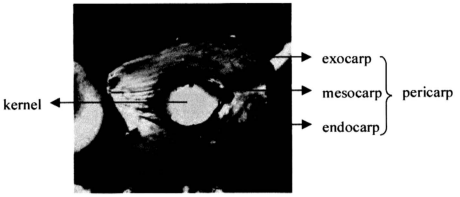
CHAPTER TWO

BIOCHEMICAL CHANGES IN THE DEVELOPING OIL PALM MESOCARP

2.1 INTRODUCTION

Palm fruits which consist of a pericarp, made up of exocarp (or skin), mesocarp and endocarp (or shell) surrounding usually one kernel. The fruits take about 20 weeks to ripe after anthesis. This maturation process proceeds with the noticeable morphological and biochemical changes in the palm fruits. The oil deposition in palm fruits occur *ca.* 16 weeks after anthesis (WAA) (Hartley, 1977).



Previous workers (Bafor and Osagie, 1988; Ariffin, 1990; Arffin *et.al.* 1990; Ilango *et al.*, 1988; Ilango *et al.*, 1989; Ikemefuna and Adamson, 1984) reported a lot of changes during the fruits development process. In this study, we report the analyses and quantification of phospholipids and selected minor components e.g., tocopherols, tocotrienols, carotenoids, sterols, squalene, free fatty acids and partial acylglycerols.

This investigation was correlated to the maturation process of palm fruits. The analyses were carried out at five different stages of the fruits development, namely 4, 8, 12, 16 and 20 WAA. The results enable us to explain the changes and biosynthesis pathway of these minor components at different maturation stages of palm fruits.

2.2 EXPERIMENTAL

2.2.1 Materials

Four bunches of oil palm fruits at different stages of ripeness, namely 4, 8, 12, 16 and 20 WAA were obtained from Research Plantation of Malaysian Palm Oil Board (MPOB). The flowers were not hand-pollinated and thus fruit age was estimated (Accuracy: ± one week). The fruits were chosen randomly and from *tenera* (DxP) species.

Florisil and phospholipid standards were purchased from Sigma Chemical Company: L-α Phosphatidylcholine (PC) from bovine liver; L-α phosphatidylethanolamine (PE) and L-α phosphatidyl-L-Serine (PS) from bovine brain; L-α phosphatidylinositol (PI) from soyabean; L-α phosphatidyl-D, L-glycerol (PG) from egg yolk lecithin. Thin layer chromatography (TLC) plates were purchased from Merck (Darmstardt, Germany). All solvents and chemicals were either HPLC or analytical grades, supplied by Merck, Sigma, J.T.Baker and Scharlau.

Visible absorption spectra were recorded on Hitachi U-2000 spectrometer in 10mm cell. Mechanical vessel shaking carried out using Vortex shaker.

Gas chromatography (GC) was performed on Chemstation, Agilent Technology 1990-2000 and Perkin Elmer Autosystem XL, both equipped with FID detector. Vitamin E analyses by high performance liquid chromatography (HPLC) was carried out using Chemstation, Agilent Technology 1990-2000 equipped with fluorescence detector, whereas phospholipids determination was carried out using Millenium Workstation 2000, Waters coupled with evaporative light scattering detector. Carotene profile was analysed using Hewlett Packard HPLC coupled with photodiode array detector.

2.2.2 Extraction of Palm Mesocarp Oil

After surface washing with water, half of each fresh fruit bunch was sterilised using autoclave at 120°C for 30 minutes. Both sterilised and unsterilised oil palm fruits was peeled off and flushed with nitrogen gas and kept in cold (5°C) when not in use. 500g each of sterilised and unsterilised peeled mesocarp were dried in the oven at 60°C. The moisture content was recorded. 500g mesocarp was blended with 500 ml of chloroform-methanol (1: 2 v/v) mixture. The blended material was transferred into a large conical flask whereby an additional 1.5 litre of the mixed solvent was added and the mixture was allowed to stand for 24 hours. The extract was filtered and the residue was re-extracted once with another litre of the same solvent mixture. The combined extracts were then rotary-evaporated and pump-dried under vacuum. Following this procedure, the following mesocarp oils were prepared:

1. Sterilised and dried mesocarp oil (SD4, SD8, SD12, SD16 and SD20)
2. Sterilised and wet mesocarp oil (SW4, SW8, SW12, SW16 and SW20)
3. Unsterilised and dried mesocarp oil (UD4, UD8, UD12, UD16 and UD20)
4. Unsterilised and wet masocarp oil (UW4, UW8, UW12, UW16 and UW20)

The number 4, 8, 12, 16 and 20 depicted the number of weeks after anthesis.

2.2.3 Purification of Palm Mesocarp Phospholipids

Purification of phospholipids was carried out using open column packed with acid-treated Florisil. Acid-treated Florisil was prepared according to the procedure of Carrol (1963) with slight modification as follow: 400g of Florisil was completely immersed *ca.* 2 cm below the surface of the concentrated hydrochloric acid. The mixture was heated over hot plate with frequent stirring using a glass rod for six hours. The acid was decanted and fresh acid was added. The contents were left to stand for 18 hours, after which they were further heated for another two hours. The acid was decanted and distilled water was added, stirred and decanted. The Florisil was transfer into Buchner funnel and washing with distilled water was continued with the aid of suction pump until the filtrate was neutral to the litmus paper. Methanol, acetone and chloroform (800ml each) were added consecutively. The acid-treated Florisil was dried in air, and then activated in an oven (100°C) for 18 hours.

The oil samples (*ca.* 1g) of palm fruits at 4, 8, 12 and 16 WAA were dissolved in minimum amount of chloroform-methanol mixture. Acid-treated Florisil (30g) was made into slurry using chloroform and packed into a 1.5 cm diameter column to a height of *ca.* 30 cm. A thin layer of anhydrous sodium sulphate (*ca.*0.25 cm) was added on the top of the Florisil. Excess chloroform was eluted down until the chloroform level was just above the column material and then the oil solution was carefully loaded. Purification of phospholipids was achieved by eluting the column with chloroform (100 ml), acetone (50 ml) and methanol (100 ml). Phospholipids were found to be

concentrated in methanolic layer and was rotary-evaporated to dryness at *ca.* 60°C. The purified phospholipids found in the methanolic layer was then used for further analyses.

The oil samples (*ca.*10g) of palm fruits at 20 WAA were stirred magnetically and refluxed with boiling methanol (ca.20ml) for 20 minutes. The methanolic layer was separated and the oil was washed once with 10 ml of the methanol. The combined methanolic portion was rotary-evaporated to dryness and re-dissolved in chloroform prior to open column purification as stated above.

2.2.4 Determination of Total Phospholipids Content

The analysis of total phospholipids content was carried out according to Goh *et al.*, (1984) and Zinzadse's reagent was prepared according to the procedure described by Kates (1975) but was modified to become a spray solution. Sodium molybdate dihydrate (6.85g) and hydrazine sulphate (0.4g) were dissolved in 100 ml distilled water together with 250 ml of concentrated sulphuric acid added. After cooling, the mixture was diluted with 300 ml of water. The greenish solution was stored in a brown bottle and can be used as staining reagents as well as complexing reagent.

Modified Zinzades's reagent (4 ml) was added to the purified phospholipids fraction and the mixture was well shaken for 30 minutes. Then, 5ml of hexane was added to the mixture and shaken for another 1 minute to extract the molybdenum-blue complexes. A visible spectrum was recorded by using Hitachi U-2000 spectrophotometer in 10mm cell and absorbance was read at 711nm, λmax for crude palm oil phospholipids. The

absorbance of sample reagent was found to be negligible. Hence, hexane can be used as blank.

2.2.5 Analysis of Phospholipids by 2D-TLC

Qualitative analysis of phospholipids in palm mesocarp was performed on 20 cm x 20 cm silica gel G plate of 0.25 mm thickness, pre-activated in an oven at 100°C for at least 2 hours. The solvent system used were chloroform-methanol-25% aqueous ammonia (65: 30: 4 v/v/v) in the first direction and chloroform-methanol-acetic acid-water (170: 25: 25: 6 v/v/v/v) in the second direction. Both solvent tanks were allowed to be fully saturated with solvent vapours for at least four hours before the chromatogram was developed. The phospholipids sample was spotted on the lower left corner, 2 cm from edges. The plate was developed in the first solvent tank until the solvent front was 2 cm from the top of the plate. After drying in the air, the plate was rotated 90° anticlockwise and developed in the second solvent tank.

The phospholipids were detected by modified Zinzadse's reagent (Kates, 1975) as blue spot and identified by comparing with the R_f values reported in literature (Christie, 1973; Goh *et al.*, 1982) and further confirmed by co-chromatography with authentic phospholipids standards.

2.2.6 Analysis of Phospholipids by HPLC-ELSD

The quantitative analysis of phospholipids was carried out by high performance liquid chromatography coupled with evaporative light scattering detector according to Juaneda *et al.*, (1990) with slight modification. The gradient solvent system consists of

A:hexane; B:2-propanol:chloroform (4:1 v/v); C: 2-propanol:water (1:1 v/v) was used. The analyses were carried out using a normal phase LiChlosorb silica column (4.6 mm i.d. X 25 cm, 5μm) with flow rate at 0.9 ml/min. The detailed gradient solvent system is as shown in Table 2.1. The gas flow of the detector was set at 1.5 SLM and the evaporator temperature was 110°C. It was necessary to re-equilibrate the column for 10 minutes prior to subsequent injections. Solutions with known concentration of each phospholipid standard were prepared and analysed to obtain the response factor and linear region for each phospholipid.

The purified phospholipids fractions (0.01g) of oil samples were dissolved in a 2 ml mixture of chloform:methanol (1:2 v/v) and injected into HPLC for analyses.

Table 2.1: HPLC Solvent System for Phospholipids Analysis

Time/min	A (%)	B (%)	C (%)
0	42	52	6
25	32	52	16
65	32	52	16
65.1	42	52	6

2.2.7 Fatty Acid Composition Determination of Phospholipids

The purified phospholipids fractions (*ca.*100μg) were transesterified into methyl esters using sodium methoxide (1 ml). After shaking for 20 minutes, the methyl esters formed were extracted using hexane (0.5 ml) and analysed by GC-FID using a 10-feet 10% SP2330 on Perkin Elmer Autosystem XL with FID detector. The injector and carrier gas pressure was set at 260°C and 18 psi respectively, meanwhile the oven was heated from 170°C to 200°C with the rate of 1.5°C/min.

2.2.8 HPLC Analysis of Tocopherols and Tocotrienols

The analyses of tocopherols and tocotrienols were carried out by High Performance Liquid Chromatography (HPLC) using the following conditions: Lichrosorb analytical silica column (25 cm x 0.46 cm ID, stainless steel, 5 μm), solvent system of n-hexane: tetrahydrofuran: 2-propanol (1000: 50: 3 v/v/v) with the flow rate of 1.0 ml/min, and an Agilent Fluorescence detector at 295 nm excitation and 325 nm emission. The hexane-purified sample was dissolved in the mobile phase and injected into HPLC with 20 μl injector loop. The standards used to calibrate the samples were α-tocopherol, α-tocotrienol, δ-tocotrienol and γ-tocotrienol.

The oil samples (*ca.* 2 g) of palm fruits at 4 8, 12 and 16 WAA were magnetically stirred with n-hexane (40 ml) for two hours. After filtration, the residue was washed once with 20 ml of n-hexane. The combined n-hexane layer was rotary-evaporated to dryness and subjected to vitamin E determination. The oil samples (ca.0.2g) of palm fruits at 20 WAA were directly dissolved in n-hexane (5 ml) for vitamin E determination.

2.2.9 HPLC Analysis of Carotenes

The carotenes profile analyses was done using high performance liquid chromatography with photo diode array detector. The isocratic non-aqueous separation system developed by Yap *et al.* (1991) was used. The analyses were carried out using a Metaphase reverse phase C18 column (4.6 mm i.d. X 25 cm, 5μm), and the solvent system was acetonitrile:dichloromethane (89:11, v/v) at a flow rate of 1.0 ml/min.

The hexane-purified oil samples (*ca.* 0.2g) of palm fruits at 4, 8, 12 and 16 WAA were dissolved in the mobile phase (2 ml) and injected into the HPLC. The oil samples (ca. 5 g) of palm fruits at 20 WAA was saponified according to the PORIM Test Method, P2.7, (1995) prior to the HPLC injection. 5g of the well-mixed sample weighed into the round bottom flask and refluxed with 30ml of ethanol and 5ml of aqueous potassium hydroxide solution for 1 hour. Then, the reaction mixture was transfer into the separating funnel and the flask was rinse with 10ml of ethanol followed by 20ml warm and then cold distilled water. The contents were allowed to cool to room temperature and 50ml of petroleum ether was added and shook rigorously for 1 minute. The soap solution was draw off when the completely separation of two phases. The extraction of unsaponifiable matter was repeated using 50ml of petroleum ether each time (5 times). The combined extracts were washed three times with 25ml portions of 10% (v/v) ethanol before drying under vacuum.

2.2.10 Determination of Mono-, Di-, Triacylglycerols, Sterols and Free Fatty Acids

Gas chromatography analysis of oil samples was performed by Hewlett Packard 5890 Plus Series II equipped with Flame Ionization Detector and a split injector (1:100 ratio). A 15m x 0.53 mm ID bonded phase fused silica column with film thickness 0.5 micron was used in the analysis. The hexane-purified oil samples (*ca.* 0.02 g) from Section 2.3.8 was silylated with 0.20 ml *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane and 0.30 ml dichloromethane. The sample mixture was shaken, homogenised and heated at 60°C for two hours. The injector and carrier gas pressure was set at 260°C and 18psi respectively, while the heating rate of oven was set at 1.5°C/min from 200-250°C.

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2.3 RESULTS AND DISCUSSION

2.3.1 Extraction of Palm Mesocarp Oil

Fruits development and oil deposition in oil palm were described by Hartley (1977) and Thomas *et al.* (1971). At 4 WAA, the fruits were very small, greenish yellow in colour and black at the tip. The endocarp was not clear and the kernel is in liquid form. At 8 and 12 WAA, the fruits were bigger as compared to the younger fruits. The endocarp was fully formed but was not hard. The kernel was still liquid at 8 WAA and become semi-gelatinous at 12 WAA. Oil deposition in the kernel starts at 12 WAA and is almost completed by 16 WAA (Crombie, 1965). During this period, the kernel and endocarp harden slowly. At 16 WAA, while there is still little oil in the mesocarp (see Section 2.3.6), the kernel was a whitish hard tissue enclosed by hard and brown endocarp. The mesocarp was greenish-yellow and non-oily, and the exocarp was deep red at the tip with a developing orange color towards the stalk. Oil deposition in mesocarp starts *ca.* 16 WAA and continues until maturity at *ca.* 20-21 WAA. The palm fruits were bright waxy orange in color and the mesocarp was bright yellow.

Table 2.2 shows the extraction of mesocarp oil from the palm fruits at different stages of ripeness and with different treatments. The moisture content was ca.18% in the ripe palm fruits and 80% for the unripe fruits. This calculation was based on the wet weight which is in agreement with published data (Ariffin, 1990). The low moisture content of the sterilised fruits normally resulted in higher oil yield as compared to unsterilised

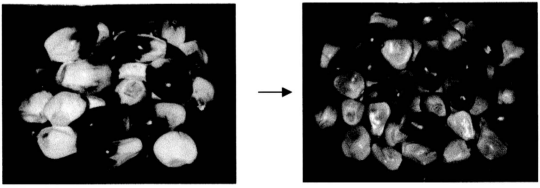
Table 2.2: Extraction of Mesocarp Oil of Palm Fruits at Different Stages of Ripeness

Mesocarp Oil	Moisture content (%)	Yield of Oil		Description
		g	%	
SD4	81.02	10.72	2.74	Greenish-yellow powder
SD8	82.54	15.86	3.17	Greenish-yellow powder
SD12	81.63	15.14	3.03	Greenish-yellow powder
SD16	82.22	15.55	3.11	Greenish-yellow powder
SD20	18.41	67.13	22.38	Orange oil
SW4	81.02	15.45	4.01	Greenish-yellow powder
SW8	82.54	15.36	4.39	Greenish-yellow powder
SW12	81.63	15.76	4.50	Greenish-yellow powder
SW16	82.22	15.68	4.48	Greenish-yellow powder
SW20	18.41	69.50	23.16	Orange oil
UD4	82.12	8.03	1.74	Purple powder
UD8	85.90	9.63	1.92	Purple powder
UD12	84.62	11.29	2.26	Purple powder
UD16	83.88	13.50	2.27	Purple powder
UD20	24.84	197.85	56.53	Orange oil
UW4	82.12	11.22	2.92	Purple powder
UW8	85.90	14.03	3.81	Purple powder
UW12	84.62	12.86	3.67	Purple powder
UW16	83.88	12.74	3.64	Purple powder
UW20	24.84	185.05	52.87	Orange oil

SD - sterilised dried mesocarp oil
SW - sterilised wet mesocarp oil
UD - unsterilised dried mesocarp oil
UW - unsterilised wet mesocarp oil
4,8,12,16,20 - weeks after anthesis

fruits. However, the extraction of oil from unsterilised ripe palm fruits exhibited opposite characteristic which doubled the yield of sterilised ripe palm fruits. This is due to the solubility problem of non-polar triacylglycerols in the solvent system (MeOH: $CHCl_3$, 2:1 v/v) employed for extraction. The enzymatic activity in unsterilised ripe palm fruits hydrolysed triacylglycerols to free fatty acids (refer to Section 2.3.6) which easily soluble in solvent system result in higher oil yield as compared to sterilised ripe palm fruits.

It is known that palm fruits contain several types of pigment, e.g. chlorophyll, carotenoids and antocyanin for certain physiological purposes (Ikemefuna *et al.*, 1984; Ahmad, 1986; Ariffin, 1990; Tan *et al.*, 1997). The extraction data revealed the fate of these pigments during the maturation of fruits and effect due to different treatments. The major pigment changes associated with ripening of palm fruits was a decrease in the chlorophyll content and increased in accumulation of carotenes. The mesocarp comprising of exocarp and pericarp changes from yellowish green to orange. This phenomenon was evidenced by bright orange color of the palm oil.



Palm fruits at 12 WAA

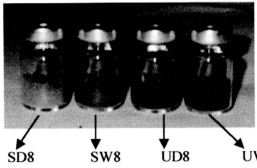
Palm fruits at 20 WAA

The presence of anthocyanin in oil palm was first reported by Ahmad *et al.* (1986). Chlorophylls and phenolics compounds including anthocyanins were obtained from the aqueous extraction of the exocarp of ripe palm fruits. The UV-visible spectrum of the purified extract showed two absorption maxima, at 276 nm and 510 nm, which were identical with the standard extracted from Hibiscus. This showed the presence of anthocyanins in oil palm fruits. Its absence in the crude palm oil should not be doubted since it is water-soluble and not miscible with the oil. The difference in colour of sterilised and unsterilised palm mesocarp oil revealed a significant amount of these compounds had been removed by sterilisation. Anthocyanins were readily dissolved in hot steam and the recovered condensate was purple in colour. In palm oil milling, these compounds were further removed by clarification and centrifugation. Thus, the palm oil mill effluent is a good source for the recovery of these valued water-soluble antioxidants.

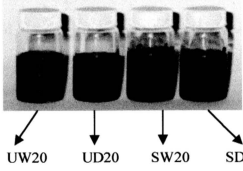
2.3.2 Phospholipids

(a) Determination of Total Phospholipids Content

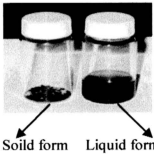
Phospholipids are usually quantified by colourimetry determination that converts phospholipids into inorganic phosphate and absorbance at certain wavelength was measured (Rouser *et al.*, 1966; AOCS Method, Ca 12-55, 1958; PORIM Test Method, P2.8, 1995). Phospholipids content is equal to the elemental phosphorous content multiplied by a conversion factor. Based on the fatty acids content and phospholipids distribution, an average molecular weight of phospholipids in palm oil is found to be 754 per phosphorous atom (Goh *et al.*, 1982). Hence the conversion factor of 24 for



Palm mesocarp oil at 8WAA with different treatments



Palm mesocarp oil at 20WAA with different treatments



Condensate collected from autoclave after sterilisation

converting elemental phosphorous content should be used instead of 30 as recommended for soybean oil.

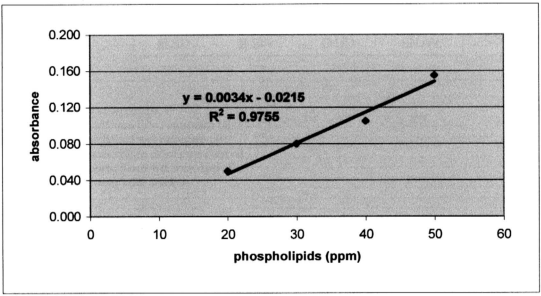
However, these methods were found to be unsuitable for total phospholipids content determination. Goh *et al.* (1984) revealed the discrepancy between phospholipids and phosphorous contents, as determined by thin layer chromatography (TLC) and a modified AOCS method. In the modified AOCS method, all organic and inorganic phosphate were ashed and determined as orthophosphate, which means that the phosphorous content should represent the total content of all phosphorous-containing compounds whereas TLC method only selectively quantify the phospholipids. Therefore, the conventional practice of using a conversion factor to convert the total phosphorous content as determined by ashing into 'phospholipids equivalent' was invalid.

In the present study, the total phospholipids content was determined according to Goh *et al.* (1984). Phospholipids in oil samples readily form the blue complexes with modified Zinzadse's reagent which is extractable by hexane. The absorbance was read at 711 nm, *viz.* λ_{max} of the reconstituted phospholipids composition of crude palm oil, i.e. *ca.* 50% phosphatidylcholine, 30% phosphatidic acid and 20% phosphatidylethanolamine. A calibration curve was plotted for standard phosphatidylcholine solutions (Figure 2.1) for the quantification of phospholipids in various mesocarp oil (Table 2.2).

The oil samples were purified by column chromatography using acid-treated Florisil to remove pigment, which otherwise would interfere directly with the colorimetric

determination as well as the complexes formation of phospholipids and molybdenum. The oil samples of palm fruits at 4, 8, 12 and 16 WAA (Table 2.2) were directly loaded onto an open column, whereas the oil samples of palm fruits at 20 WAA were refluxed with boiling methanol prior to column purification in order to eliminate the bulk quantity of triacylglycerols. This is to avoid column overloading as large quantities of oil were needed to isolate sufficient amounts of phospholipids.

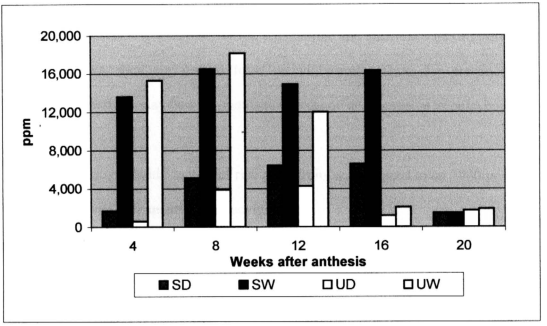
Figure 2.1: Calibration Curve of Phospholipid-molybdenum Blue Complexes



Original data refer to Appendix 1

Figure 2.2 showed that the concentrations of phospholipids in palm mesocarp oil were essentially constant until 12 WAA. A much higher phospholipids content found in the wet palm mesocarp oil and this might be due to the moisture content (see Section 2.3.1), which was believed to facilitate the phospholipids extraction from the mesocarp. Alternatively, the drying process of palm fruits in an oven may also cause chemically decomposition of phospholipids.

Figure 2.2: Concentration of Phospholipids in the Developing Palm Mesocarp



Original data refer to Appendix 1

SD	-	sterilised dried mesocarp oil
SW	-	sterilised wet mesocarp oil
UD	-	unsterilised dried mesocarp oil
UW	-	unsterilised wet mesocarp oil
4,8,12,16,20	-	weeks after anthesis

At 16 WAA, the phospholipids content of both dried and wet unsterilised mesocarp oil started to reduce dramatically, meanwhile the phospholipids content of both dried and wet sterilised mesocarp oil remained unchanged. This occurrence suggested the existence of phosphatase activity that hydrolysed the phosphoester bond and transformed phospholipids to diacylglycerols. This presumption was reassured by the upsurge of diacylglycerols content (see Section 2.3.6, Figure 2.16) in the same time.

It is known that the oil deposition of palm mesocarp started *ca.* 16 WAA. Also, the biosynthesis of phospholipids and triacylglycerols undergo the same pathway at earlier stages of fruit ripening with phosphatidic acid as the key intermediate. The phosphatidic

acids either associated with other bases, e.g. choline and ethanolamine or hydrolysed to diacylglycerols, then acylated to form triacylglycerols. Thus, the presence of the phosphatase activity may be a pre-requisite step for oil accumulation in palm mesocarp.

Lastly, the phospholipids content of ripe palm fruits was dropped to *ca.*2000 ppm by the dilution effect of bulk quantity of triacylglycerols.

(b) Analyses of Phospholipids in Palm Mesocarp by 2D-TLC

The analyses of phospholipids in palm mesocarp were performed by two dimensional thin layer chromatography (2D-TLC). The solvent systems was chloroform: methanol: 25% ammonia (65:30:4 v/v/v) at the first direction and chloroform:methanol:acetic acid: water (170:25:25:6 v/v/v/v) at the second direction. Table 2.3 shows the R_f values of phospholipid standards in the chosen solvent systems. The separation of phospholipid standards by two-dimensional TLC and illustrated Figure 2.3. The analyses of phospholipids at different stages of development of palm mesocarp are summarised in Table 2.4. The separation of phospholipids were illustrated in Figure 2.4-2.5.

Differences in the composition of phospholipids in the immature and mature palm mesocarp were observed (Table 2.4, Figure 2.4-2.5). The quantity of some components might be altered at different stages of oil palm fruit ripeness and due to different treatments before oil extraction. The major components found in palm mesocarp throughout the maturation process were phosphatidylcholine and phosphatidylinositol.

The lysophosphatidylcholine, though significant in amount until 12 WAA, showed some decline and remained at trace level when the fruit ripe. These results were in agreement with those reported by Bafor *et al.* (1988).

Table 2.3: R_f Values of Phospholipid Standards

Phospholipids	R_f Values[a]	
	S_1	S_2
Phosphatidylcholine	0.28	0.43
Phosphatidylethanolamine	0.40	0.71
Phosphatidylinositol	0.21	0.21
Phosphatidylglycerol	0.40	0.54
Diphosphatidylglycerol	0.59	0.87
Phosphatidic acid	0.18	0.79
Phosphatidylserine	0.15	0.29
Lysophosphatidylcholine	0.12	0.11
Lysophosphatidylethanolamine	0.20	0.31
Phosphatidylmethanol[b]	0.64	0.84

[a] TLC on silica gel G.
[b] Only detected in unsterilised ripe palm mesocarp oil
S_1: chloroform: methanol: 25% ammonia (65:30:4 v/v/v)
S_2: chloroform: methanol: acetic acid: water (170:25:25:6 v/v/v/v)

At 16 WAA, notable changes were observed in the composition of phospholipids in palm mesocarp. Phosphatidic acid, phosphatidylethanolamine and phosphatidylglycerol which were not detected in earlier stages appeared and became major components at 20 WAA. Trace amount of lysophosphatidylcholine, lysophosphatidylethanolamine and phosphatidylserine were noticeable only in sterilised palm mesocarp oil.

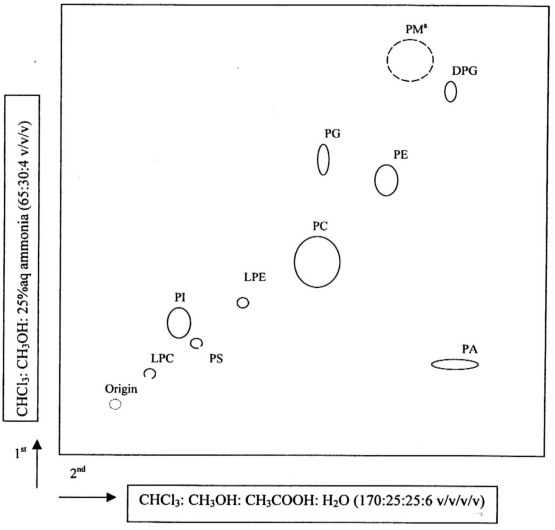
Table 2.4: Phospholipids at Different Stages of Development of Palm Mesocarp

Mesocarp Oil	Phospholipid Components
SD4	PC, PI, LPC, PA
SD8	PC, PI, LPC
SD12	PC, PI, LPC
SD16	PC, PI, LPC, PA, PE, PG
SD20	PC, PI, LPC, PA, PE, PG, DPG, PS, LPE
SW4	PC, PI, LPC, PA
SW8	PC, PI, LPC
SW12	PC, PI, LPC
SW16	PC, PI, LPC, PA, PE, PG
SW20	PC, PI, LPC, PA, PE, PG, DPG, PS, LPE
UD4	PC, PI, LPC, PA
UD8	PC, PI, LPC
UD12	PC, PI, LPC
UD16	PC, PI, LPC, PA, PE, PG, PM
UD20	PC, PI, PA, PE, PG, DPG,PM
UW4	PC, PI, LPC, PA
UW8	PC, PI, LPC
UW12	PC, PI, LPC
UW16	PC, PI, LPC, PA, PE, PG, PM
UW20	PC, PI, PA, PE, PG, DPG, PM

PC phosphatidylcholine
PE phosphatidylethanolamine
PI phosphatidylinositol
PG phosphatidylglycerol
DPG diphosphatidylglycerol
PA phosphatidic acid
PS phosphatidylserine
LPC lysophosphatidylcholine
LPE lysophosphatidylethanolamine
PM phosphatidylmethanol

SD sterilised and dried mesocarp oil
SW sterilised and wet mesocarp oil
UD unsterilised and dried mesocarp oil
UW sterilised and wet mesocarp oil
4,8,12,16,20 weeks after anthesis

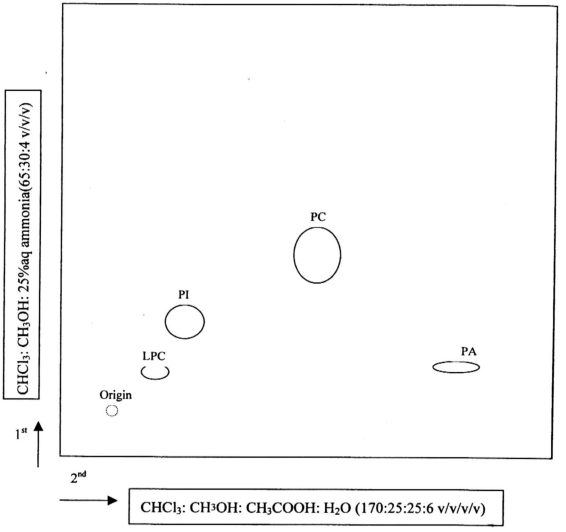
Figure 2.3: Phospholipid Standards on 2D-TLC Silica Gel Plate



[a] Only detected in the unsterilised palm mesocarp oil at 16 and 20 weeks after anthesis

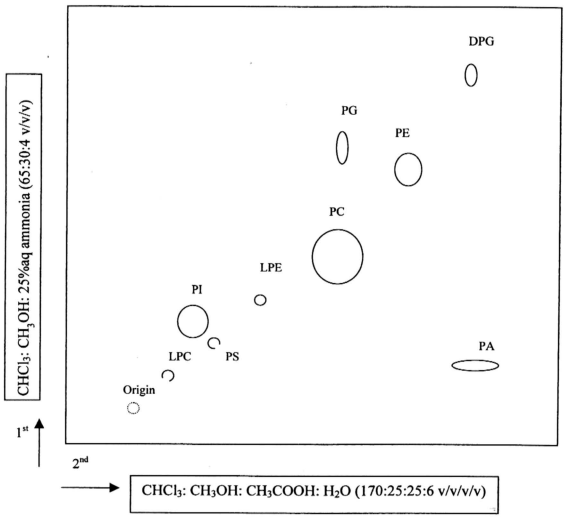
PC	phosphatidylcholine	PE	phosphatidylethanolamine
PI	phosphatidylinositol	PG	phosphatidylglycerol
DPG	diphosphatidylglycerol	PA	phosphatidic acid
PS	phosphatidylserine	LPC	lysophosphatidylcholine
PM	phosphatidylmethanol	LPE	lysophosphatidylethanolamine

Figure 2.4: Phospholipids in SW4 by 2D-TLC



PC	phosphatidylcholine
PI	phosphatidylinositol
PA	phosphatidic acid
LPC	lysophosphatidylcholine
SW4	sterilised wet mesocarp oil at 4 weeks after anthesis

Figure 2.5: Phospholipids in SW20 by 2D-TLC



PC	phosphatidylcholine	PE	phosphatidylethanolamine
PI	phosphatidylinositol	PG	phosphatidylglycerol
DPG	diphosphatidylglycerol	PA	phosphatidic acid
PS	phosphatidylserine	LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine	SW20	sterilised wet mesocarp oil at 20 weeks after anthesis

The early preliminary study (Tan, 1977) of palm mesocarp phospholipids revealed an anomalous component and later confirmed as artifact, phosphatidylmethanol by Goh *et al.* (1982). Phosphatidylmethanol was detected in unsterilised palm fruits at 16 WAA and was the most abundant component at 20 WAA. Phosphatidylmethanol was produced by enzymatic transphosphatidylation of natural phospholipids during the oil extraction of unsterilised fruits using methanolic solvents. This observation suggests that increase in enzymatic activities started in palm mesocarp since 16 WAA.

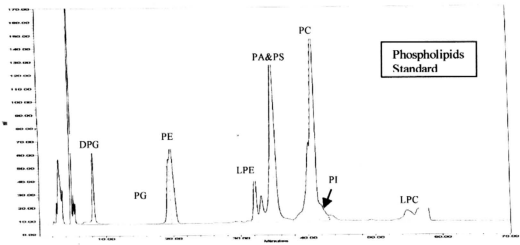
Phospholipase D (PLD) hydrolyses the terminal phosphoester bond of various phospholipids to yield phosphatidic acid and a water-soluble head group where water is in abundance. Certain primary alcohols can replace water as substrate in PLD-catalyzed reactions and the resultant lipids product is a phosphatidylalcohol rather than phosphatidic acid (Heller, 1978) which is similar to the present study. Phosphatidic acid derived from PLD has a variety of potential metabolic fates. Phosphatidic acid can be reacted with CDP-diacylglycerol synthase to form CDP-diacylglycerol which is an intermediate in the synthesis of acidic phospholipids such as phosphatidylglycerol, phosphatidylinositol, phosphatidylserine and diphosphatidylglycerol in plants (Moore *et al.*, 1973 and Munnik *et al.*, 1998). Phosphatidic acid can be dephosphorylated by acid phosphatase to form diacylglycerols (Kent, 1995), an intermediate in the synthesis of phosphatidylcholine, phosphatidylethanolamine and triacylglycerols. Thus, in view of the physiological role of PLD, the presence of the phosphatase activity may be a pre-requisite step for oil accumulation in palm mesocarp.

(c) Analysis of Phospholipids in Palm Mesocarp by HPLC-ELSD

The quantitative analyses of phospholipids in palm mesocarp were carried out by high performance liquid chromatography (HPLC) coupled with evaporative light scattering detector (ELSD) with slight modification (Juaneda *et al.*, 1990). The chromatographic separation of standard phospholipids mixture and calibration curves of each standard were shown in Figure 2.6 and Figure 2.7 respectively. Linear relationships ($r > 0.95$) between peak areas in integrator counts and phospholipids in μg region were phosphatidylinositol, phosphatidylserine and lysophosphatidylethanolamine (20-65μg), 20-85μg for phosphatidylethanolamine, phosphatidic acid, phosphatidylglycerol, diphosphatidylglycerol and lysophosphatidylcholine, and 20-130μg of phosphatidylcholine.

The HPLC-ELSD analyses quantified the composition of phospholipids in the developing palm mesocarp and their results are tabulated in Table 2.5 (Figure 2.8). Phospholipids profile at different stages of development of palm mesocarp oil was shown in Figure 2.9. Results were expressed in term of concentration and mole percentage with an average molecular weight of phospholipids in palm mesocarp oil was found to be 754 per phosphorous atom. It is found that total phospholipids by the UV method gave lower amount as compared to the HPLC method. This is because absorbance of phospholipid-molybdenum blue complexes was recorded at 711nm, λ_{max} for crude palm oil phospholipids. In fact, the λ_{max} for each phospholipid-molybdenum complex differ from each other, e.g. the λ_{max} for phosphatidic acid- and phosphatidylcholine-molybdenum blue complex are at 700nm and 725nm respectively.

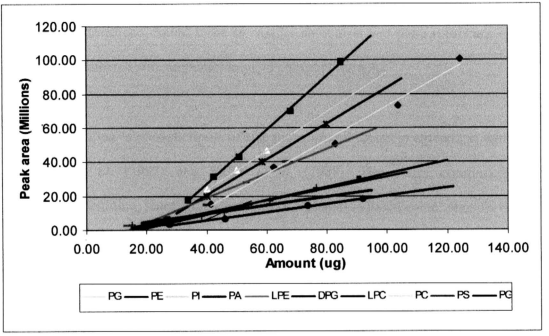
Figure 2.6: Chromatogram of Phopsholipid Standards by HPLC-ELSD



PC	phosphatidylcholine	PE	phosphatidylethanolamine
PI	phosphatidylinositol	PG	phosphatidylglycerol
DPG	diphosphatidylglycerol	PA	phosphatidic acid
PS	phosphatidylserine	LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine		

Generally, there is a slight increase in the concentration of phosphatidylcholine and phosphatidylinositol in palm mesocarp from 4 to 12 WAA. On the other hand, the lysophosphatidylcholine content reduced gradually and phosphatidic acid was vanishing at the same period. The phosphatidic acid, known as the key intermediate in the biosynthetic pathway of phospholipids might be converted to phosphatidylcholine and phosphatidylinositol due to the cellular proliferation of palm fruit. All mesocarp oil with different treatments demonstrated similar phospholipids composition with only the wet palm mesocarp extracts seem to give higher amount of phosphatidylcholine.

Figure 2.7: Calibration Curve of Individual Phospholipid Standard



Original data refer to Appendix 2

PC	phosphatidylcholine	PE	phosphatidylethanolamine
PI	phosphatidylinositol	PG	phosphatidylglycerol
DPG	diphosphatidylglycerol	PA	phosphatidic acid
PS	phosphatidylserine	LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine		

At 16 WAA, a remarkable changes in the composition of phospholipids were observed. Phosphatidylethanolamine and phosphatidylglycerol which were not detected earlier accounted for over 10% and 1% respectively of the phospholipids. Phosphatidic acid appeared again and accounted for more than 20% of the total phospholipids. The enzymatic product, phosphatidylmethanol was also detected in the unsterilised palm mesocarp oil. The phosphatidylmethanol content of the present study was estimated by deducting the amount of known phospholipids from the total that was determined by UV spectrophotometry in Section 2.3.2(a). It not uncommon for

phosphatidylmethanol found to be in smaller amount in wet palm mesocarp oil where the water competed with methanol to yield phosphatidic acid.

The composition of phospholipids of ripe mesocarp oil seems in agreement with reports by Goh *et al.* (1982) and Bafor *et al.* (1988). The major components were phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, phosphatidic acid and phosphatidylglycerol whereas the minor component was diphosphatidylglycerol. Lysophosphatidylethanolamine, lysophosphatidylcholine and phosphatidylserine remained at trace levels and were not detected in unsterilised mesocarp oil.

The present study of phospholipids in different stages of development of mesocarp oil in agreement with Moore (1982), Kates and Marshall (1975) implicating phosphatidylcholine as an intermediate both in the synthesis of membrane phospholipids and triacylglycerols and phosphatidic acid as a central metabolite in the synthesis of other polar lipids.

Table 2.5: Phospholipids Composition in the Developing Palm Mesocarp

Mesocarp Oil	Concentration /ppm (mole %) [a]											Total PL [b]
	DPG	PG	PE	PI	PS	PA	LPE	PC	LPC	PM	Total	
SD4	N.D.	N.D.	N.D.	699 (35.87)	N.D.	169 (10.16)	N.D.	877 (43.88)	122 (10.09)	N.D.	1,867	1,690
SW4	N.D.	N.D.	N.D.	6,157 (33.17)	N.D.	1,407 (8.45)	N.D.	8,443 (45.27)	1,583 (13.11)	N.D.	17,590	16,494
UD4	N.D.	N.D.	N.D.	245 (36.71)	N.D.	73 (12.31)	N.D.	308 (40.96)	41 (10.02)	N.D.	667	589
UW4	N.D.	N.D.	N.D.	5,822 (34.59)	N.D.	1,473 (10.32)	N.D.	7,353 (42.16)	1,344 (12.93)	N.D.	15,992	15,305
SD8	N.D.	N.D.	N.D.	2,149 (39.13)	N.D.	N.D.	N.D.	2,834 (46.78)	502 (14.09)	N.D.	5,485	5,126
SW8	N.D.	N.D.	N.D.	6,634 (35.13)	N.D.	N.D.	N.D.	9,112 (47.88)	2,086 (17.05)	N.D.	17,832	16,494
UD8	N.D.	N.D.	N.D.	1,651 (38.84)	N.D.	N.D.	N.D.	2,227 (47.84)	367 (13.32)	N.D.	4,245	3,884
UW8	N.D.	N.D.	N.D.	7,175 (36.05)	N.D.	N.D.	N.D.	10,228 (50.28)	1,697 (13.67)	N.D.	19,100	18,159
SD12	N.D.	N.D.	N.D.	2,718 (35.19)	N.D.	N.D.	N.D.	3,896 (49.23)	745 (15.58)	N.D.	7,359	6,412
SW12	N.D.	N.D.	N.D.	5,374 (33.96)	N.D.	N.D.	N.D.	8,783 (50.03)	1,651 (16.01)	N.D.	15,808	14,704
UD12	N.D.	N.D.	N.D.	1,898 (37.43)	N.D.	N.D.	N.D.	2,531 (48.65)	441 (13.92)	N.D.	4,870	4,259
UW12	N.D.	N.D.	N.D.	4,252 (31.83)	N.D.	N.D.	N.D.	7,085 (52.17)	1,315 (16.00)	N.D.	12,652	12,015

SD16	N.D.	176	1,098	1,899	N.D.	1,342	N.D.	2,895	271	N.D.	7,681	6,578
		(2.31)	(15.03)	(24.69)		(19.73)		(32.81)	(5.43)			
SW16	N.D.	652	2,256	4,894	N.D.	1,986	N.D.	6,526	816	N.D.	17,130	16,331
		(3.52)	(13.38)	(27.46)		(12.32)		(36.27)	(7.05)			
UD16	N.D.	13	228	365	N.D.	324	N.D.	399	32	117[c]	1,478	1,478
		(0.91)	(16.91)	(22.52)		(24.54)		(23.11)	(3.34)	(8.67)		
UW16	N.D.	31	372	505	N.D.	520	N.D.	463	77	144	2,112	2,112
		(1.50)	(10.94)	(23.87)		(27.60)		(23.00)	(5.62)	(7.47)		

SD20	137	275	291	275	T	166	T	537	T	N.D.	1,681	1,467
	(4.55)	(18.31)	(17.36)	(14.46)		(14.49)		(30.87)				
SW20	128	169	386	393	T	50	T	613	T	N.D.	1,739	1,484
	(4.00)	(10.01)	(23.85)	(23.04)		(3.30)		(35.80)				
UD20	160	21	177	180	N.D.	204	N.D.	276	N.D.	716	1,734	1,734
	(4.89)	(1.23)	(10.71)	(10.34)		(13.16)		(15.79)		(43.88)		
UW20	154	28	214	242	N.D.	240	N.D.	324	N.D.	695	1,897	1,897
	(4.30)	(1.50)	(11.87)	(12.72)		(14.20)		(16.96)		(38.45)		

N.D. not detected

[a] Amount quantified by HPLC-ELSD

[b] Amount quantified by UV-visible spectrophotometry

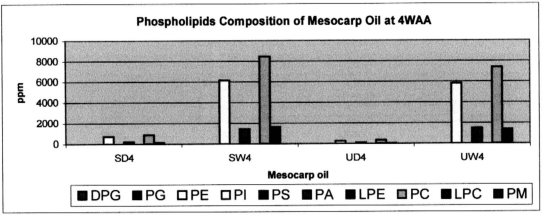
[c] Amount of PM was estimated by reducing the amount of known phospholipids from the total that was determined by UV spectrophotometry (Section 2.2.2(a)).

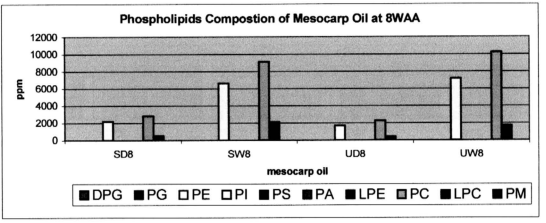
T trace amount

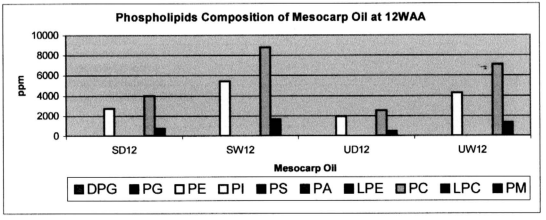
PC phosphatidylcholine
PE phosphatidylethanolamine
PI phosphatidylinositol
PG phosphatidylglycerol
DPG diphosphatidylglycerol
PA phosphatidic acid
PS phosphatidylserine
LPC lysophosphatidylcholine
LPE lysophosphatidylethanolamine
PM phosphatidylmethanol

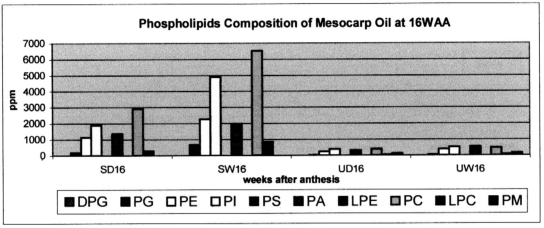
SD sterilised and dried mesocarp oil
SW sterilised and wet mesocarp oil
UD unsterilised and dried mesocarp oil
UW unsterilised and wet mesocarp oil
4,8,12,16,20 weeks after anthesis

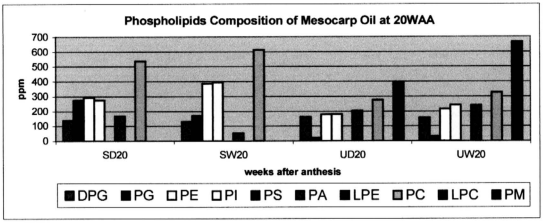
Figure 2.8: Phospholipids Composition in the Developing Palm Mesocarp







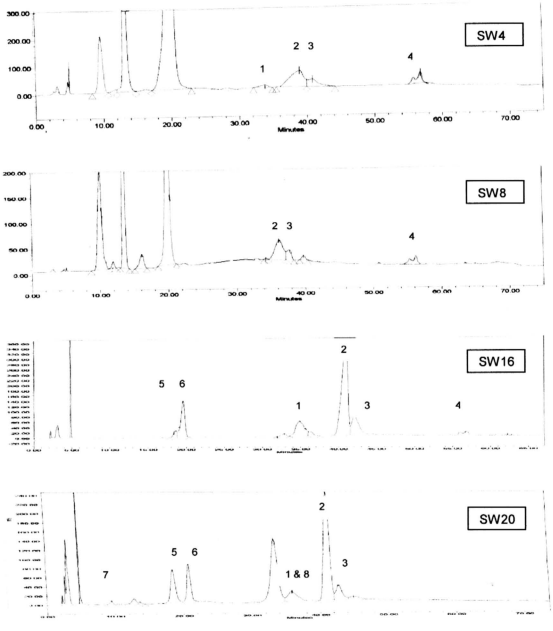




PC	phosphatidylcholine
PE	phosphatidylethanolamine
PA	phosphatidic acid
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
PM	phosphatidylmethanol
DPG	diphosphatidylglycerol
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine

SD	sterilised dried palm mesocarp oil
SW	sterilised wet palm mesocarp oil
UD	unsterilised dried palm mesocarp oil
UW	unsterilised wei palm mesocarp oil
4,8,12,16,20	weeks after anthesis

Figure 2.9: Phospholipids Profile in Developing Palm Mesocarp



1	phosphatidic acid	5	phosphatidylglycerol
2	phosphatidylcholine	6	phosphatidylethanolamine
3	phosphatidylinositol	7	diphosphatidylglycerol
4	lysophosphatidylcholine	8	phosphatidylserine

(c) Fatty Acid Composition of Palm Mesocarp Phospholipids

The fatty acid composition was determined after methanolysis of phospholipids to methyl esters which were quantified by gas chromatography (GC) and the results are listed in Table 2.6.

There were some changes in the fatty acid composition of phospholipids in the developing palm mesocarp due to the treatment prior to the extraction. Generally, the major fatty acids of the phospholipids in immature fruits are palmitic and linoleic acids. In the mature fruit mesocarp, the major fatty acids of the phospholipids are palmitic, oleic and linoleic acid. It is interesting to note that the level of oleic acid in phospholipids is lower in the immature fruits but it become prominent in the ripe fruits. The amount of arachidic acid increased slightly until 12 WAA and reduced to *ca.* 3% in the ripe fruits, whereas myristic acid remained in trace amount throughout the fruits maturation process.

The percentage unsaturation of phospholipids from unsterilised fruits was *ca.* 50%. Also, the same observation was noted in the sterilised and dried fruits extraction, except for SD4.

Table 2.6: Fatty Acid Composition of Phospholipids in Palm Mesocarp

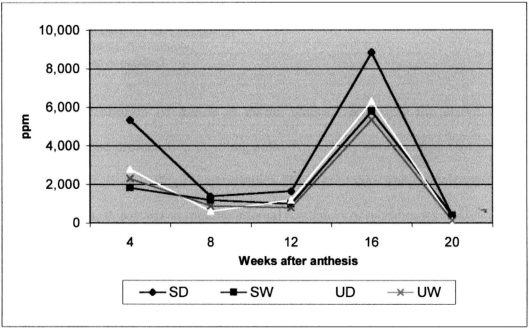
Mesocarp Oil	Fatty Acid Composition (area %)						Percent of Unsaturation
	C14	C16	C18:0	C18:1	C18:2	C20	
SD4	0.16	31.12	2.85	13.57	47.74	4.58	61.31
SD8	0.15	32.64	4.10	15.86	38.24	9.01	54.10
SD12	0.20	33.27	4.31	17.46	33.85	10.91	51.31
SD16	0.79	43.62	2.54	27.56	15.43	10.06	42.99
SD20	0.54	42.29	2.58	25.80	26.21	2.59	52.01
SW4	0.28	32.73	2.65	24.29	36.26	3.59	60.55
SW8	0.18	34.78	4.11	13.82	38.50	8.61	52.32
SW12	0.21	33.11	3.80	22.00	32.37	8.51	54.37
SW16	0.16	32.72	2.25	26.80	35.11	2.96	61.91
SW20	0.19	28.49	1.59	36.78	31.98	0.97	68.76
UD4	0.62	42.63	4.28	14.76	29.51	8.20	44.27
UD8	0.22	40.12	4.10	13.16	34.03	8.37	47.19
UD12	0.35	42.83	5.21	11.07	25.55	14.99	36.62
UD16	0.39	40.69	2.95	33.02	17.59	5.35	50.61
UD20	0.50	42.26	3.27	30.00	20.55	3.40	50.55
UW4	0.18	40.14	3.26	9.08	40.31	7.03	49.39
UW8	0.20	41.30	3.14	12.98	34.76	7.61	47.74
UW12	0.21	33.23	4.37	15.72	34.75	11.72	50.47
UW16	0.35	43.80	3.34	28.77	15.78	7.96	44.55
UW20	0.58	43.91	3.74	30.51	19.40	1.86	49.91

SD - sterilised dried mesocarp oil
SW - sterilised wet mesocarp oil
UD - unsterilised dried mesocarp oil
UW - unsterilised wet mesocarp oil
4,8,12,16,20 - weeks after anthesis

2.3.3 Sterols

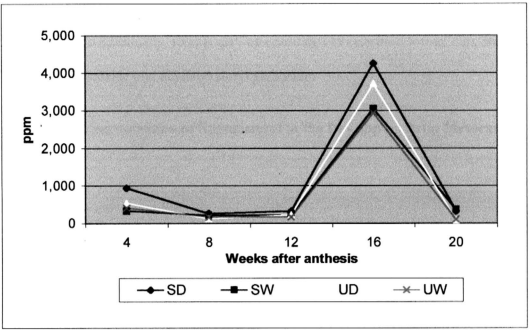
The major sterols in palm oil are common to other edible oil, namely β-sitosterol, campesterol, stigmasterol and minor amount of cholesterol. Their concentrations in the developing palm mesocarp were quantified by gas chromatography and the results are presented in Figures 2.10-2.13. All the sterols except cholesterol showed similar pattern in the distribution during palm fruits development process. The significant amount of sterols was noticeable at 4 WAA, then dropped and remained constant for the next two months. The prominent increment was found at 16 WAA and again diminished in the ripe fruits. All these observations might be closely related to the physiological function of this compound in plants.

Figure 2.10: Concentration of β-Sitosterol in the Developing Palm Mesocarp



SD - sterilised dried mesocarp oil
SW - sterilised wet mesocarp oil
UD - unsterilised dried mesocarp oil
UW - unsterilised wet mesocarp oil
4,8,12,16,20 - weeks after anthesis
Original data refer to Appendix 3

Figure 2.11: Concentration of Campesterol in the Developing Palm Mesocarp



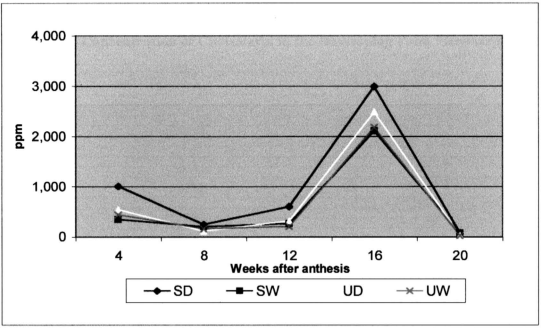
SD - sterilised dried mesocarp oil
SW - sterilised wet mesocarp oil
UD - unsterilised dried mesocarp oil
UW - unsterilised wet mesocarp oil
4,8,12,16,20 - weeks after anthesis
Original data refer to Appendix 3

The high concentration of sterols in earlier stages may imply the active cellular differentiation and proliferation in young palm fruits. They play an important structural role in the lipid core of biological membranes. Because of their unique hydrophobic and steric properties, sterols act as specific internal regulators of membranes fluidity (Demel and DeKruyff, 1976).

The active synthesis of sterols, which occurred at 12 WAA might be due to its metabolic function. Sterols served as precursors of brassinosteroids (Meudt, 1987), a special class of plant growth substances. They also act as substrates for a wide variety

of secondary metabolites such as glycoalkaloids, cardenolides and saponins. The conversion to these compounds may be the cause for the decreasing amount of sterols in the ripe fruits. Additionally, sterols may be converted to esterified forms, e.g., steryl ester, steryl glycoside and acylated steryl glycoside.

Figure 2.12: Concentration of Stigmasterol in the Developing Palm Mesocarp

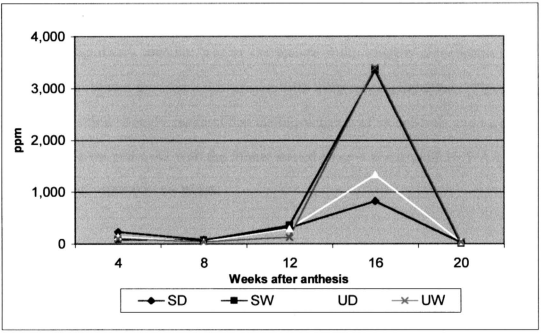


SD - sterilised dried mesocarp oil
SW - sterilised wet mesocarp oil
UD - unsterilised dried mesocarp oil
UW - unsterilised wet mesocarp oil
4,8,12,16,20 - weeks after anthesis
Original data refer to Appendix 3

Cholesterol which is present in negligible amount in normal crude palm oil was found to be actively synthesized at 12 WAA. Its concentration was even higher than stigmasterol at 16 WAA. Cholesterol showed greatest decline as compared to other sterols to about 30ppm in ripe palm fruits.

The major sterols showed similar extraction rate in descending order SD, UD, SW and lastly UW, whereas cholesterol totally opposed this order. The extraction of the more polar cholesterol is facilitated by high moisture content in wet palm fruits. On the other hand, relatively lower moisture content in SD palm fruits gave larger amount of sterols which were shown in Figures 2.10-2.13.

Figure 2.13: Concentration of Cholesterol in the Developing Palm Mesocarp



SD - sterilised dried mesocarp oil
SW - sterilised wet mesocarp oil
UD - unsterilised dried mesocarp oil
UW - unsterilised wet mesocarp oil
4,8,12,16,20 - weeks after anthesis
Original data refer to Appendix 3

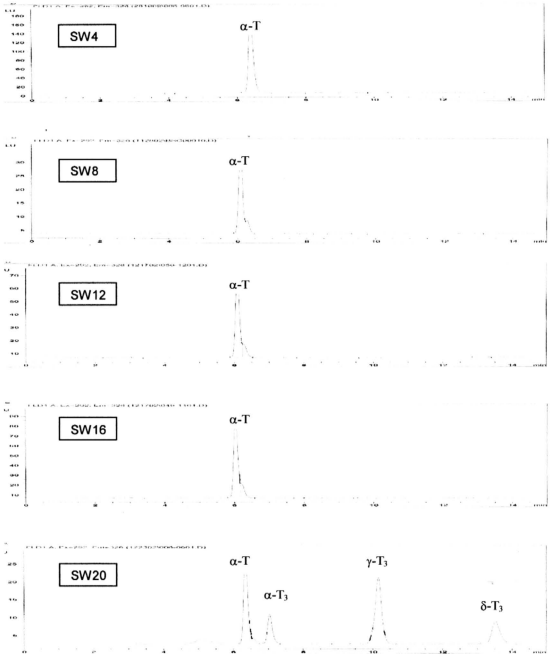
2.3.4 Tocopherols and Tocotrienols

Crude palm oil consists of 600-1000 ppm vitamin E with 20-30% tocopherols and 70-80% tocotrienols. Quantification of vitamin E in the developing palm mesocarp was carried out by HPLC analysis using fluorescence detector. The chromatograms of the analyses are shown in Figure 2.14 and the results tabulated in Table 2.7.

The most interesting observation in the present study is that tocotrienol isomers were found in significant amount only in the mature fruit, whereas α-tocopherol can be detected throughout the maturation process with different concentrations (Figure 2.14). This observation strongly suggests that the biosynthesis of tocopherols and tocotrienols are via different pathways with the former started since 4 WAA until 16 WAA and the latter was activated after 16 WAA.

The possible biosynthesis pathway of tocopherols and tocotrienols is shown in Scheme 2.1 and Scheme 2.2 respectively (Pennock, 1983). The key intermediate, homogentisic acid associated with phytyl pyrophosphate to form 2-methyl-6-phytyl-benzoquinol which is then transforms to δ-tocopherol (δ-T). The methylation of δ-T will yield γ-T or β-T and further methylate to yield α-T. The high concentration of α-T throughout the fruits maturation process may imply a very rapid conversion of δ-T to α-T.

Figure 2.14: Vitamin E Profile for SW Mesocarp Oil



α-T	α-tocopherol	SW	sterilised dried mesocarp oil
α-T_3	α-tocotrienol	4,8,12,16,20	weeks after anthesis
γ-T_3	γ-tocotrienol		
δ-T_3	δ-tocotrienol		

Table 2.7: Concentration of Vitamin E Isomers in the Developing Palm Mesocarp

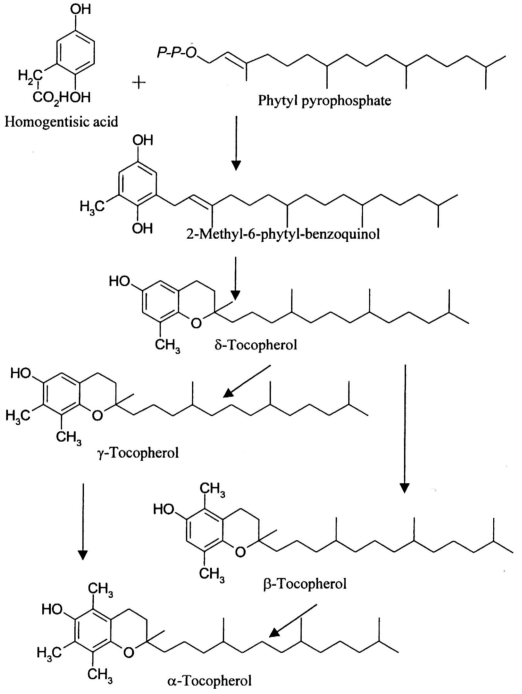
Mesocarp	Concentration / ppm				
Oil	α-T	α-T_3	γ-T_3	δ-T_3	Total
SD4	532	N.D.	N.D.	N.D.	532
SD8	607	N.D.	N.D.	N.D.	607
SD12	900	N.D.	N.D.	N.D.	900
SD16	3,514	N.D.	N.D.	N.D.	3,514
SD20	771	530	1,811	431	3,543
SW4	460	N.D.	N.D.	N.D.	460
SW8	1,356	N.D.	N.D.	N.D.	1,356
SW12	1,384	N.D.	N.D.	N.D.	1,384
SW16	4,650	N.D.	N.D.	N.D.	4,650
SW20	1,049	644	1,905	451	4,050
UD4	165	N.D.	N.D.	N.D.	165
UD8	331	N.D.	N.D.	N.D.	331
UD12	1,036	N.D.	N.D.	N.D.	1,036
UD16	1,485	N.D.	N.D.	N.D.	1,485
UD20	238	126	463	130	957
UW4	57	N.D.	N.D.	N.D.	57
UW8	138	N.D.	N.D.	N.D.	138
UW12	436	N.D.	N.D.	N.D.	436
UW16	1,145	N.D.	N.D.	N.D.	1,145
UW20	207	118	488	134	947

N.D. - not detected
SD - sterilised dried mesocarp oil
SW - sterilised wet mesocarp oil
UD - unsterilised dried mesocarp oil
UW - unsterilised wet mesocarp oil
4,8,12,16,20 - weeks after anthesis

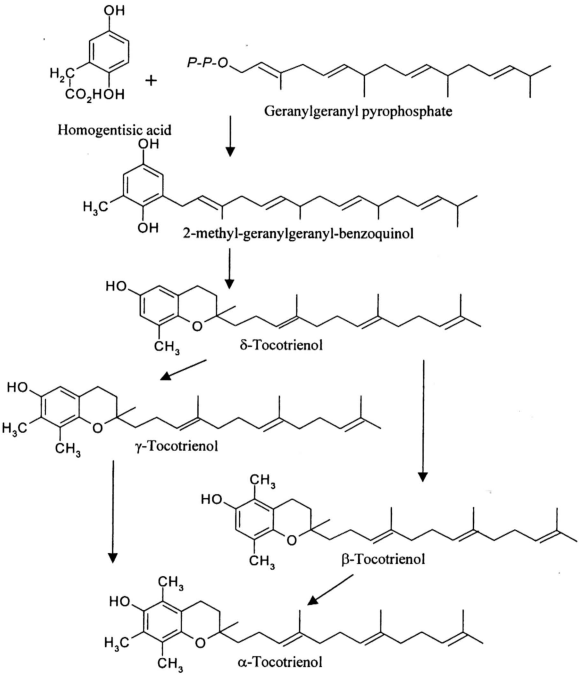
On ripening, the biosynthesis of tocotrienols might be activated by an increasing chloroplast degeneration that leads to the loss of chlorophyll, the synthesis site of tocopherols (Threlfall, 1967; Botham and Pennock, 1971). The homogentisic acid is now turned to combine with geranylgeranyl pyrophosphate to form 2-methyl-6-geranylgeranyl-benzoquinol that is converted to δ-tocotrienol. Further methylation is needed to yield β-, γ- and α-tocotrienol.

The sterilised palm fruits extracts gave much larger amount of α-T suggesting the existence of oxidative enzymes in the developing palm fruits. For example, the phenolase enzymes may be present and promoting tissue browning (David, 1970) in unsterilised palm fruit subsequent to peeling. α-T acts as a strong antioxidant retarding the phenolase activity. Also, water may assist in the extraction of α-T as can be seen in the SW fruit extraction.

Scheme 2.1: Biosynthesis Pathway of Tocopherols



Scheme 2.2: Biosynthesis Pathway of Tocotrienols



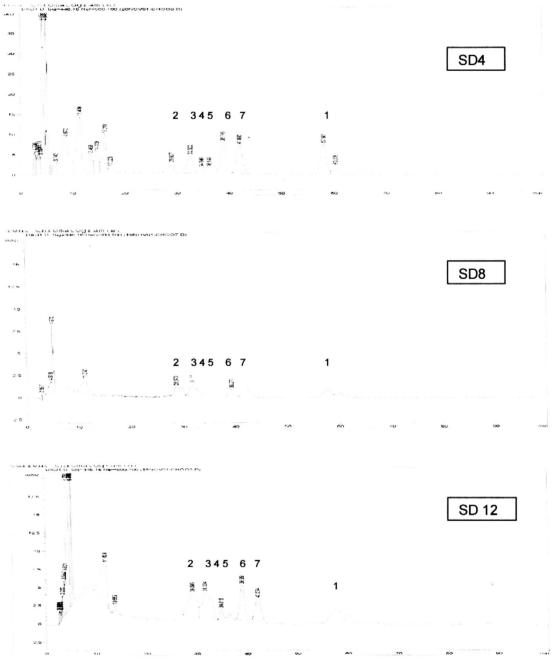
2.3.5 Carotenes

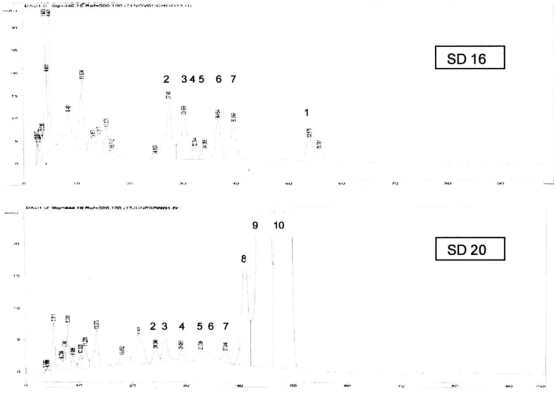
Palm carotenoids consist of hydrocarbon (carotenes) and oxygenated carotenoids (xanthophylls). The present study focus on the carotenes changes during palm fruits development. The composition of carotenes in the developing palm fruits was determined qualitatively using HPLC coupled with photodiode array detector (HPLC-PDA). The carotene profile in sterilised dried (SD) mesocarp oil of different stages of palm fruits ripeness is shown in Figure 2.15. All, namely SD, SW, UD and UW mesocarp oils exhibited similar carotene profile pattern.

The chromatograms revealed that the major components of crude palm oil, namely α- and β-carotenes were detected only in the ripe mesocarp oil. Contrarily, the minor carotenes, e.g. phytoene, lycopene, neurosporene, α- and β-zeacarotene, δ- and γ-carotene were detected throughout the maturation process. The observation was in agreement with previous report (Ilango *et al.*, 1988 and 1989; Ikemefuna and Adamson, 1984), which stated the gradual biosynthesis of carotene in the early stages of fruits development but with α- and β-carotenes' massive accumulation after 16 WAA. This concur with the observed changes of greenish-yellow pericarp of immature fruit to bright waxy orange pericarp of mature fruit.

It is believed that the biosynthesis of carotenes in palm mesocarp was similar to higher plants, as described in Section 1.1.4(d) and the possible pathway was illustrated in Scheme 2.2. The first basic carotene, phytoene was formed and later undergo sequential desaturation to produce phytofluene, ζ-carotene, neurosporene and lycopene.

Figure 2.15: Carotene Profile for SD Mesocarp Oil of Developing Palm Fruits

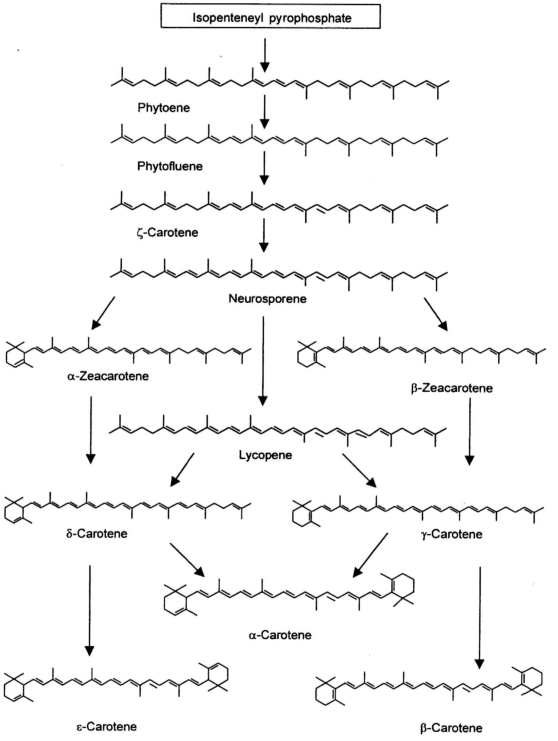




1	Phytoene	6	δ-Carotene
2	Lycopene	7	γ-Carotene
3	α-Zeacarotene	8	cis-α-Carotene
4	β-Zeacarotene	9	α-Carotene
5	Neurosporene	10	β-Carotene
SD	sterilised dried mesocarp oil	4,8,12,16,20	weeks after anthesis

Then, cyclisation takes place to form α- and β-zeacarotene, δ- and γ-carotene, and eventually to the end products, namely α- and β-carotenes. However, enzymes which controll cyclisation steps in the formation of α- and β-carotenes seem to appear only after 16 WAA. This phenomenon might be correlated to the chloroplast degeneration leading to the loss of chlorophyll started at 16 WAA (Ikemefuna and Adamson, 1984). α- and β-carotenes were synthesised and served compensatory function as light harvesting complex, or else as photoprotective agent (Ooquist *et al.*, 1980).

Scheme 2.3: Biosynthesis Pathway of Carotenes in Higher Plants



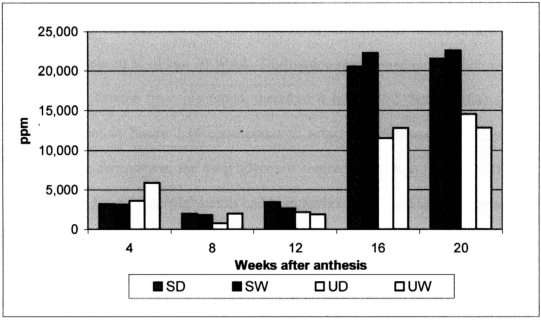
2.3.6 Diacylglycerols, Triacylglycerols, Monoacylglycerols and Free Fatty Acids

The concentration of diacylglycerols, triacylglycerols, monoacylglycerols and free fatty acids at different stages of fruit ripening, i.e. 4, 8, 12, 16 and 20 WAA were quantified by GC-FID and the results are shown in Figures 2.16-2.19.

Diacylglycerols: Diacylglycerols were found in very low concentration in early stages of maturation of palm fruits and until 12 WAA, they increased by *ca.* seven times and five times of the previous quantity in sterilised and unsterilised palm mesocarp oil respectively at 16 WAA (Figure 2.16). This observation was in good agreement with the results of Siew and Ng (1997) which reported that the composition of diacylglycerols in palm oil was dependent on the degree of fruit ripeness and the extent of hydrolytic degradation. Thus, the tremendous increase in diacylglycerols content can be attributed either as endogenous compounds (1,2-diacylglycerol) or as partial hydrolysed acylglycerols (1,3-diacylglycerol).

It is well established that oil accumulation started *ca.* 16 WAA and the significant increase of diacylglycerols content in palm mesocarp can be anticipated as a preparation of this process. Phosphatidic acid phosphatase (also called phosphatidate phosphohydrolase) catalyzes the dephosphorylation of phosphatidic acid to generate diacylglycerols and then eventually forming triacylglycerols by further acylation; or for the zwitterionic phospholipids biosynthesis. There might be a compromise between the biosynthesis of phospholipids and triacylglycerols at the moment. According to the Stymne *et al.* (1987) and Mcmaster and Bell (1997), the incorporation of phosphorylcholine into diacylglycerols which was the final reaction in the Kennedy

Figure 2.16: Concentration of Diacylglycerols in the Developing Palm Mesocarp



SD - sterilised dried mesocarp oil
SW - sterilised wet mesocarp oil
UD - unsterilised dried mesocarp oil
UW - unsterilised wet mesocarp oil
4,8,12,16,20 - weeks after anthesis
Original data refer to Appendix 3

pathway for phosphatidylcholine biosynthesis in safflower microsomes can be suppressed, reversible and lead to the formation of diacylglycerols from phosphatidylcholine under the conditions of active triacylglycerols biosynthesis. Thus, we could anticipate that the same mechanism might occur in the palm mesocarp in the active phase of triacylglycerols biosynthesis. There is no massive accumulation of phosphatidic acid content as shown in Table 2.5, Section 2.3.2(c) which suggests the rapid turnover from phosphatidic acid to diacylglycerols in the palm mesocarp at 16 WAA. The results in Table 2.5 merely represent the phospholipids composition in membrane phospholipids.

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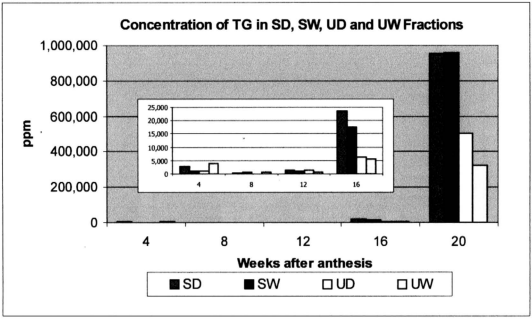
Contrarily to expectation, higher diacylglycerols content was found in the sterilised palm mesocarp oil at 16 and 20 WAA. Sterilisation was carried out as soon as possible after fruit collection from plantation, therefore it is believed that the diacylglycerols content shown in Figure 2.16 corresponds to actual diacylglycerols quantity in palm mesocarp. In comparison, the diacylglycerols content is lower in the unsterilised palm mesocarp oil because diacylglycerols had been further hydrolysed to free fatty acids.

Triacylglycerols: Obviously the active phase of triacylglycerols accumulation in palm mesocarp started *ca.*16 WAA. This observation is in good agreement with the increase of diacylglycerols at the same period since they serve as precursors of triacylglycerols biosynthesis. Triacylglycerols content was found to be less than 1% for all types of mesocarp oil during the first 12 weeks of fruit development and thereafter rose rapidly to 95% on the wet weight basis in sterilised palm mesocarp oil. The lipases activity which is not stopped in unsterilised mesocarp oil eventually decreased the triacylglycerols amount to less than 35% as can be seen in UW20. Meanwhile the free fatty acid content for UW20 is more than 60%.

Monoacylglycerols: Monoacylglycerols are not involved in the triacylglycerols biosynthesis and their presence in palm mesocarp oil is probably a consequence of lypolitic activity. From Figure 2.18, the monoacylglycerols content of the sterilised palm mesocarp oil is low and constant throughout the maturation process. On the other hand, the amount of monoacylglycerols in unsterilised mesocarp oil is relatively lower than our expectations because it has been hydrolysed to free fatty acids as can be seen in UD16 and UW16.

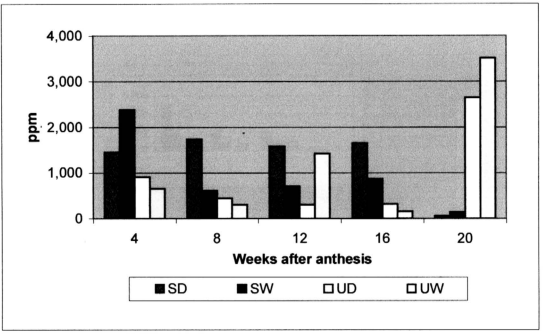
Figure 2.17: Concentration of Triacylglycerols in the Developing Palm Mesocarp



SD - sterilised dried mesocarp oil
SW - sterilised wet mesocarp oil
UD - unsterilised dried mesocarp oil
UW - unsterilised wet mesocarp oil
4,8,12,16,20 - weeks after anthesis
Original data refer to Appendix 3

Free fatty acids: The presence of endogenous lipases activity in oil palm mesocarp had been contradictory and was finally firmly established by two reports (Henderson and Osborne, 1991; Sambanthamurthi *et al.*, 1991). The formation of free fatty acids from acylglycerols in palm oil is due to the catalytic action of the enzyme lipase. This formation is also pronounced when the palm mesocarp is bruised. The free fatty acids level in sterilised palm mesocarp oil is low throughout the development of the palm fruits (Figure 2.19). However, the level of free fatty acids in unsterilised palm mesocarp oil increases extensively at 16 WAA. Thus, lipases activity in the oil palm mesocarp is synchronised with oil accumulation and reaches maximum at 20 WAA. The presence of lipases at 16 WAA is probable therefore an inducible agent for fruit ripening.

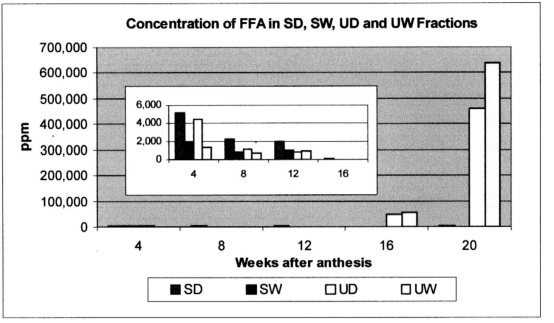
Figure 2.18: Concentration of Monoacylglycerols in the Developing Palm Mesocarp



SD - sterilised dried mesocarp oil
SW - sterilised wet mesocarp oil
UD - unsterilised dried mesocarp oil
UW - unsterilised wet mesocarp oil
4,8,12,16,20 - weeks after anthesis
Original data refer to Appendix 3

In spite of these, lipases are powerful tools for catalyzing not only hydrolysis but also esterification and transesterification reactions. The possible roles of mesocarp lipases in transesterification during oil accumulation was supported by synchronisation of lipases activity with triacylglycerols biosynthesis.

Figure 2.19: Concentration of Free Fatty Acids in the Developing Palm Mesocarp

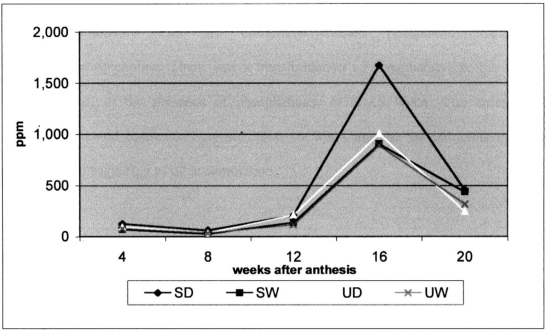


SD - sterilised dried mesocarp oil
SW - sterilised wet mesocarp oil
UD - unsterilised dried mesocarp oil
UW - unsterilised wet mesocarp oil
4,8,12,16,20 - weeks after anthesis
Original data refer to Appendix 3

2.3.7 Squalene

Squalene as precursor in the biosynthesis of sterols showed similar pattern in distribution during oil palm fruits maturation process. The highest concentration is *ca.* 1600 ppm formed in SD palm mesocarp oil at 16 WAA as shown in Figure 2.20.

Figure 2.20: Concentration of Squalene in the Developing Palm Mesocarp



SD - sterilised dried mesocarp oil
SW - sterilised wet mesocarp oil
UD - unsterilised dried mesocarp oil
UW - unsterilised wet mesocarp oil
4,8,12,16,20 - weeks after anthesis
Original data refer to Appendix 3

2.3.8 Summary of Present Findings

The biochemical changes, e.g., phospholipids, tocopherols and tocotrienols, carotenoids, triacylglycerols, partial acylglycerols, free fatty acids, sterols and squalene in the developing palm mesocarp have been investigated. The distribution pattern of these compounds revealed some of their possible roles during maturation process and their fates resulted from different treatments before oil extraction.

The increase in phospholipids and sterols contents due to cellular proliferation at earlier stages of fruits ripening has been observed. The major phospholipids components found

in the immature fruit are phosphatidylcholine, phosphatidylinositol and lysophosphatidylcholine. There was a transformation of phosphatidylcholine to other phospholipids in the presence of phosphalipase D at 16 WAA. The existence of phosphatases and lipases activity started at 16 WAA may be the inducible agent for ripening and triggering of oil accumulation.

The biosynthesis of tocopherols and tocotrienols might follow different pathway with the former activated at the earlier stages of fruits ripening, whereas the latter was activated after 16 WAA. At the same time, there was a massive biosynthesis of carotenoids followed by degradation of chlorophyll mainly due to the biosynthesis of α- and β-carotenes.

The sterols were found in significant amount at 4 WAA and 16 WAA. This observation suggests the active cellular proliferation at 4 WAA and the sterols accumulation precede the oil accumulation and reached the maximum at 16 WAA. Squalene showed similar distribution pattern as sterols in the developing palm mesocarp.

The maturation and ripening of palm fruits is a very complicated process which involve numerous biochemical and chemical changes. 16 WAA seems to be the most important growth stage where many significant changes were found in the palm fruits.