 24 h.

15.2 Crystallization using extraction by organic solvent with temperature control

After continuous MAG production in PBR, residual glycerol was removed from the product mixture by separation with separating funnel. The organic solvent in the product mixture was removed by evaporation using rotary evaporator. Crude MAG product was used for further study.

15.2.1 Effect of organic solvents on MAG crystallization

Crystallization of MAG from crude MAG product was carried out by dissolving crude MAG product in several organic solvents: hexane, isooctane and acetone with 10% w/v concentration and kept at 0 °C for 8 h. Afterward, the mixture was filtered under reduced pressure to remove uncrystallized oil.

The MAG product was dried in desiccator for 8 h. The composition of product mixture and MAG product were determined by TLC/FID analyzer. The purity and yield of MAG product were calculated using the formula in section 15.1.1.

15.2.2 Effect of crude MAG product concentration in organic solvent on MAG crystallization

Crude MAG product was dissolved in suitable organic solvents (section 15.2.1) with varying concentration of crude MAG product in organic solvents at 2.5, 5, 10, 15, 20 and 25 % (w/v). The temperature was controlled at 0 °C for 8 h.

15.2.3 Effect of temperature on MAG crystallization

Crude MAG product was dissolved in suitable organic solvents (section 15.2.1) with suitable concentration of crude MAG product in organic solvents (section 15.2.1) and varying the temperature at 10, 5, 0, -5 and –10 °C for 8 h.

15.2.4 Effect of time on MAG crystallization

Crude MAG product was dissolved in suitable organic solvents (section 15.2.1) with suitable concentration of crude MAG product in organic solvents (section 15.2.2), suitable temperature (section 15.2.3) and varying time for crystallization at 2, 4, 8, 12, 18 and 24 h.

15.3 Fractionation by silica gel 60

The silica gel 60 column was used for recovery of MAG. The column (0.68 cm ID, 25 cm long) contained 5 g silica gel 60. One mL crude MAG product dissolving in hexane (50 %,w/v) was passed through the column and rinsed with hexane to remove glycerides and fatty acid present in the void volume. Afterwards, the column was eluted with 5 % ethanol in hexane (Padt *et al*., 1992). The composition of each fraction was determined by TLC/FID analyzer.