CHAPTER 3
EXPERIMENTAL

3.1 EXTRACTION OF PALM OILS

3.1.1 Materials

Oil palm fruits from *E. guineensis* [Dura (D), Pisifera (P) and Tenera (Te)], *E. oleifera* (O) and their hybrids O x D, O x P as well as the backcross OD x P were collected from Johore Labis Estate, Johore, Malaysia from Nov. 88 - Feb 89. Oil from the Albescens species was kindly supplied by Chemara. All chemicals and solvents used were of analytical grade.

3.1.2 Oil Palm Species and Varieties

Fresh oil palm bunches were cut into small spikelets and autoclaved at 1.032 bars steam pressure (120°C) for 15 minutes. The mesocarp was then separated from the nut and dried at 50°C for about an hour. The oil was extracted from the dried mesocarp with *n*-hexane in a Soxhlet apparatus for 5 hours, *n*-hexane was then rotary-evaporated and the oil was further vacuum dried and kept in refrigerator before analysis. It was found that the total carotene content was not affected significantly using the Soxhlet extraction due to the presence of natural antioxidants (i.e. tocopherol & tocotrienols) in the palm oil.

3.1.3 Residual Oil from Screw-Pressed Fibres

(a) Solvent Extraction

Freshly pressed palm-fruit fibres, collected from several mills immediately after being discharged from the nut/fibre separator, were dried at 50 - 60°C for 1 hour and the residual oil was extracted with solvents such as *n*-hexane and chloroform using a Soxhlet apparatus. The weight of the residual oil was taken after the solvent was removed by a rotary evaporator and pumped dry under vacuum.

(b) High Pressure Liquid CO_2 Extraction

High Pressure Soxhlet Extractor from J&W Scientific Inc. USA was also used to extract the residual oil from fibres using liquid CO_2. The extractor chamber as well as the internal glass Soxhlet extractor is shown in Fig. 3.1. The dried fibres (about 5-6 g) were loaded into the soxhlet assembly where the inlet of the siphon tube was inserted with cotton wool. The soxhlet assembly was then placed inside the pressure chamber and about 160±10 g of dry ice were placed into the shell but outside of the glass assembly. The pressure cap was installed carefully to ensure that the cold finger was positioned right on top of the Soxhlet. All the machine screws were then quickly installed, tightened firmly and the needle valve was closed. The extractor assembly was then placed into the water bath (40-45°C) and the cooled water (5-8°C) was connected to the condenser. After a pressure rise to 800-850 psi followed by commencement of condensation, the pressure of 700-750 psi was obtained because the condensation and extraction cycle had been established and extraction was continued for 2 hours.

Other extraction conditions were also experimented on, e.g. by changing the pressure (by changing the quantity of dry ice), the water bath temperature and the extraction time.

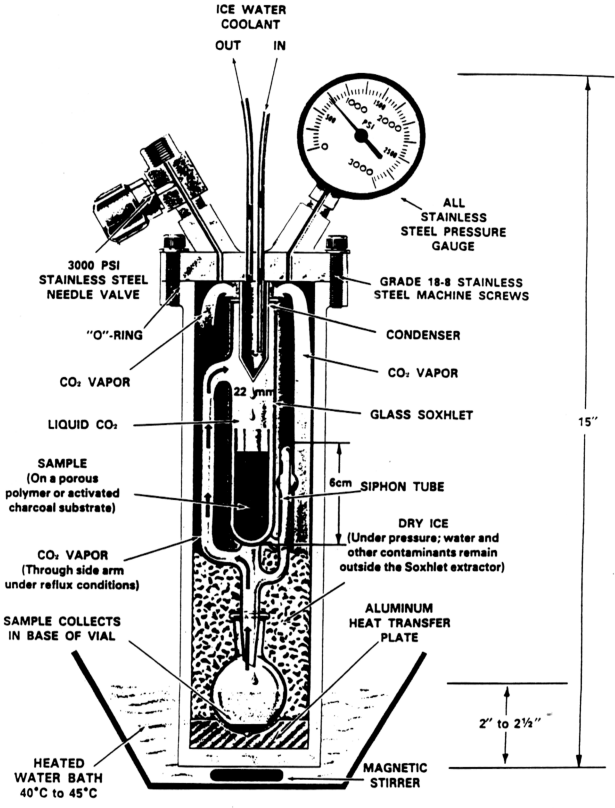

Figure 3.1 High pressure Soxhlet extractor

3.1.4 Double-Screw Pressing of Fibres

The double-pressing process was carried out in the palm oil mill. In the conventional process, fruitlets from fresh fruit bunches after being sterilised, threshed and separated from the stalk, were conveyed into the digester and the oil was then extracted (pressed out) by a single pressing of the screw press. In the double-pressing process, the oil from the fruitlets was first extracted at a low pressure (16 rpm, <90 bars), the fibres were then separated from the nuts in the pressed cake and a second extraction was carried out at higher pressure (12 rpm, >90 bars) on the fibres alone. The second-stage pressed oil was washed and dried separately from the first-stage pressed oil.

3.1.5 Exocarp and Mesocarp of the Oil Palm Fruits

The oil palm fruit spikelets (Tenera variety) were sterilised at 1.032 bars steam pressure (120°C) for 15 minutes. The exocarp (skin) of the sterilised oil-palm fruits was peeled out from the fruits, dried at 50°C for an hour and then extracted with *n*-hexane for about 2 hours using a Soxhlet extractor. The solvent was then rotary-evaporated off and the oil extract was further dried under vacuum and stored in a refrigerator before analysis. Similar procedures were used for the oil extraction of mesocarp and pericarp (mesocarp and exocarp) after they were peeled off from the nut.

A comparative study was also carried out on the fresh oil-palm fruits without sterilisation.

3.2 ANALYSIS OF CAROTENES BY NON AQUEOUS REVERSED-PHASE HPLC

3.2.1 Materials

Lycopene, α- and β-carotenes, used as authentic standards in this study, were from Sigma (St. Louis, USA) and the HPLC-grade acetonitrile and dichloromethane were from Merck (Germany). Petroleum ether (b.p. 40 - 60°C) and ethanol used for saponification were of analytical grade from Merck. All other chemicals and solvents used were of analytical grade.

3.2.2 Saponification Procedure

About 5 g of the oil extracts were weighed accurately into a 150 ml round bottom flask and dissolved in 50 ml of ethanol. The oil was then saponified with 5 ml of 50% ethanolic KOH heated at 50°C in the dark on a water bath under a stream of nitrogen for 45 minutes. The saponified sample was then cooled to room temperature and 2x50 ml of distilled water was then added, unsaponified matter was then extracted with 50 ml portions of petroleum ether until the supernatant became colourless. The combined petroleum-ether extract was washed 4 times with 50 ml portions of distilled water and dried over sodium sulphate. A portion of the extract was brought to dryness in a rotary evaporator at 30°C. The residue was dissolved in a suitable volume of mobile phase, 100 µl of which were injected into the HPLC.

3.2.3 HPLC Analysis

The isocratic separation was performed on a ZORBAX ODS column (4.6 mm ID x 25 cm, stainless steel, 5 µm spherical particles) protected with a Du Pont guard column (20 µm ZORBAX ODS). A solvent system of acetonitrile (89%) and dichloromethane (11%) was used and the flow rate was 1 ml/min.

Analysis and detection of carotenes were carried out using a Varian 5000 HPLC instrument equipped with a variable wavelength (190 - 900 nm) UV-100 detector and a SP 4270 integrator. Detection was recorded at various absorption maxima and attenuated to display the various carotenes present. An external standard of β-carotene was used for quantification based on the peak heights.

A non-aqueous solvent system with 11% of dichloromethane in acetonitrile was chosen to provide separation of the carotenoids as well as to allow for sample solubility. It has been reported that non-aqueous reversed-phase liquid chromatography can enhance chromatographic efficiency, recovery, sample capacity as well as column lifetime.[83]

Individual separated carotene was collected and the absorption spectrum of each carotene was recorded using a Hitachi 150-20 spectrophotometer.

3.2.4 Spectrophotometric Determination of Carotenoids

The total carotenoid content in oil was determined spectrophotometrically at 446 nm as described previously.[202]

Basically, the oil sample was melted at 50-60°C and about 0.1-0.5 g (depending on the carotenoid concentration) of oil was weighed accurately into a 25 ml volumetric flask. The oil sample was dissolved in *n*-hexane and the absorption at 446 nm was determined in a 1-cm cuvette after the base line correction of the solvent blank.

The carotenoid content is expressed as ppm β-carotene based on the following calculation:

$$\text{Carotene content (ppm)} = \frac{478.75\,(A_s - A_b)}{W}$$

where A_s = absorbance of the sample,

A_b = absorbance of the solvent blank,

W = weight of the sample in gram.

3.2.5 Iodine-Catalysed Photoisomerisation of Carotenes

Photoisomerisation of the carotene was carried out by exposing the solutions of the carotenes collected from HPLC [redissolved in *n*-hexane (0.1-1.0 mg/ml) after removal of the HPLC mobile phase] to diffused daylight under nitrogen for 1 hour in the presence of iodine (2% of the wt. of carotene).[46] Iodine was then removed by washing the mixture with 2% aqueous $Na_2S_2O_3$ followed by distilled water and drying over anhydrous Na_2SO_4. The solvent was then removed under N_2 and the products were redissolved in the mobile phase for HPLC analysis.

3.3 HPLC ANALYSIS OF TOCOPHEROLS AND TOCOTRIENOLS

Detailed analyses of the content of tocopherols and tocotrienols in various oil samples were carried out using normal-phase HPLC using the following conditions:- a Lichrosorb analytical column (25 cm x 0.46 cm ID, stainless steel, 5 μm) protected by a guard column (1.5 x 0.46 cm, 10 μm), a solvent system of *n*-hexane : THF : 2-propanol (1000:50:3, v/v/v) with a flow rate of 1 ml/min, and a Water 470 Fluorescence detector at 295 nm excitation and 325 nm emission. Oil samples were dissolved directly in the mobile phase without any pretreatment and 20 μl of the solution was injected using a 20 μl injector loop.

The chromatograms and peak areas were recorded using a Maxima 820 Chromatogphy Workstation (Waters, USA) and quantification was carried out based on the peak areas with reference to an external standard.

3.4 DETERMINATION OF STEROLS

3.4.1 Materials

The cholesterol standard was purchased from Sigma and 0.2% of ethanolic solution of 2,7-dichlorofluorescein was prepared by dissolving 0.2 g of 2,7-dichlorofluorescein (AR grade) in 100 ml of absolute ethanol. The silica gel for preparative thin layer chromatography (TLC) was from Merck (No.7731). All other chemicals and solvents used were of analytical grade.

3.4.2 Saponification

Oil samples, 3-5 g, were weighed accurately into a 150 ml round bottom flask and were dissolved in 50 ml of ethanol. The mixture was heated to about 60°C and 5 ml of 50% potassium hydroxide aqueous solution were added and refluxed for about 1 hour. The saponified mixture was then transferred to a separating funnel and 100 ml of distilled water were then added. The unsaponified matter was extracted with 3 x 50 ml diethyl ether. The pooled diethyl ether layers were washed with 50-ml portions of distilled water until the washings (minimum of 3 times) were no longer alkaline. The diethyl ether extract was then dried over Na_2SO_4, rotary-evaporated, vacuum dried and kept in the freezer prior to TLC purification.

3.4.3 Isolation of Sterols by TLC

The sterols were isolated from the unsaponifiable matter by preparative TLC (20 x 20 cm plates, 0.75 mm thickness) with a solvent system of chloroform : diethyl ether : acetic acid,

(99:5:1, v:v:v). The sterol band was visualized under UV light after spraying with 0.2% 2,7-dichlorofluorescein in ethanol. The sterol band was scraped out and extracted by chloroform at 60°C. The filtered chloroform extract was rotary-evaporated, dried under vacuum and transferred to a volumetric flask for gas chromatography (GC) analysis.

3.4.4 Quantification of Sterols

The detailed composition of sterols was determined using gas chromatography. A HP 5790 gas chromatograph, equipped with a 5 feet x 1/4" 3% OV-17 glass column was used under the following conditions: column temperature at 265°C, FID detector at 280°C, injector at 200°C and He carrier gas at 30 ml/min. The chromatograms, peak areas and the retention times were recorded using a HP 3396 II intergrator. Cholesterol was used as an external standard in quantitative determination.

3.5 FATTY ACID COMPOSITIONS OF VARIOUS PALM OIL AND PALM OIL PRODUCTS

3.5.1 Preparation of Fatty Acid Methyl Esters

The oil sample (~0.3 g) was weighed in a small round bottom flask and 6 ml of 0.5N methanolic NaOH solution was then added and refluxed for 20-30 minutes. Seven milliliters of 20% methanolic boron trifluoride (Merck) was then added and the mixture was refluxed for another 1 minute. *n*-Heptane (3 ml) was then added and the mixture was stirred gently and was allowed to cool to room temperature. The *n*-heptane layer was then transferred into a graduated vial for GC analysis.

3.5.2 Analysis of Fatty Acid Composition by Gas Chromatography

The fatty acid methyl esters were analysed on a HP 5970A gas chromatograph equipped with a capillary column (30-meter, 0.5 μm ID, DB-23) from J&W Scientific USA. The following chromatographic conditions were used :- injector at 200°C, FID at 280°C, oven temperature programme : 180°C for 2 minutes, 4°C/min to 250°C and maintained for 9 mins, carrier gas : Helium at 1.2 ml/min

The chromatograms, peak areas and retention times were recorded by a Hitachi model D-2500 chromato-intergrator.

3.6 PREPARATION OF CAROTENE CONCENTRATE

3.6.1 Materials

Crude palm oil samples were obtained from a few palm oil refineries. All chemicals and solvents used were of analytical grade. Phenolphthalein solution (1%) was prepared by dissolving 1 g of phenolphthalein (BDH) in 100 ml of 2-propanol. "Neutralised 2-propanol" was prepared by titrating 2-propanol with 0.1 N sodium hydroxide using phenolphthalein as indicator.

Sodium hydroxide reagent (~0.1 N) was prepared by dissolving 4 g of NaOH in 1 liter of distilled water. The concentration was standardised with dry potassium hydrogen phthalate (heated at 120°C for 2 hours and cooled in a desiccator). This was carried out by dissolving 0.4 g (weighed to 4 decimal place) of potassium hydrogen phthalate in 50 ml of distilled water and using 2 drops of phenolphthalein as indicator. The mixture was placed on a hot

plate and was swirled until the salt has completely dissolved. The solution was titrated with NaOH solution to the first appearance of a permanent pink colour.

$$\text{Normality of the NaOH} = \frac{W \times 10^3}{t \times 204.2}$$

where W = weight of potassium hydrogen phthalate

t = volume of KOH in ml

3.6.2 Determination of Free Fatty Acid Content (VOTC method)

The oil sample (3-5 g) was weighed to the precision of two decimal points into a conical flask. Neutralised 2-propanol (50 ml) was added and the mixture was heated to gentle boiling on a hot plate. Three drops of phenolphthalein indicator was added and the content was titrated with standardized sodium hydroxide (0.1 N) with constant magnetic stirring and heating. The end point was indicated by the appearance of a faint pink colour persisting for at least 30 seconds.

The free fatty acid content is calculated from the formula below:

$$\text{For palm oil, \% FFA as palmitic acid} = \frac{25.6 \times t \times N}{W}$$

where t = volume (ml) of sodium hydroxide solution

N = normality of sodium hydroxide solution

W = weight (g) of the oil

3.6.3 Laboratory Scale Transesterification

Crude palm oil was transesterified with methanol at a weight ratio of oil to methanol 2:1, catalysed by 0.5% excess sodium hydroxide in methanol after the free fatty acids have been neutralised based on the following calculation:

$$\text{Weight of NaOH used to neutralise the FFA} = \frac{\%\text{FFA} \times \text{M.wt of NaOH} \times \text{wt of the oil (g)}}{256}$$

The reaction mixture was stirred and heated to reflux. The progress of the reaction was monitored by TLC (silica gel, solvent chloroform/*n*-hexane, 1:1) until all the triglycerides were converted to methyl esters. The ester layer was then separated from glycerol layer, washed with warm distilled water until the washings became neutral. The esters were then dried with Na_2SO_4 and the solvent was removed under reduced pressure.

3.6.4 Molecular Distillation of Methyl Esters

The carotenes retained in the esters after transesterification of crude palm oil were concentrated by the removal of volatile methyl esters, which were first distilled under high vacuum using a 2" wiped-film molecular still (Sibata, Japan) at a pressure of 40-60 mtorr with temperatures ranging from 100 to 130°C. The carotene concentrate was collected after the residue (undistilled esters and carotenes) from the first distillation was recycled at higher temperature (130-150°C) to distill off the remaining esters.

3.6.5 Pilot Plant Production of Esters

Crude palm oil (17 kg) was mixed with 15 litres of methanol or ethanol in a multi-purpose pilot plant reactor. The mixture was stirred and heated up to about 65°C. Adequate amount of KOH was used to neutralise the free fatty acids (based on the equation described in Section 3.6.3) and excess 0.5% of KOH in methanol/ethanol was then added to the stirred solution. The conversion was monitored by TLC plate using a solvent system of *n*-hexane : chloroform (1:1; v:v).

The reaction product was then left to settle at room temperature. The glycerol layer settling at the bottom of the product was drained out by gravity. The esters were then washed 3 times with an equal volume of warm water and dried under vacuum at a temperature of about 70-80°C.

3.6.6 Pilot Plant Production of Carotene Concentrate

The esters (methyl/ethyl) of crude palm oil were distilled using a pilot plant wiped-film molecular distillation unit. The remaining volatile solvent in the esters was first distilled off at 100°C, 200-300 mtorr, at a flow rate of 20 kg/hr. Most of the esters were distilled at 130°C at 10 kg/hr under 40-60 mtorr as distillate. The residual esters and other minor components collected at residue vessel were then further distilled at 150°C at similar flow rate to remove most of the esters under low vacuum (~10 mtorr). The carotene concentrate collected as residue was recycled at the same temperature to ensure that all esters were distilled over. The final carotene concentrate collected was flushed with N_2 and kept in the refrigerator for further analyses.

3.7 CAROTENE CONCENTRATE IN POWDER FORM

3.7.1 Materials

All the components used for powder formulation were of food grade. All other chemicals and solvents used for analyses were of analytical grade.

3.7.2 Preparation of Palm Carotene Powder

Carotene powder was prepared from palm carotene using a mixture of corn syrup, dipotassium phosphate, caseinate and Famodan with various composition as shown in the following Table.

Formulae of different samples of carotene powder (weight %)

Type of sample	10%	20%	30%	35%	40%	50%
Palm carotene	10	20	30	35	40	50
Corn syrup	82.5	72.9	63.3	58.6	55.0	44.4
Dipotassium phosphate	1.5	1.4	1.3	1.3	1.2	1.1
Caseinate	5.7	5.4	5.1	4.8	4.5	4.2
Famodan	0.3	0.3	0.3	0.3	0.3	0.3

A total of 200 g of the materials was mixed with an equal weight of water. The sample was then homogenised by stirring. While stirring the mixture, the sample was pumped through the air-pump spray dryer which operated at 110°C. The dried powder was then collected for further analysis.

3.7.3 Determination of Carotene Content

A powder sample (3 g) was weighed accurately into a small beaker, water (30 ml) was added gradually and the sample was made into smooth paste. The mixture was warmed at 50-60°C to dissolve the sample in water. About 3.5 ml of ammonia solution (NH_4OH) was added and the mixture was transferred into a separating funnel. Ethanol (30 ml) was then added to the mixture and mixed gently. The oil was extracted with 2 x 50 ml of diethyl ether. The combined diethyl ether extract was then washed with 2 x 50 ml of distilled water and dried over Na_2SO_4. After removal of the solvent by rotary evaporating, the sample was vacuum-dried. The carotene content of the oil was then determined spectrophotometrically as described in Section 3.2.4.

3.7.4 Stability of Carotene Powder

The stability of carotenes in the prepared powder form had been monitored for a period of one year at 40°C, ambient (28-30°C) and refrigerated (4°C) temperatures; these were carried out by determining the carotene concentration in the oil samples extracted from the powder samples at monthly intervals.

3.8 PREPARATION OF DEACIDIFIED AND DEODORISED RED PALM OIL

3.8.1 Materials

The CPO used in this study was obtained from several local palm oil mills. Hybrid palm oil was obtained from United Plantation (M) Sdn. Bhd. Commercial bleaching earths were used and the phosphoric acid (20% concentration) was from Ajax Chemicals. All chemicals and solvents used were of analytical grade.

3.8.2 Pretreatment of Crude Palm Oil

Crude palm oil was pretreated with phosphoric acid (0.5% wt. of oil) at 90°C for 10 minutes, followed by bleaching earth (0.5% wt. of oil) at 110°C for 30 minutes. The oil was then filtered to remove the bleaching earth. This refining process was carried out with different types of bleaching earths and under various conditions.

3.8.3 Deodorisation and Deacidification

The pretreated palm oil was heated to a temperature of 80°C and was allowed to pass through a 2" wiped-film molecular distillation unit (Sibata, Japan) at a rate of 24 gram per hour and under a vacuum of 20-25 mtorr. The temperature of the molecular still was maintained at 150°C throughout the distillation process. The volatile odourous components and the free fatty acids were distilled and collected as distillate. The deodorised and deacidified oil was collected as residue. Deodorisation and deacidification were also carried out under various temperatures and pressures.

3.8.4 Pilot Plant Production of Deodorised and Deacidified Red Palm Oil

The pretreated bleached and degummed palm oil/palm olein was collected from palm oil refinery using the conventional bleaching and degumming process; the deodorisation and deacidification was carried out using a POPE 6" wiped-film molecular distillation unit. Pretreated oil (17 kg) was first degassed at 80°C at 200 mtorr at a flow rate of about 15 kg/hr. The volatile components and free fatty acids were then distilled at 160°C still-body temperature at a flow rate of 7-10 kg/hr under an vacuum of about 10-12 mtorr. The oil collected as residue was further recycled at 165°C to remove most of the free fatty acids under a lower vacuum (6-8 mtorr).

3.8.5 Determination of Induction Period

The oxidative stability of the oil was determined by the measurement of the induction period using a Rancimate 679 intrument (Metrohm, Swiss).[227] Basically, 6 x 2.5 g of the oil samples were weighed into the reaction vessels and placed into the heating block of the instrument which was set at 120°C. Air was pumped through the oil sample at a flow rate of 20 litre/hr and was flowed through another measuring vessel which was filled with 75 ml of distilled water. The rate of oxidation was measured by the measuring cells based on the conductivity in the water. The schematic description is shown in Fig. 3.2(a), the conductivity *vs* time was recorded and the "induction period" results were printed on the built-in printer as shown in Fig. 3.2(b).

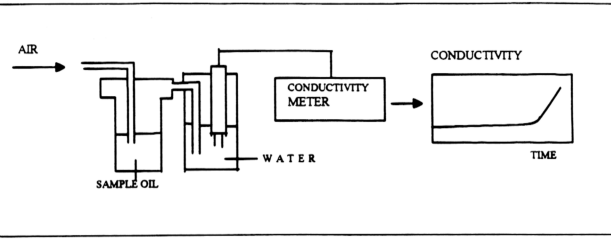

Figure 3.2(a) Schematic diagram of the Rancimate.

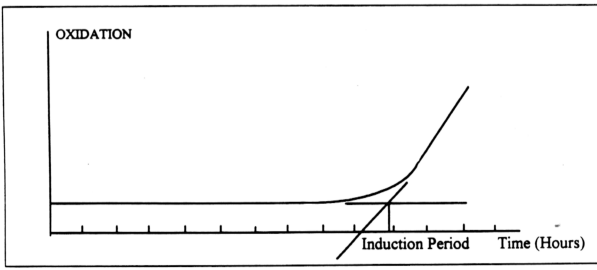

Figure 3.2(b) Conductivity curve and the Induction Period (IP).

3.8.6 Analytical Methods

The following analyses were carried out on the palm oil samples using AOCS (1974) and IUPAC (1979) methods:-

i. peroxide value

ii. iron content

iii. phosphorus content

iv. absorbance at 233 and 269 nm

3.9 PHOTOPROTECTIVE AND ANTIOXIDANT ACTIVITIES OF PALM CAROTENES

3.9.1 Materials

The Soybean oil was from Socma (M) Sdn. Bhd., methylene blue was from Merck, 2,4-dinitrophenylhydrazine (DNPH) was from BDH. Preparative TLC (20 X 20 cm) plates were prepared from silica gel (MERCK 7731) with 1 mm thickness. All other chemicals and solvents used were of analytical grade.

3.9.2 Preparation of Methyl Esters of Soybean Oil

Soybean oil (100 g) was mixed with 30 ml of methanol in a 2-neck flask, the mixture was heated to about 70-80°C and 0.6 g of NaOH in 20 ml of methanol solution was then added. The reaction was completed after 15-20 minutes as monitored by TLC (silica gel, solvent : chloroform/*n*-hexane, 1:1, v/v).

The reaction product was transferred to a separating funnel and the glycerol layer was separated from the ester layer. The ester layer was washed with warm distilled water until the washings became neutral. The solvent was then rotary-evaporated off and the esters were further dried under vacuum.

3.9.3 Photosensitised Oxidation of Fatty Acid Methyl Esters

Photoxidation was carried out by using methylene blue (4 mg) as a sensitiser in a solution of 2 g methyl ester of soybean oil in 50 ml dichloromethane. Oxygen was bubbled into the mixture which was exposed to a 100 watt tungsten sun lamp. The apparatus was set up as shown in Fig. 3.3. Two samples were taken at different time intervals and stored in the freezer before further analysis. A similar oxidation was also carried out with 200 ppm of palm oil carotenes added to the solution.

3.9.4 Analysis of the FAC of Photosensitised Oxidation Products

Heptadecanoic acid (0.3 ml, 0.012 g/ml) was added to the collected fractions as an internal standard. The mixture was blown dried under a stream of N_2 and the residue was redissolved in 0.5 ml of a mixture of diethyl ether : *n*-hexane (1:8). The solution was applied to a silica gel column (4.0 cm x 1.0 cm ID) and the esters were eluted with a solvent system of diethyl ether : *n*-hexane (1:8). The ester fraction (first 20 ml) was rotary-evaporated and redissolved in 1 ml of dichloromethane for the determination of fatty acid composition (FAC) by GC as described in Section 3.5.2

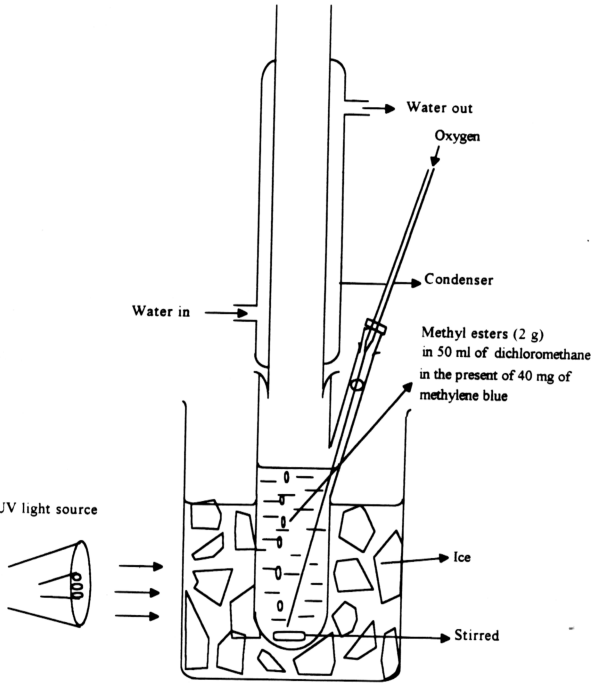

Figure 3.3 Reaction apparatus for photosensitised oxidation of methyl esters.

3.9.5 Determination of Total Carbonyl Compounds with 2,4-Dinitrophenylhydrazine

(a) Preparation of Carbonyl Derivatives

DNPH (50 mg) was dissolved in 100 ml of 1 M hydrochloric acid and the solution was extracted twice with 50 ml of *n*-hexane to remove impurities. The solution (10 ml) was added into a mixture of 9.0 ml water and 0.4 ml of glacial acetic acid containing about 0.02 g of oxidation products. The mixture was vigorously shaken at room temperature for five minutes and extracted six times with 6.0 ml of dichloromethane. The organic extracts containing 2,4-dinitrophenylhydrazones were combined and the solvent was rotary-evaporated off.

(b) Separation of Carbonyl Derivatives by Preparative TLC

The separation method for various dinitrophenylhydrazone derivatives of carbonyl compounds was essentially that used by Esterbauer.[239] Basically, the 2,4-dinitrophenyl-hydrazones were initially separated into two groups using preparative TLC (silica gel : thickness 1.0 mm, plate dimensions 20 cm x 20 cm). TLC plates was first developed with dichloromethane to about 5 cm from the origin followed by benzene as second developer for about 15 cm.

The zone between origin and R_f 0.12 was referred as polar group. All hydrazones with R_f values greater than or equal to 0.12 (the R_f of 4-hydroxynonenal) were scraped off and extracted with methanol (2 x 5 ml). The solvent was rotary-evaporated and the extract was rechromatographed using preparative TLC (silica gel, with benzene as developing solvent).

The dinitrophenylhydrazone derivatives (DNPs) of 4-hydroxyalkenals (mostly due to 4-hydroxynonenal), 2-hydroxylcarbonyl compounds (osazones) and non-polar carbonyl compounds were isolated from the rechromatographed TLC. The zone of the 4-hydroxyalkenals-DNPs was identified as R_f equal to 0.12; the 2-hydroxylcarbonyl

compound-DNPs (osazones) bands were detectable through the characteristic change of their colour (orange-red to blue), which were developed after spraying with a methanolic solution of KOH (10 g KOH dissolved in 100 ml of methanol). The excess DNPH zone was located and removed using a reference sample. The zones above 2-hydroxylcarbonyl compound-DNPs (osazones) was referred as the non-polar compound-DNPs.

(c) Quantitation of Carbonyl Compounds

TLC zones from DNPs of 4-hydroxyalkenals, 2-hydroxylcarbonyl compounds (osazones) and non-polar carbonyl compounds were scraped off and extracted with methanol (2 x 5 ml) and made up to 25 ml. The absorbance was measured at UV 365 nm using a Hitachi 20-150 Spectrophotometer.

3.10 EFFECT OF PALM CAROTENES ON LDL OXIDATION

3.10.1 Materials

The membrane dialysis tubing was from Spetrapor (L.A., USA), the bovine serum albumin was from Sigma and the folin phenol reagent was from Merck. All other chemicals and solvents used were of analytical grade.

Fresh blood samples were collected from overnight fasting "New Zealand White" rabbits. Each blood sample was collected from the ear vein into a bottle containing concentrated disodium ethylenediaminetetraacetic acid (Na_2EDTA) solution (as anticoagulant, 1/100 dilution, w/v 15% Na_2ETDA) with a final concentration of 1.5 mg/ml blood. The blood was then centrifuged at 4°C for 20 minutes using a R.C.S.C. bench top

centrifuge at 2,000 rpm. The plasma was then aspirated as the top layer from the red blood cells and was stored at 4°C for further analysis.

Phosphate Buffer Solution (PBS) 0.01 M, pH 7.4, was prepared from the following two stock solutions:-

A : 0.2 M solution of monobasic sodium phosphate $NaH_2PO_4.2H_2O$ was prepared by dissolving 27.8 g of $NaH_2PO_4.2H_2O$ in 1 litre of distilled water and,

B : 0.2 M solution of dibasic sodium phosphate Na_2HPO_4 was prepared by dissolving 28.4 g of Na_2HPO_4 in 1litre of distilled water.

The 0.01 M PBS was prepared by mixing 19 ml of A and 81 ml of B from the stock solutions and then made up to 2 litre with distilled water in a 2 litre volumetric flask. The pH was determined by a pH meter and was adjusted to pH 7.4 by adding either solution A or B depending on the pH value.

3.10.2 Isolation of Low Density Lipoprotein

Pooled fresh plasma from the rabbits was used for the isolation of low density lipoprotein (LDL). Total LDL (density 1.019-1.063 g/ml) was prepared by sequential ultracentrifugation from the plasma according to a method by Havel *et al.*[289] In brief, the plasma was adjusted to 1.019 g/ml by adding 0.0169 g of KBr in 1 ml of plasma based on the following calculation:

$$M_{KBr} = \frac{V_i\, d_f - d_i}{1 - \nu_{KBr}}$$

where, M_{KBr} = weight of solid KBr to be added,

V_i = initial volume of solution to be adjusted,

d_f = final density desired,

d_i = initial density,

ν_{KBr} = partial specific volume of KBr.

The KBr was dissolved gently in the plasma and ultracentrifuged at 50,000 rpm for 24 hours in the 70 Ti rotor (6 x 26.3 ml, polycarbonate tube) at 4°C, in a Beckman Ultracentrifuge. After ultracentrifugation, the top 8 ml in each ultracentrifuge tube was removed. The remaining solution was made up to 1.063 g/ml by adding 8 ml of 1.162 g/ml KBr solution to each tube. The solution was mixed and ultracentrifuged at 50,000 rpm again for another 24 hours at 4°C. After ultracentrifugation, the LDL fraction was aspirated from the top 8 ml of the tube. Fresh LDL samples were reserved at 4°C before dialysis.

3.10.3 Dialysis of Isolated LDL Solution

Each LDL sample was dialysed using membrane dialysis tubing from Spectrapor. The dialysis tube was boiled for 30 minutes in boiling water prior to use. All isolated lipoprotein fractions were placed in the dialysis tubing and dialysed for 24 hours at 4°C in the dark against nitrogen-bubbled PBS (0.01 M, pH 7.4) containing 0.16 M NaCl.

The PBS was changed 4 times during the 24-hour dialysis. After the final dialysis, the LDL solution was transferred to a sample vial, kept under N_2 at 4°C and was used within 24 hours.

3.10.4 Protein Determination (Lowry Method)

Total protein contents of LDL samples were determined according to the method by Lowry *et al.*[290] Fatty acid-free Bovine serum albumin (BSA) was used as the standard.

Reagents

1. Bovine serum albumin standard : 1 mg/ml in 0.1 M NaOH was prepared and was diluted to 0.1 mg/ml with 0.1 M NaOH (4 g of NaOH was dissolved in 100 ml of distilled water).

2. Solution "A": 2% Na_2CO_3 in 0.1 M NaOH. This is stable at room temperature for 1-2 weeks or until it became cloudy.
3. Solution "B": 0.5% $CuSO_4$ in 1% citric acid. This is stable at room temperature for 2 weeks.
4. Solution "C": 1 part solution "B" was mixed with 50 parts solution "A". This was prepared immediately before use.
5. Folin phenol reagent was diluted with distilled water 1:1 (v:v) before use.

Standard Curve

A series of tubes (in duplicate) was set up and the BSA standard solutions were prepared based on the following ratio:

Tube	BSA standard (μl)	0.1 M NaOH (μl)	(μg BSA)
0	0	200	0
1	50	150	5
2	100	100	10
3	150	50	15
4	200	0	20

Unknown Samples

LDL samples were diluted to the final concentration of protein of approximately 25-100 μg/ml, 200μl of the diluted LDL samples (in duplicate) were transferred to the tubes for analysis.

Procedure

Solution "C" (1 ml) was added to each tube and was allowed to stand at room temperature for 15 minutes. Folin reagent (10 μl) was then added, vortexed and allowed to stand for 20 minutes at room temperature. The absorbance of the mixture was then measured spectrophotometrically at 750 nm against the reagent blank.

A plot of absorbance at 750 nm *vs* concentration of BSA was made for a series of standards used and the protein content of the LDL was determined from the plot.

3.10.5 Oxidation of LDL Supplemented with Palm Carotenes

(a) Photoxidation

LDL isolated from rabbit's plasma was diluted to about 0.2 mg protein/ml with oxygen-saturated 0.01 M PBS (pH 7.4, 0.16 M NaCl). Palm carotenes were added externally into the lipoprotein in an ethanolic solution, in which about 20 μl of ethanolic carotene solution (270 μM) was added and mixed gently to the LDL solution to give a final carotene concentration of 4.5 μM. A similar volume of ethanol was added to the pure LDL (without palm carotenes) as control. Equal amounts of methylene blue in PBS solution were added to both solutions to give a final concentration of 1.2 μM.

An LDL solution without carotene and methylene blue was also included to monitor whether any oxidation occured which was not due to photoxidation process. All the samples were kept in temperature between 0-4°C under ice-cooled water. The photoxidation was initiated by irradiating the samples with 100 watt tungsten sun lamp. The absorption at 234 nm was recorded at every 15-minute time interval over a period of 5-6 hours.

(b) Autoxidation

Similar experiments were also carried out for LDL with and without palm carotenes, copper (II) chloride ($CuCl_2$, act as prooxidant) was added to initiate autoxidation in place of the methylene blue for photoxidation.

In experiments on Cu^{2+}-induced oxidations, LDL solutions (with or without palm carotenes) were supplemented with $CuCl_2$ (4.0 μM) using a 1-mM $CuCl_2$ stock solution in distilled water. The samples were incubated at 37°C and the oxidations were monitored by the UV absorption at 234 nm at every 15-minutes time interval over a period of 5-6 hours.

3.10.6 Autoxidation of LDL Supplemented with Vitamin E

Vitamin E (4.5 μM) containing palm tocopherol and tocotrienols was incorporated externally into LDL solution (0.2 mg protein/ml) using 20 μl of an ethanolic vitamin-E stock solution (270 μM) previously transferred to the walls of the vessel, and the LDL solution was shaken gently.

The treated LDL solution was supplemented with $CuCl_2$ (4.0 μM) to initiate the oxidation. The sample was incubated at 37°C and oxidation was continuously monitored by the UV absorption at 234 nm at every 15-minutes time interval over a period of 5-6 hours.

3.11 EFFECT OF CAROTENES, VITAMIN E AND VITAMIN C ON THE OXIDATION OF PLASMA AND LDL

3.11.1 Materials

Human plasma and LDL (1.019 g/ml < d < 1.063 g/ml) samples isolated from the serum of the normalipidemic subjects were obtained from the Department of Biology, University of California, Berkeley, USA. The LDL was dialysed for 18 hours against three changes of 0.01M sodium phosphate buffer (pH 7), immediately prior to use. The dialysed solution of LDL was adjusted to a concentration of 1 mg/ml (expressed as protein concentration) by dilution in the same buffer. Dihydrolipoic acid was a gift from Asta Medica (Offenbachh, Germany), *N*-2-hydroxylethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) was from Calbiochem Corporation (La Jolla, CA), and 2,2'-azobis(2-amidinopropane)hydrochloride (AAPH) was from Polysciences Inc. (Warrington, PA). Isoluminol, microperoxidase and ascorbic acid were from Sigma (St. Louis, MO). Dodecyltriethyl-ammonium phosphate solution (0.5 M) of chromatography grade was from Regis Chemical Company (USA). All HPLC solvents were of HPLC grade, other chemicals and solvents used were of analytical grade.

3.11.2 Palm Carotene Supplementation

Purified palm carotene sample was prepared by saponifing the carotene concentrate obtained as described in Section 3.6.4 and the carotenes in the unsaponifiable matter were column chromatographed through a silica Sep-Pak (Supelco) with *n*-hexane. The first coloured fraction (carotenes) to be eluted was collected and blown dry under a stream of N_2. An ethanolic palm-carotene stock solution was prepared by dissolving the purified palm

carotenes in minimum amount of ethanol. The concentration of stock solution was determined spectrophotometrically in *n*-hexane solution at 446 nm using the formula as described in Section 3.2.4. LDL and plasma suspensions were pretreated with palm carotenes to a final concentration of 18 μM of carotenes (using a 4.7 mM of ethanolic palm-carotene stock solution) for 24 hours. The plasma and LDL suspensions were then centrifuged to remove the excess carotenes in the solution.

3.11.3 Azo-Initiated Oxidation

Oxidations of plasma and LDL samples were induced by the thermolytic decomposition of the water-soluble azo-initiator, 2,2'-azobis(2-amidinopropane)hydrochloride (AAPH), at a concentration of 5 mM in 10 mM of HEPES buffer (pH 7.3), for 6 hours at 37°C. Samples were collected every 30 minutes for various analyses and the oxidation was arrested by the addition of 40 μM butylated hydroxytoluene (BHT) followed by refrigeration.

3.11.4 Determination of Conjugated Dienes in LDL

The oxidised samples collected (100 μl) were diluted to 2 ml using 0.05 M saline Tris buffer solution (pH 7.4, Sigma) and the concentration of conjugated-diene compounds formed during the oxidation were monitored spectrophotometrically at UV absorption at 234 nm.

3.11.5 Determination of α-Tocopherol in Plasma and LDL

(a) Extraction of Lipid Components from Plasma/LDL

The plasma or LDL sample (50 μl) collected was diluted to 1 ml with distilled water, followed by 1 ml of 0.1 M SDS and vortexed for 30 seconds. Ethanol (2 ml) was then added and vortexed for another 30 seconds. Finally 2 ml of *n*-hexane was added, vortexed again for

90 seconds and centrifuged at 4500 rpm for 3 minutes. The upper *n*-hexane layer (lipid extracts, 1 ml) was transferred out and *n*-hexane was removed by a stream of N_2. The non-volatile compounds in small vial were redissolved in 0.2 ml of methanol/ethanol (1:1, v/v) mixture for HPLC analyses.

(b) HPLC Analysis of α-Tocopherol

α-Tocopherol was assayed by reversed-phase HPLC using a C18 column (5 μm, 25.0 x 0.46 cm ID. Waters, Inc.) with an in-line electrochemical detector (LC 4B, Bioanalytical System). The eluant was a mixture of methanol/ethanol (1/9, v/v) containing 20 mM lithium perchlorate as electrolyte. The flow rate was 1 ml/min and the injected volume was 20 μl. The detector setting was 0.5-0.7 V, 5-50 nA for full recorder scale (nAFS) for the electrochemical detector. An external standard of α-tocopherol was used for quantification based on the peak areas.

3.11.6 Determination of β-Carotene in Plasma

(a) Extraction of Carotenes from Plasma

The extraction of carotenes from the plasma sample was carried out as follows :- A phosphate buffer solution (190 μl) was added to 10 μl of plasma suspension, after which 200 μl of ethanol containing 0.025% butylated hydroxytoluene (BHT) was added. The sample was extracted twice with 6 volumes of *n*-hexane containing 0.025% BHT. The *n*-hexane was evaporated to dryness under a stream of N_2 and the residue was reconstituted in 250 μl of a mixture of toluene/methanol (1:3, v/v) for HPLC analysis.

(b) HPLC Determination of β-Carotene

β-Carotene concentrations were determined by HPLC using a Beckman/Altex system with an Ultrasphere ODS, 5 μm, 15.0 cm x 4.6 cm ID, reversed-phase column (Beckman) and an elution solvent mixture of 82.5% methanol, 17.5% toluene, and 0.5% ammonium acetate was used at a flow rate of 1 ml/min. The eluent was monitored at 450 nm by a spectrophotometric detector. An external standard of β-carotene was used for quantification based on the peak areas.

3.11.7 Quantification of Lipid Hydroperoxides

(a) Extraction of Lipid Hydroperoxides

Extraction of lipid hydroperoxides from plasma or LDL was essentially the same as the method described in Section 3.12.5(a) for vitamin E extraction.

(b) Preparation of Isoluminol and Microperoxidase Solutions

Aqueous sodium borate (100 mM) was prepared by dissolving 38.14 g of sodium borate ($Na_2B_4O_7.10H_2O$) in about 900 ml of distilled water and its pH was adjusted to 10 with 50% aqueous sodium hydroxide. Isoluminol (177.2 mg) was dissolved in a mixture of 700 ml methanol and 300 ml of the above borate buffer followed by the addition of 25 mg of microperoxidase. This mixture was used as the reaction solution for the post-column HPLC assay.

(c) HPLC Analysis of Lipid Hydroperoxides

Lipid hydroperoxides were separated by HPLC and detected by chemiluminescence as described previously.[291] The lipid hydroperoxides were separated by a C18 reversed phase column (5 μm, 25.0 cm x 0.46 cm ID, Beckman) with a solvent system of *n*-butanol/methanol

(1/4, v/v). The flow rate was 1 ml/min and the injected volume was 50 μl. The reaction solvent (isoluminol and microperoxidase solution) for the post-column assay was pumped at a flow rate of 1.5 ml/min. The separated lipid hydroperoxides were allowed to undergo reaction with the reagents at a T-mixing joint preceding the chemiluminescence detector. A Soma Chemi Lumi Detector S-3400 was used to detected chemiluminescence. This assay has a detection limit for lipid hydroperoxide of 10 nM in plasma, and measures the hydroperoxy groups themselves, rather than indirect indices of lipid peroxidation such as diene conjugation or thiobarbituric acid-reactive substances (TBARS).

3.11.8 Quantification of Ascorbic Acid

(a) Extraction of Ascorbic Acid

Plasma sample (200 μl) was mixed with 0.98 ml of methanol and 20 μl of 0.05 M Na_2ETDA and vortexed for 90 seconds. The mixture was then centrifuged at 500 g for 8 minutes, about 30-40 μl of the supernatant was injected into HPLC.

(b) HPLC Analysis

The level of ascorbic acid was determined by paired-ion reversed-phase HPLC coupled with electrochemical detection, as described previously.[292]

Briefly, the HPLC mobile phase consisted of 40 mM sodium acetate ($NaOAc.3H_2O$), 0.54 mM Na_2EDTA, 7.5% methanol and 1.5 mM dodecyltriethylammonium phosphate at pH 4.75 in aqueous solution. This was prepared by dissolving 5.443 g of sodium acetate and 0.2010 g of Na_2ETDA in about 900 ml of water. Methanol (50 ml) and 3 ml of 0.5 M dodecyltriethylammonium phosphate were then added and made up to 1 litre with water. The pH of the final solution was adjusted to pH 4.75 with acetic acid. The mobile phase was filtered through 0.22 μm filter and sonicated prior to use.

The ascorbic acid was separated by a 5 μm, 25.0 cm x 0.46 cm ID, C18 reversed-phase column (Beckman) at a flow rate of 1 ml/min. The ascorbic acid was detected by an electrochemical detector (LC 4B BAS) which was set at +0.5 V. External standard of ascorbic acid was prepared (2 μM) in methanol solution, the concentration was determined spectrophotometrically using an extinction coefficient of 14500 $M^{-1}cm^{-1}$ at 265nm.

3.11.9 Carbonyl Assays

Carbonyl compounds were determined by a modification of the procedure described by Levine *et al.*[293] using dinitrophenylhydrazine (DNPH) dissolved in HCl, accompanied by blanks in HCl alone. Briefly, after the DNPH reaction, proteins were precipitated with an equal volume of 20% (w/v) trichloroacetic acid and the pellets were washed once with 4 ml of 10% (w/v) trichloroacetic acid and three times with 4 ml of an ethanol/ethyl acetate mixture (1:1). Washings were achieved by mechanical disruption of the pellets in the washing solution using a small spatula and re-pelleting by centrifugation at 6000 rpm for 5 minutes. Finally, the precipitates were dissolved in 6 M-guanidine-HCl solution and the absorbance peak at 320-400 nm was determined by a spectrometer. Protein contents were determined on the HCl blank pellets using a BSA standard curve in guanidine-HCl and reading the absorbance at 280 nm.

3.12 CAROTENE DISTRIBUTION AND OXIDATIVE STABILITY OF LDL

3.12.1 Materials

All the synthetic diet components and cholesterol were purchased from United States Biochemical (USB) Corp. (USA). Sodium dodecyl sulphate (SDS) was from BDH. Phosphate-buffered solution (PBS) was prepared essentially following the method described in Section 3.10.1. All other chemicals and solvents used were of analytical grade.

Refined, bleached and deodorised palm olein (RBDPOL) and crude palm olein (CPOL) were obtained from Federal Flour (M) Bhd., coconut oil and soybean oil were from Wahi & Sons (M) Sdn. Bhd. and Socma (M) Sdn. Bhd. respectively.

RBDPOL without palm vitamin E (tocopherol and tocotrienols) was prepared by eluting 5% of oil in *n*-hexane through an alumina oxide (Merck 1077) column. Vitamin E was trapped inside the column and the earlier fraction of vitamin-E free oil eluted from the column was collected. The *n*-hexane was removed by a rotary evaporator and the vitamin-E free oil was vacuum dried and kept at 0°C before use.

3.12.2 Animals and Diets

"New Zealand White" rabbits (6 months old) were randomly divided into four groups and were fed *ad libitum* semisynthetic diets (Table 3.1) containing 5% (w/w) commercial pellets, 95% (w/w) of purified substances consisting of (a) 15% refined, bleached and deodorised palm olein (RBDPOL) plus 2000 ppm palm carotenes (CE group), (b) 15% RBDPOL without palm vitamin E plus 2000 ppm palm carotenes (CX group), (c) 15% RBDPOL (PO), or (d) 15% RBDPOL without vitamin E (PX). All the feed materials were supplemented with cholesterol (5 g/kg).

Similarly, another study was carried out with four groups of rabbits fed on different dietary fats consisting of (a) 13% coconut oil plus 2% corn oil (CNO group), (b) 15% crude palm olein (CPOL group), (c) 15% soybean oil (SO group), all supplemented with cholesterol (5 g/kg), and (d) a control group which was fed RBDPOL-diet without supplementation of cholesterol (PO_{wc} group).

Blood samples were collected after a 12-week dietary treatment as described in Section 3.10.1. The animals were then sacrificed. The organs were removed immediately and stored at -40°C before analysis.

Table 3.1 Formulation of semisynthetic feed*

Components	Composition g/kg
Casein-vitafree	200
DL-Methionine	3
Dextrose monohydrate	150
Corn-starch	240
Celufil (Non-nutritive base)	200
AIN mineral mixture	45
AIN vitamin mixture	10
Choline dihydrogen citrate	2
Dietary fat	150

*50 g of commercial-rabbit feed pellets was mixed with 950 g of a mixture of the aboved mentioned feed materials (from US Biochemical Corp.) and dietary fats. The mixture together with cholesterol (5g/kg) was blended and repelletised.

3.12.3 Oxidation of LDL

LDL (density 1.019-1.063 g/ml) was isolated from the plasma and dialysed essentially following the procedure of Section 3.10.2. Dialysed LDL samples were diluted with 0.01 M oxygen-saturated, phosphate-buffered solution (pH 7.4, 0.16 M NaCl) to 0.1 mg protein/ml and incubated at 37°C. Oxidation was initiated by addition of copper (II) chloride to a final concentration of 4.0 μM. Absorption at 234 nm was continuously monitored by a U-200 Hitachi spectrophotometer over a period of 5-6 hours.

3.12.4 Total Fatty Acid Composition

(a) Extraction of Lipids

Lipids from plasma and LDL were extracted using a method by Folch *et al*[294] with some modification. Briefly, plasma or LDL samples (1 ml) were added to 3 ml of methanol, chloroform (6 ml) and 50 μl of 1% butylated hydroxytoluene (BHT) in chloroform were then added. The mixture was vortexed for 3 minutes and 1 ml of 0.8% of KCl was then added, after which the mixture was vortexed again for another 2 minutes. The mixture was centrifuged at 2000 rpm for 3-5 minutes using a bench top centrifuge (Speedfuge, HSC 10AC, Savant), the bottom chloroform layer was siphoned into a round bottom flask and the residue was reextracted with 2 x 3 ml of chloroform. The chloroform extracts were pooled, rotary evaporated to remove the solvent and vacuum dried before esterification.

(b) Transesterification/Esterification of Esters/Fatty acids

The lipid sample was saponified with 8% ethanolic potassium hydroxide in vacuum-sealed tube for 2 hours at 100°C, the unsaponifiable matter was washed away with 3 x 1 ml of *n*-hexane and the saponified matter was acidified with 1 ml of 10% HCl. The free fatty acids

were extracted with 3 x 1 ml of *n*-hexane. After the *n*-hexane was removed by a stream of N_2, the residue was further vacuum dried and the free fatty acids were then esterified with 1 ml of boron trifluoride-methanol (20%) in a vacuum sealed tube for 2 hours at 100°C. The reaction mixture was then mixed with 1 ml of distilled water and esters were extracted with 2 x 2 ml of *n*-hexane. The *n*-hexane was removed from the extracts by a stream of N_2 and the esters were purified through a small silica gel column (2 cm x 0.5 cm ID, Merck 7734) with 2% diethyl ether in *n*-hexane before analysis by gas chromatography.

(c) Gas Chromatographic Analysis

The total fatty acid composition of the plasma or LDL's lipid composition was analysed using gas chromatography as described in Section 3.5.2.

3.12.5 Carotenes, Retinol and Retinyl Esters in Rabbit's Plasma and Organs

(a) Extraction of Carotenes, Retinol and Retinyl Esters

Plasma sample (1 ml) was mixed with 1 ml of phosphate-buffered saline (pH 7.4, 0.16 M NaCl) in a centrifuge tube and vortexed for 30 seconds. Ethanol (2 ml) containing 0.025% butylated hydroxytoluene (BHT) was then added and the mixture was vortexed again for another 90 seconds. Each sample was then extracted with 2 ml of *n*-hexane containing 0.025% BHT. The mixture was vortexed for 2 minutes and then centrifuged at 4500 rpm for 3 minutes at room temperature. The *n*-hexane layer was then siphoned out and the aqueous layer was re-extracted with 2 x 2 ml of *n*-hexane.

Organ tissues were weighed (approximately 1-2 g for liver, kidney, lung, adipose, muscle and heart, whereas for the adrenal, pancreas and spleen, whole organs were used) and homogenised in 2 ml of phosphate-buffered solution (PBS). Another 2 ml of PBS was used to rinse the homogeniser and then 1 ml of 0.1 M sodium dodecyl-sulphate (SDS) was added and

vortexed for 30 seconds. Ethanol (2 ml) was then added and vortexed for 30 seconds. Finally 2 ml of *n*-hexane was added, the mixture was vortexed for 90 seconds and then centrifuged at 4500 rpm at room temperature for 3 minutes. The *n*-hexane layer was then siphoned out and the aqueous layer was re-extracted with 2 x 2 ml of *n*-hexane.

The *n*-hexane extracts from both plasma or organ tissues were pooled and blown dry under a stream of N_2 in a small vial. After that, 0.2 ml of 20% dichloromethane in acetonitrile was introduced. The mixture was sonicated and the solution obtained was injected into the HPLC.

(b) HPLC Analysis

The HPLC system used included a Waters Model 501 solvent pump and a photodiode array detector (PDA 991) which was equipped with a computer data collecting system for recording absorption spectra from 280 to 550 nm.

Isocratic separation was performed on a Zorbax ODS column (25 cm x 0.46 cm ID, stainless steel, 5 μm spherical particles) protected with a Du Pont guard column (20 μm, ZORBAX ODS). A solvent system containing 20% dichloromethane in acetonitrile was used and the flow rate was set at 1 ml/min. The PDA detector's wavelength was set at a range from 250 to 550 nm to record all the possible carotenes, retinol and retinyl esters present in the injected samples. An external standard of β-carotene was used for quantification based on the peak areas.

3.12.6 Analysis of Plasma/LDL Vitamin E

The extraction of lipids from plasma sample was essentially the procedure described in Section 3.11.5 (a) and the vitamin E content was analyzed essentially following the method described in Section 3.3