CHAPTER 2

Lipid Constituents of Oil Droplets from Palm Oil Sludge

2.1 INTRODUCTION

The term "LIPID" has traditionally been used to denote a variety of natural products including fatty acids and their derivatives, steroids, terpenes, carotenoids and bile acids which are soluble in common organic solvents. A more specific definition generally restricted them to fatty acids and their derivatives or metabolites (1).

The principal lipid classes of fatty acid (long chain aliphatic monocarboxylic acid) moieties linked by an ester bond to an alcohol, mainly the trihydric alcohol, glycerol, or by amide bond to long-chain bases. Also, they may contain phosphoric acid, organic bases, sugars or more complex components that can be liberated by various hydrolytic processes. Lipids may be subdivided into two broad classes of 'simple' and 'complex' lipids depending on the number of hydrolysis products produced when hydrolysed. The terms 'neutral' and 'polar' respectively are used to define these classes.

The number of fatty acids detected in plant tissues approaches 300 but most of them only occur in a few plant species (2). The major fatty acids are either saturated or unsaturated monocarboxylic acids with an unbranched, even numbered carbon chain. The saturated C12:0-C18:0 and the unsaturated C18:1, C18:2 and C18:3 together account for almost all of the fatty acid content of higher plants.

By far the largest group of plant acyl lipid is based on the trihydric alcohol glycerol and includes acyl glycerol, glycerophospholipids and glycosylglycerides illustrated in Fig. 1a-c respectively. Various tissues of the plant may contain various lipids in different quantities.

2.1.1 Palm Oil Lipids

Oil palms in Malaysia is mainly derived from the *Elaeis guineensis* which is a cross breed of *Dura x Psifera*. The Malaysian palm oil and palm kernel oil composition are fairly consistent as the majority of the plantations are commercially cultivated with such palms.

The lipids present in the palm fruits can be categorised mainly into two major classes of triglycerides depending from which part of the fruit they are derived. From the mesocarp where palm oil is

derived, the major triglycerides consists of the C50 and C52 (3) while the kernel oil which is derived from the kernel mainly consists of C36 and C38 (Table 1) (4). The major fatty acids in palm oil are the C16:0 palmitic and C18:1 oleic but kernel oil consists of shorter chain fatty acids of mainly C12:0 lauric and C14:0 myristic acids and unsaturated C18:1 oleic (Table 2).

Palm oil contains about 1% of a mixture of minor components (5). This includes the carotenoids which impart the typical orange reddish colour of crude palm oil. They range from 500-700 ppm in crude palm oil. Tocopherols and tocotrienols range from 600-1000 ppm in crude palm oil and possess anti oxidant properties suitable for food applications. In view of some of their positive health related findings (6,7) and their relatively high level in palm oil, extraction of these two natural resources from palm oil is being actively pursued on a commercial scale (8,9). Sterols (326-527 ppm), where the major ones have been identified as sitosterol and campesterol have also been well characterised (10).

Phospholipids have been widely studied due to their vital role in biomembrane structure of living cells. All living cells are bound by a plasma membrane which basically consists of a bilayer of phospholipids (11). Embedded in this bilayer are cholesterol and proteins

while the carbohydrate component of glycoprotein and glycolipids occur on the external surface of the membrane. In plant cells, further fortification is provided by a layer of cellulose cell wall.

Phospholipids are amphiphilic compounds and exhibit various mesomorphic forms with water under different conditions (12). They are also good emulsifiers and in view of their surface active properties, phospholipids are commonly used in various food and non-food products (13). Phospholipids derived from soya bean, commonly known as lecithin are available commercially. Glycolipids have been extracted from plant leaves and wheat endosperm and can form liquid crystals with water (14,15) but their commercial potential and possiblity in food application is not known.

Crude palm oil extracted from the mesocarp of *Elaeis guineesis* by chloroform-methanol solvent mixture was found to contain 1000-2000 ppm of phospholipids while crude palm oil obtained from conventional milling process contained only 20-80 ppm of phospholipids (16). 0.37% w/w glycolipids were reported to be present in crude palm oil (17). 3.8% w/w glycolipids was found in the oil from spent earth discarded in the refining process (18). Both phospholipids and glycolipids were detected in palm oil extracted from the mesocarp of the dura and psifera

varieties of palm although the quantity in relation to the oil was not given (19).

Typical palm oil contains mainly triglycerides but partial glycerides like monoglycerides and diglycerides are important intermediates in the biosynthesis of the oil and are thus present in palm oil. In fact palm oil is an unusual oil in that it posesses a high concentration of diglycerides (5.0-7.9%) compared to other vegetable oils (20). Monoglycerides are amphiphilic and exhibit various mesomorphic states at different temperatures and water content (21). They rank among the most commonly used emulsifiers in the food industry while the use of diglycerides singly as an emulsifier could not be found in references surveyed but Kako and Kando (22) found that a critical ratio of monoglycerides and diglycerides gave a stable emulsion of the soya bean oil droplet in water. These two partial glycerides at certain ratios were also determined to be highly surface active in displacing and replacing the proteins interface formed in corn oil emulsions (23).

Although the presence of certain minor components including phosphorus containing compounds has been identified as playing a significant role in refinability and stability of palm oil (24), the diglycerides have a profound effect on the crystallisation behaviour in fractionation (25). They could possibly have a strong

influence on the stability of oil droplets found in palm oil mill effluent especially the sludge discharge.

Should the oil droplets be stabilised by a layer of such amphiphiles,they will be stable against coalescence and flocculation, rendering their recovery difficult in the present milling process which is mainly mechanical. A study to determine whether such substances are present in the oil droplets from the sludge could provide a better understanding of the milling process.

2.2 MATERIALS AND METHODS

2.2.1 Separation of Oil Droplets from Centrifuged Sludge

Fresh sludge was collected from the separator of a 60 tonne-per-hour palm oil mill and used immediately the next day. The sludge was warmed to at least 60°C and centrifuged at 10,000 rpm for 20 minutes at 30°C using a Sorvall High Speed centrifuge with a fixed angle rotor GSA which is capable of centrifuging 6 x 200 ml of sample in one run. About 15 litres of sludge was centrifuged within a day.

The creamed oil droplets together with some supernatant were scooped off from the surface of the sludge in the centrifuge tubes. The oil droplets were pooled together and then divided into 2 portions. Each

portion was washed by hand shaking with 200 ml of distilled water at 60°C and re-centrifuged to separate the oil droplets. The washing was repeated and the free oil droplets by now would have concentrated to form a dense layer of yellowish cream which could be lifted easily by a flat spatula from the centrifuge tube.

The concentrated oil droplets were weighed into round bottom flask and vacuum dried at 60°C. The moisture content was determined.

The phosphorus and nitrogen contents of the oil droplets were determined before and after every water washing. Phosphorus determination was carried out using the ashing and colorimetric method (26). Nitrogen was determined using the modified Kjedahl method (27).

2.2.2 Microscopy

Optical microscopy was carried out using an Olympus BH microscope and microphotographs taken with a Nikkon camera.

2.2.3 Particle Sizing

The top layer of oil droplets and sedimented solids respectively were redispersed in distilled water and their particle size determined using a Malvern Mastersizer with a 100 mm lens.

2.2.4 Extraction of Lipids from Dried Oil Droplets

About 1 g of the two-times water-washed, dried oil droplets was weighed into a small beaker. 10 ml of warm methanol was added and stirred for 1 minute using a magnetic stirrer followed by 20 ml chloroform and further stirring for another 2 minutes. The methanol/chloroform was filtered into a pre-weighed flask.

The residual solids were extracted with 30 ml of a $CHCl_3$: MeOH (2:1) mixture and filtered. Further extraction of the solids were carried out using 20 ml of chloroform and finally another 10 ml of methanol. The combined filtrate was concentrated by evaporation, spotted on to TLC plates, developed in a solvent system consisting of ($CHCl_3$:MeOH: CH_3COOH:H_2O) (170:25:25:6 v/v) and charred for preliminary observations.

2.2.5 Column Chromatography of Lipid Extract

Preliminary separation of the above lipids mixture into simple and complex lipids was carried out using column chromatography. 10 g of silicic acid (previously conditioned at 110°C) were slowly packed into a 1.5 cm x 30 cm glass column and sealed with some glass wool near the teflon stopper.

About 0.3 g of the lipid dissolved in chloroform was eluted stepwise with 200 ml $CHCl_3$ (Fraction 1), 800

ml CH_3COCH_3 (Fraction 2) and 200 ml MeOH (Fraction 3) at a flow rate of about 3 ml per minute. Initially, volumes of 10 ml eluant were collected regularly to determine the required volume of each solvent needed to completely elute each class of lipids. The eluant collected was rota-evaporated and the dried lipid fraction weighed.

2.2.6 Thin Layer Chromatography

Glass plates measuring 20 cm x 20 cm were coated with a millimeter thick layer of silica gel G (Merck) using a spreader. 70 g of silica gel was made into an aqueous slurry with 140 gm of water and poured on the first plate and quickly spread to the rest of the plates with a roller.

The plates were air dried overnight, then conditioned at 110°C for at least 2 hours and stored in desiccators until needed.

The dried fraction eluted with chloroform (Fraction 1) was dissolved with a little chloroform and spotted on to the TLC plate using a microsyringe. Elution of the plates was carried out in a solvent tank consisting of hexane:diethyl ether:formic acid (80:20:2) v/v. The solvent tank was prepared the previous day to ensure saturation of the vapour. A piece of filter paper was also placed in the tank for the same reason.

The dried fraction eluted with acetone (Fraction 2) and with methanol (Fraction 3) were also dissolved in acetone and methanol respectively and spotted on to TLC plates. Elution of Fractions 2 and 3 were carried out in separate tanks using the solvent system $CHCl_3$:MeOH:CH_3COOH:H_2O (170:25:25:4) v/v.

2.2.7 Identification of Lipids

Identification of lipids was by visualiser sprays and by comparing the retention time with authentic compounds wherever possible. The simple lipids were compared with those of bulk palm oil where the lipids are known.

2.2.8 Preparation of Visualiser Sprays

a) Zinzade reagent - 6.85 g of sodium molybdate dihydrate and 0.4 g hydrazine sulphate were weighed into a flask. 100 ml of water was added. Another 250 ml of concentrated sulphuric acid was slowly added and then further diluted with 300 ml water. The mixture was cooled constantly under running water. The reagent is dark greenish brown and phospholipids are stained blue instantly, if present.

b) Orcinol-sulphuric mixture - 200 mg of orcinol was dissolved in 100 ml of 75% sulphuric acid.

Glycolipids appeared as reddish purple spots when heated.

c) 'Dragendorf' reagent - Solution A was prepared from 40 g potassium iodide dissolved in 100 ml water. Solution B consists of 1.7 g bismuth substrate in 100 ml of 20% acetic acid. 5 ml of solution A was mixed with 20 ml of solution B. The mixture was diluted with 75 ml of water. Choline containing compounds are stained orange red with this reagent.

d) 2',7'-Dichlorofluorescein spray - 0.1 g of 2'-7' dichlorofluorescein were dissolved in 100 ml of 95% methanol. The spray is bright orange in colour. Simple lipids become obviously fluorescent when viewed under UV light.

e) Ferric chloride spray - 50 mg of $FeCl_3.6H_2O$ were dissolved in 90 ml of water, 5 ml acetic acid and 5 ml of sulphuric acid. The spray is colourless but stained cholesterol or cholesterol ester to pinkish purple color.

Qualitative identification of the compounds was carried out on narrow bands of the mixture spotted near the base of the plate and eluted.

For quantitative purpose, a long band was spotted on the plate and, after elution, the plate was covered with a narrower clean glass leaving only a small margin on

both sides of the plate. The visualiser was then sprayed on the uncovered part of the plate and heated with a blower, if necessary. The required band was then marked and scrapped for further analysis.

2.2.9 Quantification of Glycolipids

The acetone fraction (Fractions 2) was separated into 6 individual bands by TLC, visualised by Orcinol spray, marked and scraped into tubes. Quantification of the hexose units in glycolipids was according to Yamamota and Rouser (28). A solution consisting of 2% anthrone in 98.9% H_2SO_4 (10 ml) was diluted with 87.5% H_2SO_4 (90 ml). 4 ml of this reagent was added to the separated TLC plate scrapings in 1 ml of dimethylformamide and heated in a boiling-water bath for 4 minutes. The samples were then cooled instantly by immersing the tubes in ice-cold water. The complex formed is dark brownish in colour and its absorbance measured at 652 nm in a Hitachi U-2000 UV spectrophotometer. Calibration was carried out with standard solutions containing 10, 20, 40 and 100 μg glucose/ml.

2.2.10 Quantification of Phosphorus

The methanol fraction (Fraction 3) was separated into 5 bands by TLC and scraped into crucibles and its phosphorus content determined according to the PORIM

method (21) where the sample was ashed with 0.1g magnesium oxide at 500°C and dissolved in 5 ml of 6M HNO_3. 20 ml each of aqueous ammonium molybdate containing 50 g dm^{-3}, and 5 g dm^{-3} ammonium vanadate solution in 20% dilute nitric acid were used to form a blue phosphorus complex. The absorbance was measured at 400 nm using the Hitachi U-2000 UV spectrophotometer. Calibration was carried out with aqueous solutions containing 4,8,12,16 μg/ml of phosphorus (potassium hydrogen phosphate, KH_2PO_4).

2.2.11 Fatty Acid Composition Analysis

Methylation of Lipids

Methylation of simple lipids was carried out by adding 1 ml of 0.5 M sodium methoxide to 3 drops of the sample dissolved in 2 ml of hexane. The methyl esters were washed with water and dried with anhydrous sodium sulphate before injecting into the gas chromatograph.

Free fatty acids and phospholipids were converted to the methyl esters of the fatty acid using methanolic Boron trifluoride. The sample was refluxed with 4ml methanolic sodium hydroxide followed by 5ml methanolic boron trifluoride. 3ml heptane was introduced into the flask and then floated to the neck of the flask with saturated sodium chloride. The heptane fraction was pipetted off and dried with a little anhydrous sodium

sulphate. The purified ester when ready was injected into the gas chromatograph.

Gas Chromatography

Methyl esters were identified using a HP 5890 Series II chromatograph using a SP-2340 fused silica capillary column (60 m, 0.25 mm ID). The injector, oven and detector temperatures were at 240°C, 180°C and 240°C respectively. The carrier gas, helium, was regulated at 1 ml/min.

The relative percentage of each fatty acid as methyl ester was calculated with the HP integrator 3396. Retention time of each component was based on a secondary standard of palm oil glycerides analysis. The primary standard used was RM6 from Sigma Chemicals.

2.2.12 Glycerides Analysis

Silylation of Glycerides

About 0.5 g of the glycerides was dissolved in 0.5 ml of dichloromethane. 0.5 ml of N_1O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) was added. The mixture was capped and heated (40-50°C) for 15 minutes.

Gas Chromatography

0.1 μl of the above silylated compound was injected into a Hitachi G-3000 gas chromatograph fitted with a fused silica capillary column of PEG-20M Bonded liquid phase (15 m, 0.53 mm ID). The injector and detector temperatures were at 270°C and 340°C respectively. The oven temperature was programmed to maintain at 100°C for the first minute and subsequently raised to 320°C within 20 minutes.

The relative percentage of the glycerides was calculated with the integrator. Retention time of each component was based on a palm oil standard with an added internal standard, tricaprin.

*Summary of analytical techniques used in the Lipid Characterisation of Oil Droplets is attached as Appendix I.

2.3 RESULTS AND DISCUSSION

2.3.1 Separation of Oil Droplets and Phosphorus Determination

Sludge from the separator in the clarification station of a palm oil mill is viscous and dark brown in colour. When it is centrifuged at high centrifugal force of 7,600 G, oil droplets will float to the surface as a yellowish

cream (Fig. 2). This layer of oil droplets has a phosphorus content of 2972-5506 ppm. On washing the oil droplets with water, a small amount of dark brown solids sedimented while most of the oil droplets remained floated as a more condensed cream layer. After the second washing of the oil droplets with water and centrifuged, they become compact and could be easily lifted up by a spatula while some residual solids sedimented. Immediately after centrifugation, especially the first two centrifugations, the oil droplets tend to redisperse rapidly back into the supernatant and therefore they must be collected immediately after centrifugation.

After each washing, the phosphorus content of the oil droplets increased (Table 3), possibly due to the removal of the heavier plant cell materials which contained lower phosphorus content on a dry basis. The sediment did not contain as high a concentration of phosphorus as the oil droplets from the top layer. The high phosphorus content of the dried supernatant is due to the resuspension of the oil droplets from the cream layer. Less than 40 ppm were present on a wet basis. After recentrifugation, oil droplets made up the major portion of the total weight of the top layer on a dry basis. Thus the phosphorus is associated with the oil droplets which are retained in the top layer after centrifugation. The droplets contained a smaller amount

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of nitrogen which are possibly proteins associated with the plasma membrane. The nitrogen content is extremely high in the sedimented solids.

2.3.2 Microscopy and Particle Sizing

Optical microscopy shows that the top cream layer separated by centrifugation contained spherical free oil droplets (Fig. 3) and some plant cells with entrapped big oil droplets. The sedimented solids were mainly plant cells with little or no oil droplets inside. Masses of plant cells fragments were also present (Fig. 4a-b).

Particle sizing confirms the presence of these two types of particulates in the cream and sediment respectively. The cream layer consists of fragments of plant cells and mainly oil droplets ranging from less than 1 μm to 10 μm (Fig. 5). About 15% of the fragments are whole plant cells with entrapped large globule of oil. These oil-bearing cells range from 20 to 120 μm in diameter. The sedimented solids are similar in dimension to the oil-bearing plant cells but the majority do not have any enclosed oil droplet. The dense aggreggates of plant fragments are easily redispered in water.

After washing and re-centrifugation twice with water, some unruptured oil-containing cells are still retained in the top cream layer as seen from the particle size distribution (Fig. 6). More plant cells and

fragments of plant cells sediment on centrifugation. The smaller particulates are the cell organelles and ruptured membranes. Cell organelles and membrane are denser than water (29) and they sediment when centrifuged while the majority of the small oil droplets and cells with bigger entrapped oil droplets of lower density will cream.

The varied distribution of oil droplets, plant cells, plant cells with entrapped oil droplets, fragments of plant cells and organelles possibly contributed to the great variation but high phosphorus content of the different layers of sludge when centrifuged. Generally about 40-50% of the weight of membrane is contributed by the main lipid classes of phospholipids, glycolipids, sterols and neutral lipid (29). Next is proteins which account for 35-40% of it while the third major component is carbohydrates. Nitrogen, an integral part of proteins associated with plasma membrane was also found in high concentration in the washed oil droplets. The ratio of phospholipid to protein at 10:1 (wt:wt) on the two times washed oil droplets compared with a lower ratio of 1:1.6 determined in the spherosomes (oil bodies) of peanuts (30) indicates that the oil droplets separated are probably not natural oil bodies of the palm fruit but oil droplets emulsified during the milling process.

2.3.3 Chemical Analysis of Oil Droplets

The oil droplets from the centrifuged sludge, after water washing and recentrifugation twice, contained 55 wt % water, 33 wt % $CHCl_3$/MeOH extractable and 12 wt % residual non-lipid solids (Table 4). The plant cells contributed to the nonlipid solids. The $CHCl_3$/MeOH extract contained 3800-4080 ppm of phosphorus equivalent to 8-10 wt % phospholipids in the total extract.

Further chemical analysis were carried out on the $CHCl_3$/MeOH extract. Elution on TLC plates indicated the presence of a total of at least twelve compounds (Fig. 7). Five were positive to Zinzade spray and seven positive to Orcinol showing the presence of phospholipids and glycolipids respectively. 2 bands appeared brown when sprayed with Orcinol.

Further separation of the $CHCl_3$/MeOH extract on a column of silicic acid fractionated the lipids into 3 fractions according to the polarity of eluant used (Table 5). Neutral lipids eluted by chloroform made up the major portion of the extract. Separation of these into the various fractions and classes of complex lipids of neutral lipids, glycolipids and phospholipids was confirmed by thin layer chromatography (Fig. 8 and 9).

The chloroform fraction consisted of at least 7 compounds which were identified by visualiser sprays and

comparison with the retention time of the lipids of crude palm oil (Fig. 8). Gas chromatography of the chloroform fraction showed that triglycerides made up about 83.5% of the total glycerides (Table 6). A relatively high percentage of diglycerides (8%) and free fatty acid (8%) were found. Less than 1% monoglycerides were detected. Carotene and sterols were identified from thin layer chromatography. These probably make up the major portion of unsaponifiables in which 0.6-0.8% was found in the extract. Gas chromatography of the unsaponifiables confirmed the presence of the various sterols commonly found in palm oil (Fig. 10). The fatty acid composition of the various lipids of the chloroform fraction was determined by gas chromatography (Table 7). Palmitic (C16:0) and oleic (C18:1) made up the major acids of the triglyceride fraction, similar to those of commercial palm oil. The other partial glycerides consisted of mainly C16:0 and C18:1 fatty acids too.

The acetone fraction made up 6% of the total weight of the $CHCl_3$/MeOH extract. At least six types of glycolipids (labelled 1-6) were present, being positive to Orcinol and Napthol sprays (Fig. 9). Digalactosyldiacylglycerol (DGDG) was confirmed with authentic sample spotted side by side on the same plate. The calculated retention factors of the other four glycolipids were comparable to those obtained by Yamaoko et al. (17,18) from oil palm leaf (Table 8). The

various glycolipids present in the sludge droplets were similar to those extracted from the palm oil from spent earth used in the refining of crude palm oil (18). The major ones from the oil droplets are esterified steryl glycoside (ESG), monogalactosyl diglyceride (MGDG) and digalactosyl diglycerides (DGDG).

The methanol fraction which made up about 10% by weight of the $CHCl_3$/MeOH extract, contained five distinct blue bands (labelled a-e) when sprayed with Zinzade reagent indicating at least 5 types of phospholipids (Fig. 9). Identification was initially made by spraying with specific spray reagents. Band e and b were stained orange with Ninhydrin indicating the presence of free amino group while band c was positive to Dragendrof reagent indicating presence of phosphatidylcholine.

The various phospholipids were further confirmed with authentic phospholipids spotted on TLC plates beside the methanol fraction. The retention factors are shown in Table 9. Bands a,b, c, d, and e were identified as phosphatidylserine/phosphatidylinisitol (PS/PI), phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) respectively. Band a and b were usually very close and were thus quantified as one phospholipid type.

The phospholipid composition of the plasma membrane from different plant tissues or of various organelles are

different but generally PC and PE are the major phospholipids followed by PI, PS and PG (31). The amount of phospholipids present as a total of the glycerolipids varies widely too. The proportions of the different types of phospholipids separated from the oil droplets were about equal within the range of 20-32% individually (Table 9). The main fatty acids of the phospholipids associated with the oil droplets were C16:0 (41.1-55.7%), C18:1 (26.9.-42.9%) with less than 10% of C18:2 and trace quantity of C18:3. The three major fatty acid of C16:0, C18:1 and C18:2 made up the major components of phospholipids extracted from crude palm oil by Goh (16) and Kulkarni (19). The fatty acid composition of the individual phospholipids vary from different parts of the plants and also on the types of organelles from which they are extracted but generally C16:0, C18:2 and C18:3 are the main fatty acids (29). In a compilation of the fatty acid composition of various seed phospholipids and triglycerides, it is noted that the fatty acids composition of these two lipids are usually in the same proportion (32). This is in agreement with the present results where C16:0, C18:1 and C18:2 being the major fatty acids of both the triglycerides in crude palm oil are also the major fatty acids of the phospholipids extracted from the oil droplets.

The quantity of phospholipids extracted from palm mesocarp oil by solvent extraction from crude palm oil

by Goh (16) was only 0.1-7 wt % but was 1.4 wt % from the centrifuged gummy residue. George (33), working on the lipid profile of the various streams during milling, found relatively high concentrations of phospholipids and glycolipids from the lipid extracts of the components of the palm fruits when they were separated. The press fibres contained the highest concentration of glycolipids and phospholipids at 2.6 wt % and 3.0 wt % respectively of the total lipids extracted. In the present study, the $CHCl_3$/MeOH extract contains even higher amount of glycolipids (6 wt %) and phospholipids (10 wt %) with respect to the total lipid extracted.

As glycolipids and phospholipids are complex polar lipids, different solvents and extraction conditions may affect their solubility and thus the extraction efficiency but in the present study similar solvents were used but in different ratios or order. Goh (16) had even immersed the fibres in $CHCl_3$/MeOH (2:1) mixture overnight in their extraction. Chloroform, methanol or its mixtures are commonly used in the extraction of lipids from plant tissues but the exact procedure and ratio of solvents varied.

In view of the highly variable amount and composition of the phospholipids obtained and the lack of reports regarding the specific biochemical composition of palm oil organelles in cells/membranes/fibres, the origin

of the phospholipids found associated with the oil droplets could not be exactly identified. The milling process involves mainly the rupturing of the oil bearing cells by mechanical force to release the oil when the digested fruit mash is screw pressed. The fate of the fragmented mesocarp cells and membranes have not been biochemically characterised but since phospholipids are generally known to be constituents of membranes and organelles of plant cells, their origin has to be from the mesocarp cells. In the intimate mixing of the aqueous and oil phases during processing, the amphiphilic phospholipids may become concentrated at the oil/water interface of the oil droplets.

Although phospholipids are one of the main constituents of the cell membranes and organelles, views differ as to whether oil droplets from various oil seeds and plants are surrounded by a membrane. In the early stage of development of the palm fruit, spherical oil bodies in the mesocarp are sparsely distributed throughout the cytoplasm. In mature fruits, the cells are completely filled with oil and become the only predominant organelle of the cell (34). The spherical configuration could be a result of the phenomenon of cohesion where the surface area is a minimum in relation to the minute volume. But very often the oil was also observed to follow the contour of the cell wall suggesting a sort of flexible "membrane" surrounding them;

The high concentration of 10 wt % phospholipids found associated with the neutral lipids in the $CHCl_3$/MeOH wash extract cannot be accounted for only by adsorption of a monolayer of phospholipids on the droplets. Based on the surface area of the oil droplets determined of 1.61 m^2 per gram of oil droplets (data derived from particle size distribution) only 0.49% phospholipids is required for a monolayer. If they could be covered by multilayers of phospholipids as Larsson (38) has postulated in the stabilisation of oil droplets by bilayer formation more phospholipids indeed can be present. Another possibility for the high concentration of phospholipids is that phospholipids has been extracted from the sludge debris in the process of centrifugation and extraction using $CHCl_3$/MeOH which is a polar solvent system.

The fatty acid composition of the palm oil at different periods of maturity are significantly different (39). Young fruits before maturity (approx. 14 weeks) have a high level of C18:2 (>19.3%) and C18:3 (72.7%). In this study, the triglycerides composition of the oil droplets was similar to that of commercial palm oil with 9.7% of C18:2 and insignificant amount of C18:3. Thus it is deduced that the majority of the oil droplets found in the sludge did not originate from unripe oil cells but were emulsified from the expressed oil during the milling process.

Plant cells containing oil do not rupture easily when they are pumped to the sludge pit where the temperature is at ambient. Palm oil containing partially saturated fatty acids would partially crystallise within the plant cell and in the absence of any mechanical shear, rupturing of such oil cells would not occur. Rupturing by osmotic pressure is unlikely as the aqueous sludge contains a high concentration of soluble inorganic salt. Oil retained in oil cells thus constitutes an oil loss when discharged while free oil droplets can be separated by high-speed centrifugation but as this study shows they are stabilised by the natural surface active compounds such as phospholipids, monoglycerides, sterols or proteins which are present in the systems.

2.4 CONCLUSION

The composition of the major lipids found in the oil droplets separated from the centrifuge sludge is similar to that of commercial palm oil, except that it consists of 10 wt % phospholipids and 6 wt % glycolipids. These highly surface active compounds stabilised the oil droplets rendering oil nonrecoverable from the sludge as a homogeneous phase.

Upon separating the oil droplets by high speed centrifugation, washing and extraction followed by column chromatography highly purified phospholipids and glycolipids from the palm fruit can be separated in substantial quantity. These may have commercial implication as a value added resource by-products from the palm oil milling process, just as lecithin is a valuable by-product of soya bean oil refining.

In the centrifugation process of oil droplet separation, a clear supernatant is obtained, while the oil droplets creamed and the solids sediment in a compact form. This supernatant contains numerous aqueous soluble compounds, one of which has been identified as pectin which is known to have commercial value (40). It may contain other potentially valuable compounds. After extraction of these soluble compounds, liquid effluent treatment will be rendered easier. At present the voluminous liquid sludge of high Biological Oxygen Demand (BOD) due to high concentration of soluble organic contents and suspended solids make effluent treatment extremly costly and time consuming. The sediment in a compact and drier form would make handling easier. The solids of the sludge are valuable by-products with potential applications as fertiliser and animal feed.

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Thus the potential of recovery of various valuable by-products from the three different centrifuged fractions of sludge, may well make the overall milling of palm oil from palm mesocarp an even more economical venture.

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Table 1. Triglyceride Composition w/w of Palm Oil and Palm Kernel Oil

Triglyceride	C28	C30	C32	C34	C36	C38	C40	C42	C44	C46	C48	C50	C52	C54	C56
Palm oil[a]	-	-	-	-	-	-	-	-	0.07	1.18	8.08	39.88	39.77	11.35	0.39
Palm Kernel[b]	0.55	1.25	6.34	8.43	23.33	16.96	9.79	9.10	6.56	5.14	5.79	2.30	2.17	0.45	

Source a - W.L.Siew, T.S. Tang, O.C.H.Flingoh,C.L. Chong,Y.A. Tan (1993). Identity Characteristics of Malaysian Palm Oil Products: Fatty Acid and Triglyceride Composition and Solid Fat Content, *Elaeis*,**5**, 38-46.

b - W.L.Siew, K.G. Berger (1986). Malaysian Palm Kernel Oil: Chemical and Physical Characteristics. PORIM Technology,**6**.

Table 2. Fatty Acid Composition w/w of Palm Oil and Palm Kernel Oil

Fatty Acid	C6:0	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0
Palm Oil[a]	-	-	-	0.24	1.11	44.14	4.44	39.04	10.57	0.37	0.38
Palm Kernel Oil[b]	0.3	4.2	3.7	48.7	15.6	7.5	1.8	14.8	2.6	-	-

Source: a,b - Same as Table 1.

Table 3. Phosphorus and Nitrogen Contents of the Cream Layer of Oil Droplets

Sample	[a]Phosphorous content (ppm) (Dried wt. basis)		[b]Nitrogen content (ppm) (Dried wt. basis)
Mean	Range	Average value	Average Value
Oil droplets separated by centrifugation	2972-5506	4362	3143
Oil droplets after water wash 1X	3041-14896	10,115	3858
2X	3630-31168	22,176	8478
Aqueous layer separated by centrifugation	1101-7454	3516	2896
Aqueous layer after water wash 1X	5662-8524	6948	N.D
of oil droplets 2X	14115-21350	16680	N.D
Solids separated by centrifugation	627-769	694	13720
Solid after 1X	392-1863	934	N.D
water wash 2X	599-1567	1083	N.D

a - Phosphorus determination based on at least 7 samples. To convert to phospholipids, multiply by a factor of 25.

b - Nitrogen determination based on 2 samples in duplicates. To convert to protein, multiply by a factor of 6.25

N.D.- Not determined, insufficient samples

Table 4: Composition of Twice Water-Washed Oil Droplets

Constituent	Range (%) on dried weight bases	Mean (%)
Moisture	43-70	55
$CHCl_3$/MeOH extract	62-82*	33(73)*
Residual non-lipids solids (%)	18-38*	12(27)*

* % proportion of $CHCl_3$/MeOH to residual nonlipid solids on dried basis.

Table 5 : Lipid Composition of the $CHCl_3$/MeOH Extract of the Dried Oil Droplets by Column Chromatography (w/w)

Lipid	Range %	Mean % w/w
Neutral lipids (Chloroform fraction)	74-91	84
Glycolipids (Acetone fraction)	3-9	6
Phospholipids (Methanol fraction)	5-19	10
Total		100
*Phospholipids determined by colorimetric method in the crude $CHCl_3$/MeOH Extract		8

Table 6 : Lipid Composition of Chloroform Fraction Isolated from Cream Layer Separated by Column Chromatography and Determined by Gas Chromatography

Lipid	Relative %
Triglycerides	83.5
Diglycerides	8.0
Monoglycerides	0.5
Free fatty acid	8.0

Table 7 : Fatty Acid Composition of the Neutral Lipids (Chloroform Fraction) Isolated from the Cream Layer as Determined by Gas Chromatography

Fatty Acid / Lipids	C14:0	C16:0	C18:0	C18:1	C18:2	C20:0/18:3
Triglycerides	1.1	45.2	3.7	39.9	9.7	0.2
1,2 Diglycerides	0.5	36.8	3.4	49.2	10.1	t
1,3 Diglycerides	0.5	42.2	3.3	44.7	9.3	t
Monoglycerides	1.4	67.6	1.5	23.7	5	t
Free fatty acid	1.5	45.6	9.5	30.4	2.0	t
Commercial palm oil	1.2	42.1	4.2	39.9	10.8	0.3

t = trace amount

Table 8 : Relative % w/w of Various Glycolipids (Acetone Fraction) extracted from the oil droplets (cream layer) and Retention Factors Determined from Thin Layer Chromatography in the solvent System ($CHCl_3$:MeOH:CH_3COOH:H_2O) 70:25:25:6 (v/v)

Band No[a]	Rel. %	Rfx100	Rfx100*	Glycolipid*
1	6	5	26	Sulfoguinovosyl diglyceride (SQDG)
**2 (DGDG)	22	31	27	Digalactosyl Diglyceride (DGDG)
3	17	53	47	Steryl glycoside (SG)
4	9	65	57	Cerebroside without Hydroxy fatty acid (CU)
5	20	80	81	Monogalactosyl diglyceride (MGDG)
6	26	90	87	Esterified Steryl Glycoside (ESG)

* Retention factor of identified glycolipids obtained from oil palm leaf developed in solvent system of $CHCl_3$:MeOH:CH_3COOH:H_2O (85:5:10:3 v/v) (ref. 18)

**Confirmed with authentic sample on TLC plate

[a]Refer Fig. 9 for band identification

Table 9. Fatty Acid Composition of Phospholipids from $CHCl_3$/MeOH Extract of Dried Oil Droplets

*Phospho-lipid Band	Rf x 100	Phospho-lipid Identified	% w/w (Phosphorus Analysis) on total extract	12:0	14:0	16:0	18:0	18:1	18:2	18:3	20:0	Others
a	4	PS/PI	32	0.5	1.2	45.3	5.0	37.3	9.7	0.1	0.3	0.6
b	7	PS/PI		3.0	1.4	55.7	6.1	24.8	5.1	-	0.5	3.5
c	17	PC	27	1.3	1.1	41.1	2.7	42.9	9.0	-	0.4	1.5
d	37	PG	20	0.6	1.1	53.1	5.9	34.1	2.9	0.4	-	1.9
e	43	PE	21	3.0	2.1	51.0	3.5	26.9	5.1	0.1	0.1	8.0

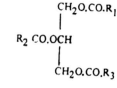
*Band a-e, refer to Fig. 9. PS-phosphatidylserine, PI-phosphatidylinositol, PC-phosphatidylcholine, PG-phosphatidyl glycerol, PE-Phosphatidylethanolamine

Fig. 1. Common Plant Lipids

1-monoglyceride (1-monoacylglycerol)

1,2-Diglyceride

(1,2-Diacylglycerol)

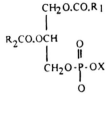


Triglyceride (Triacylglycerol)

1,3-Diglyceride

(1,3-Diacylglycerol)

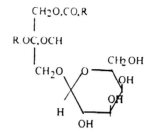
(a) Neutral glycerides (R = an alkyl group)



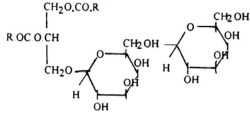
Basic structure

Base moiety	Phospholipid
X = -H	Phosphatidic acid (PA)
X = $-CH_2CH_2N(CH_3)_3$	Phosphatidylcholine (PC)
X = $-CH_2CH_2NH_2$	Phosphatidylethanolamine (PE)
X = $-CH_2CH_2NH$ CO R	N-Acyl Phosphatidylethanolamine
X = $-CH_2CH(NH_2)COOH$	Phosphatidylserine (PS)
X = - Glycerol	Phosphatidylglcerol (PG)
X = -Inositol	Phosphatidylinositol (PI)
X = $-CH_2CHOH(OH)CH_2O-P(O^-)O-CH_2CH(O-CO-R_4)CH_2O-CO-R_3$	Cardiolipin (CL)

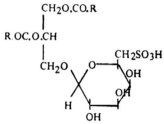
(b) The major phosphoglycerides



diacylgalactosyglycerol
(monogalactosyldiglyceride,MGD)



diacygalabiosylglycerol
(digalactosyldiglyceride,DGD)



diacylsulfoquinovosylglycerol
(sulfoquinovosyldiglyceride,SQD,
plant sulfolipid)

c) Glycosylglycerides

Source: J.L. Harwood (1986). Lipid Structure . *The Lipid Handbook*. Edi., F.D. Gunstone, J.L. Harwood, F.B. Padley, Chapman and Hall, London, 25-46.

Figure 2. Sludge After High Speed Centrifugation

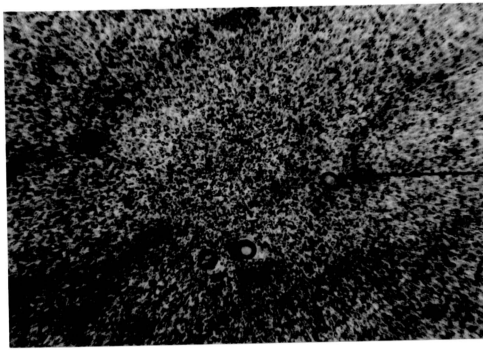
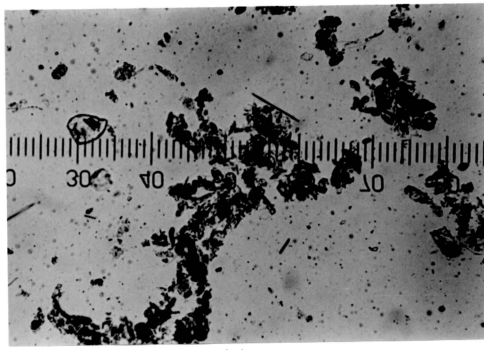
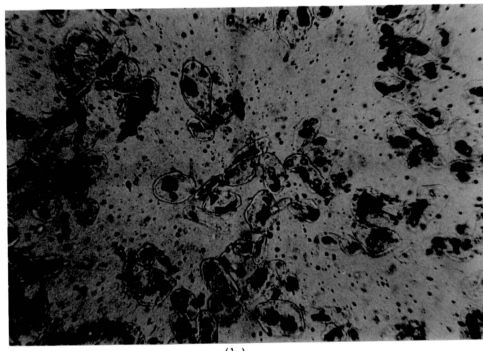


Figure 3: Oil Droplets of the Cream Layer Separated from the Sludge by Centrifugation (Mag 200x)



(a)



(b)

Fig. 4. Solids from Bottom Sediment Isolated from Sludge by Centrifugation.

a: Fragments of plant cells (Mag 100x)
b: Plant cells (Mag 100x)

Fig. 5. a: Particle Size Distribution of of Oil Droplets in the Cream Layer Isolated by Centrifugation of the Sludge.

b: Particle Size Distribution of the Solids in the Sediment Isolated by Centrifugation of the Sludge.

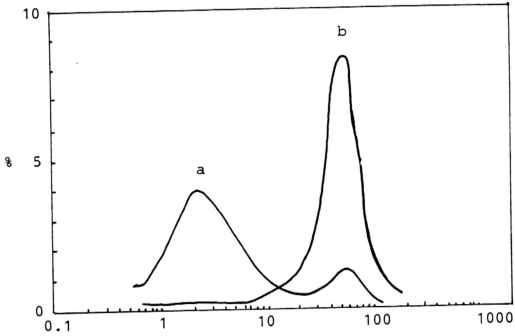
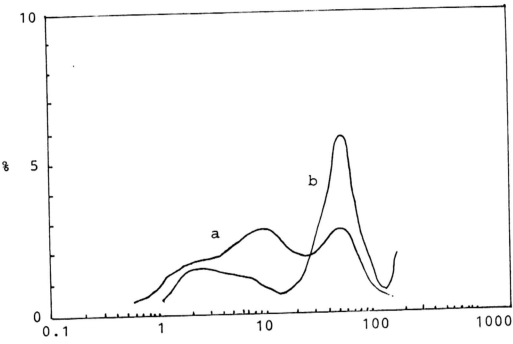


Fig. 6: a: Particle Size Distribution of the Oil Droplets in the cream layer after washing Twice with Water.

b: Particle Size Distribution of the Sediments Collected from the Oil Layer after washing Twice with water



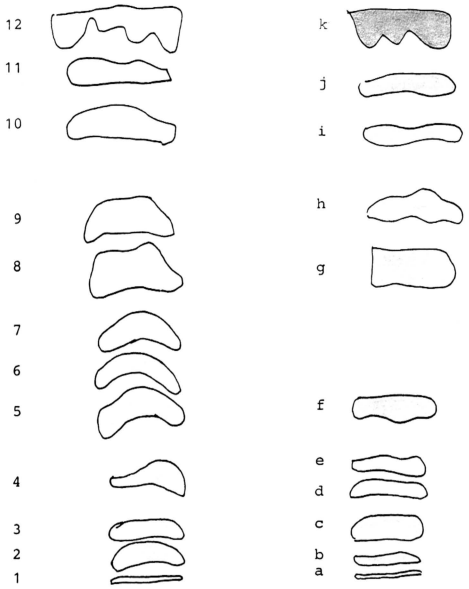


Fig. 7: Thin-layer Chromatography of $CHCL_3$/MeOH extract on Silica Gel G. Solvent System $CHCl_3$:MeOH:CH_3COOH:H_2O (170:25:25:6).

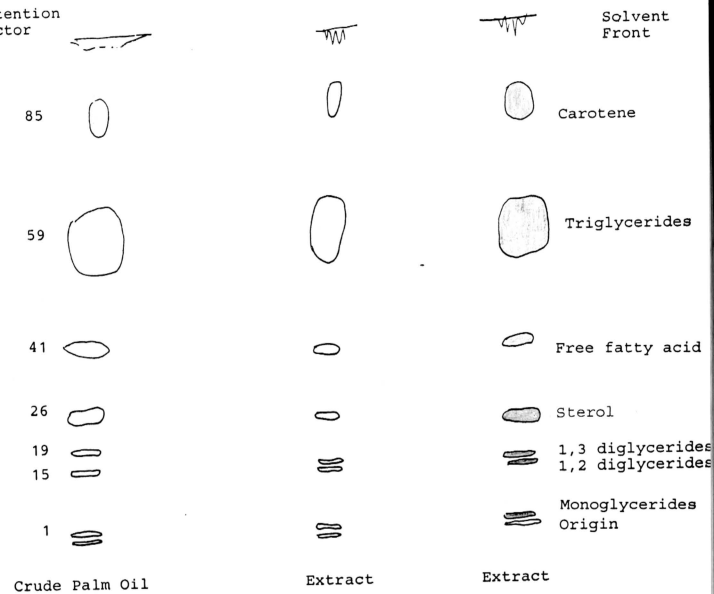
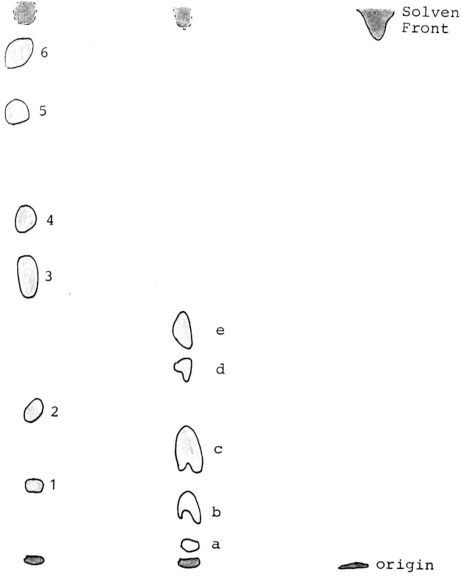


Fig. 8. Thin Layer Chromatography of the Chloroform Fraction from column chromatography. Solvent system Hexane:Diethyl ether:Formic Acid (80:20:2), visualised with 2,7'-Dichlorofluorescein spray (yellow) and Ferric Chloride spray (lipids are stained brown).



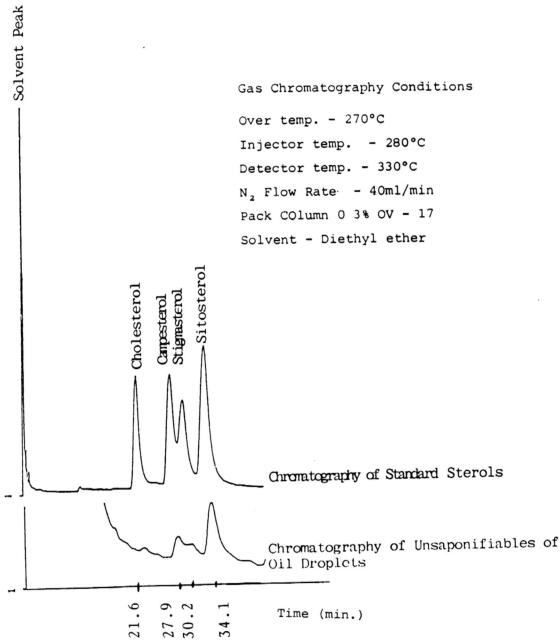
A. Acetone fraction visualised with Orcinol and Napthol

B. Methanol fraction visualised with Zinzade

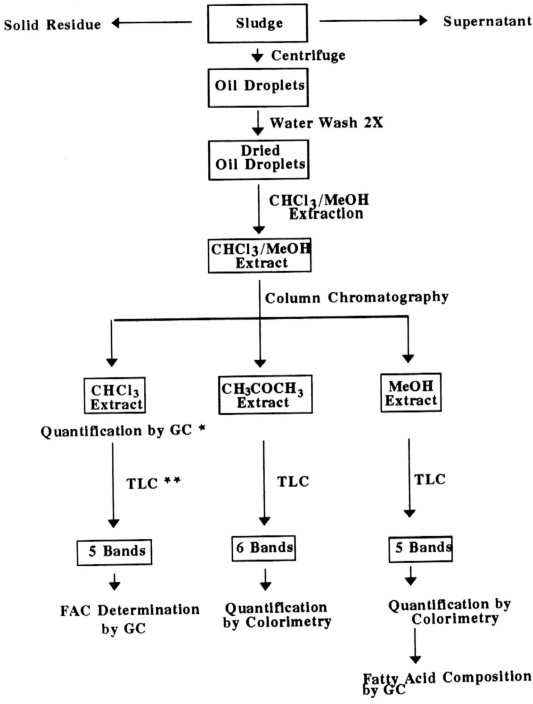
C. Chloroform fraction charred with conc. H_2SO_4

Fig. 9. TLC of the Different Fractions Separated by Silicic Acid Column Chromatography. Solvent System $CHCl_3$:MeOH:Ch_3COOH:H_2O (170:25:25:6).

Fig. 10. A Comparison of the Retention Time of the Gas Chromatogram of Some Sterol Standards and that of the Unsaponifiables from the Oil Droplets.



Summary of Analytical Techniques in Lipid Characterisation of Oil Droplets



GC-Gas Chromatography
TLC - Thin Layer Chromatography