CHAPTER THREE

RECOVERY OF PHOSPHOLIPIDS FROM PALM-PRESSED FIBRE

3.1 INTRODUCTION

Phospholipids which serve a wide variety of functions (see Section 1.2.6) were commercially available from egg yolk extracts and soyabean oil extracts. Phospholipids, a by-product from refining process of soybean oil with *ca.*1.1-3.2 wt. % had become the main source of it (Tom, 1996). Palm oil contains relatively low level of phospholipids (5-130 ppm) since the wet milling process ensures that only an estimated 4% of the phospholipids in the palm fruits remain in the crude palm oil. Large amount of phospholipids were retained in palm-pressed fibre (37,000 ppm) and sludge (40,000 ppm) (Table 3.1). The increased in the production of palm oil also resulted in an increase in the loss of phospholipids which is treated as waste by-product. In view of its being high valued products (commercial lecithins are sold at 2-10 times the price of soybean oil), it is therefore useful to conduct an in-depth study on the properties and recovery of phospholipids from palm-based products, particularly palm-pressed fibre, a by-product in the milling of palm oil.

Crude lecithin mixture exhibit weak emulsifying properties due to the phospholipid constituents. To improve its properties, it inevitably required to undergo a series of process, namely fractionation, purification and modification to fulfill these purposes.

Crude soybean lecithin contains 52% mixed phospholipids of which 12-18% is phosphatidylcholine. Deoiling with acetone generates a granular product containing

78% mixed phospholipids (20-95% phosphatidylcholine). On further extraction with ethanol can be fractionated to yield high concentrations of phosphatidylcholine (Schnieder, 1989).

Table 3.1: Phospholipid Content from Palm Oil Milling

	Weight of Oil [a] (%)	Phospholipids (ppm)	Total Phospholipids Kg (%)
Crude palm oil	22	80[b]	1.8 (4)
Oil recovered from sludge water	0.67	40,000	26.8 (55)
Oil recovered from palm-pressed fibre	0.54	37,000	20.0 (41)
(Total extractable [c])	(23.2)	(1,200)	(48.6 [100])
Solvent extracted mesocarp oil[d]		2,000	

Source: Goh *et al*, 1982
[a]Based on 100 metric tonnes of fresh fruit bunches
[b]Values of 20-80 ppm have been analysed .
[c]Sum of the above values, assuming no other oil losses.
[d]Mesocarp oil extracted by chloroform:methanol (1:2 v/v)

The objective of the present study is to recover the phospholipids from palm-pressed fibre by stepwise solvent extraction and direct solvent extraction. Both extraction methods were conducted by using soxhlet apparatus immersing method. The crude lecithin recovered was further subjected to octadecyl (C_{18}) open column chromatography purification. Phospholipids are normally purified using acid-treated Florisil. The used of octadecyl absorbent had successfully improve the purification of phospholipids.

3.2 EXPERIMENTAL

3.2.1 Materials

Palm-pressed fibre was obtained from palm oil mill in Labu. Fresh palm-pressed fibre was kept in the freezer. All chemicals and instruments mentioned in Section 2.2 were used for the analyses of phospholipids.

3.2.2 Stepwise and Direct Solvents Extraction

Palm-pressed fibre was dried in an oven at 60°C prior to extraction. The dried fibre (*ca.* 300g) was initially extracted with 3.5 litre of hexane using sohxlet apparatus at 60-80 °C for 16 hours. After filtration, the extraction was continued by 3.5 litre of acetone and the last extraction was by using 3.5 litre of 95% ethanol. The filtrate was rotary-evaporated and pumped to dryness. Following this procedure, the following palm-pressed fibre oils were prepared:

i. Palm-pressed fibre oil extracted with hexane (FOH)

ii. Palm-pressed fibre oil extracted with acetone (FOA)

iii. Palm-pressed fibre oil extracted with 95% ethanol (FOE)

Stepwise solvents extractions were repeated with another 300g dried fibre by immersing in different solvent, instead of soxhlet extraction at room temperature for 24 hours. Following this procedure, the following palm-pressed fibre oils were obtained:

iv. Palm-pressed fibre oil extracted by hexane (FOHS)

v. Palm-pressed fibre oil extracted by acetone (FOAS)

vi. Palm-pressed fibre oil extracted by 95% ethanol (FOES)

A comparative study *viz.* direct solvent extraction of palm-pressed fibre oil was also carried out. 300g dried fibre was extracted with 3.5 litre of 95% ethanol directly by immersing the fibre at room temperature and by sohxlet extraction. Hence, the following palm-pressed fibre oils were obtained:

vii. Palm-pressed oil by immersing method (FOEDs)

vii. Palm-pressed oil by soxhlet apparatus (FOED)

The palm-pressed oil was purified and analysed according to Section 2.2.3, 2.2.4, 2.2.5 and 2.2.6.

3.2.3 Purification of Phospholipids Using Octadecyl (C18) Open Column Chromatography

Reversed-phase open column chromatography (octadecyl) has been developed to recover phospholipids in high purity from palm-pressed fibre oil. The octadecyl (*ca.* 30g) was made into slurry using methanol and packed into a 3 cm diameter column to a height of *ca.* 8 cm. A thin layer of anhydrous sodium sulphate (*ca.* 0.25 cm) was added to the top of the absorbent. Excess methanol was eluted down until the methanol level was just above the column material. The oil samples of FOES (*ca.* 1g) were dissolved in minimal amount of chloroform:methanol (1:2 v/v) mixture and loaded carefully into the column. Several solvent systems had been developed and listed in Table 3.2 below.

3.2.4 Fatty Acid Composition Determination of Phospholipids

Analyses were carried out according to the procedures in Section 2.2.7.

Table 3.2: Solvent Systems for Open Column Chromatography

Solvent system	Fraction	Solvent	Volume /ml
A	1	MeOH	50
	2	MeOH:$CHCl_3$ (98:2 v/v)	100
	3	$CHCl_3$	50
B	1	EtOH:H_2O (90:10 v/v)	50
	2	EtOH:H_2O (95:5 v/v)	100
	3	Hex	100
C	1	EtOH:H_2O (90:10 v/v)	100
	2	EtOH:H_2O (95:5 v/v)	100
	3	Hex	50
D	1	EtOH: H_2O (90:10 v/v)	50
	2	EtOH: H_2O (90:10 v/v)	50
	3	EtOH: H_2O (90:10 v/v)	50
	4	Hex	50
E	1	EtOH: H_2O (90:10 v/v)	100
	2	EtOH: H_2O (95:5 v/v)	100
	3	Hex	50
F	1	EtOH: H_2O (85:15 v/v)	100
	2	EtOH: H_2O (95:5 v/v)	100
	3	Hex	50
G	1	EtOH: H_2O (80:20 v/v)	50
	2	EtOH: H_2O (95:5 v/v)	100
	3	Hex	50

MeOH - methanol
EtOH - 95% ethanol
Hex - hexane
$CHCl_3$ - chloroform
v/v - volume to volume

3.3 RESULTS AND DISCUSSION

3.3.1 Stepwise and Direct Solvents Extraction

In the present study, stepwise and direct solvents extraction processes were employed to recover phospholipids from palm-pressed fibre. The initial step involved extraction of palm-pressed fibre oil with hexane to remove non-polar lipids (triacylglycerols, diacylglycerols and monoacylglycerols). The extraction is followed by acetone to remove most of the glycolipids. Finally, extraction by ethanol to yield high concentration of phospholipids. The stepwise and direct solvent extractions were carried out by soxhlet method and immersing method. The yield of phospholipids by stepwise solvent extraction and direct solvent extraction are summarized in Table 3.3 and Table 3.4 respectively. The present stepwise solvents extraction employs three different solvents with different polarity were found to be more effective as compared to the previous published data.

Phospholipids have been concentrated in the ethanolic layer when the extraction was performed with immersing method. The concentration of phospholipids was 44,205 ppm in FOEDs by direct immersing method and 130,138 ppm in FOES by stepwise immersing method. Contrarily, the phospholipids content in FOE (54,205 ppm) was even lower as compared to FOED that containing 89,928 ppm of phospholipids. This observation can be explained by the heat instability property of phospholipids. Phospholipids will be darkened, impaired and oxidized if heated above 70°C for a long time (Rudiger, 1982; Mustafa *et al.*, 1989; Michael Foods Inc. US, 2000). Thus, prolonged heating during soxhlet extraction might severely oxidize the phospholipids and resulting in its lower content in FOE.

Table 3.3: Stepwise Solvents Extraction of Phospholipids

Extraction Method	Fibre Oil	Yield		Phospholipids	
		g	%	ppm	mg
Soxhlet	FOH (hexane)	15.39	5.13	5,450	83.88
	FOA (acetone)	4.06	1.35	1,957	7.95
	FOE (95% ethanol)	8.83	2.94	54,205	478.63
Immersing	FOHS (hexane)	11.48	3.83	1,233	14.15
	FOAS (acetone)	3.76	1.16	3,598	13.53
	FOES (95% ethanol)	7.70	2.57	130,138	1002.06

Table 3.4: Direct Solvent Extraction of Phospholipids

Extraction Method	Fibre Oil	Yield		Phospholipids	
		g	%	ppm	mg
Soxhlet	FOED (95% ethanol)	20.49	6.83	89,928	1842.62
Immersing	FOEDs (95% ethanol)	17.12	5.71	44,205	756.79

The palm-pressed fibre oil, FOHS, FOE, FOED, FOES and FOEDs, were analysed for phospholipids composition by HPLC-ELSD. The results are tabulated in Table 3.5 and the HPLC charts for FOHS and FOEDs were shown in Figure 3.1 and Figure 3.2. The major components were same as to those found in crude palm oil with major being phosphatidylcholine, followed by phosphatidylethanolamine, phosphatidylinositol and phosphatidylglycerol. The higher concentration of phospholipids obtained by HPLC quantification as compared to UV spectrophotometry. The explanation was same as stated in Section 2.3.2 (c).

Table 3.5: Phospholipids Composition of Palm-pressed Fibre Oil

Fibre Oil	Concentration of Phospholipids / ppm						
	HPLC-ELSD[a]						UV[b] Spectrophotometry
	PG	PE	PI	PA	PC	Total	
FOE	N.D.	15,336	11,656	N.D.	33,040	60,032	54,205
FOED	N.D.	23,987	16,732	N.D.	53,134	93,853	89,925
FOES	N.D.	38,086	14,703	N.D.	94,827	145,026	130,138
FOEDs	6,077	12,482	N.D.	7,355	20,886	46,800	44,205
FOHS	147	456	N.D.	N.D.	763	1,367	1,233

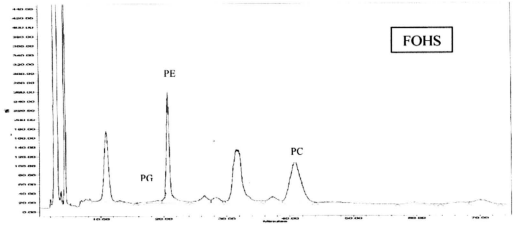
PC phosphatidylcholine
PE phosphatidylethanolamine
PA phosphatidic acid
PI phosphatidylinositol
PG phosphatidylglycerol

[a] Amount quantified by HPLC -ELSD
[b] Amount quantified by UV spectrophotometry
N.D. not detected

Fatty acid composition of phospholipids from palm-pressed fibre oil, namely FOHS, FOE, FOED. FOES and FOEDs were subjected to GC analysis and the results were tabulated in Table 3.6. Phospholipids for all palm-pressed fibre oil showed similar fatty acids composition. It is more unsaturated with a higher linoleic content compared to triacylglycerols in crude palm oil (Gee, 1985).

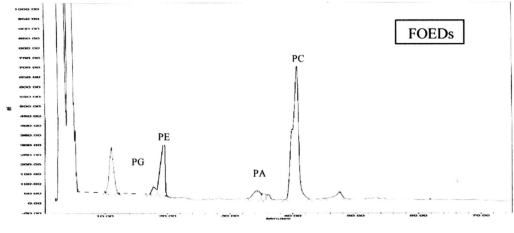
Phospholipids from palm-pressed fibre oil contain relatively lower unsaturation as compared to soyabean which comprise mainly of about 60% linoleic acid, 10% oleic acid and 5% linolenic acid (Scholfield, 1985). Thus, palm-based phospholipids might have greater antioxidant activity which enhanced with a greater proportion of saturated fatty acids (Chigozie *et al*, 1997).

Figure 3.1: Phospholipids Profile in FOHS



FOHS	palm-pressed fibre oil recovered with hexane (immersing method)
PG	phosphatidylglycerol
PE	phosphatidylethanolamine
PC	phosphatidylcholine

Figure 3.2: Phospholipids Profile in FOEDs



FOEDs	Palm-pressed fibre oil recovered with 95% ethanol (immersing method)
PG	phosphatidylglycerol
PE	phosphatidylethanolamine
PA	phosphatidic acid
PC	phosphatidylcholine

The higher levels of phospholipids in palm-pressed fibre oil is understandable because, unlike seed oils which are solvent extracted, palm oil is mechanically extracted and is separated from aqueous slurry sludge during milling. Thus, it can be expected that considerable amount of phospholipids still remain in the fibre. The present study confirms that there is considerable amount of phospholipids present palm-pressed fibre oil. Thus, this could provide a good source for phospholipids production.

Table 3.6: Fatty Acid Composition of Phospholipids in Palm-pressed Fibre Oil

Palm-pressed Fibre Oil	Fatty Acids (%)					
	C14	C16	C18:0	C18:1	C18:2	C20
FOE	N.D.	34.69	1.13	32.35	30.87	0.96
FOED	N.D.	36.25	0.52	32.59	29.78	0.86
FOES	N.D.	35.62	0.83	33.68	28.85	1.02
FOEDs	N.D.	34.58	1.03	33.75	30.05	0.59
FOHS	N.D.	33.71	2.41	32.91	30.76	0.21

3.3.2 Purification of Phospholipids by Octadecyl (C_{18}) Open Column Chromatography

The palm-pressed fibre oil (FOES) extracted by stepwise solvents extraction comprised 13% phospholipids. The remaining 87% is believed to be mostly polar and macros compounds such as lignin. In our present study, such compounds eluted together with phospholipids in methanol fraction when separation was carried out by acid-treated Florisil. To overcome this problem, a reversed-phase absorbent, octadecyl is used to separate these compounds to order to yield high purity phospholipids.

Several solvent systems were examined with some of them giving very encouraging results in terms of high purity with satisfactorily recovery as shown in Table 3.7. The unwanted polar compounds were successfully removed in the first fraction and the subsequent elution fraction yield high purity phospholipids fraction as can be seen in Experiment in the case of A and F. Phospholipids fraction (A2) with highest purity, *ca.* 55% and best recovery of 56.02% was achieved by eluting with methanol-chloroform solvent system. However, these solvents were hazardous and can be replaced with food grade solvents, e.g., hexane and ethanol. Comparable purity was obtained, *ca.* 53% (F2) but with lower recovery, 42.18% when food grade solvents were used.

HPLC analyses (Table 3.8) revealed that the concentrated phospholipids fractions, namely A2, B3, D3 and F2 comprised phosphatidylcholine and phosphatidylethanolamine. Indeed, the octadecyl open column purification of crude phospholipids mixture is essentially equivalent to the phosphatidylcholine enrichment. Phosphatidylcholine constitutes *ca.* 90% of phospholipids in A2, B3. D3 and F2. Phosphatidylinositol in FOES might be eluted to another fraction, or maybe retained significantly in the absorbent.

Table 3.7: Recovery of Phospholipids (%) from FOES by Octadecyl (C_{18}) Open Column Chromatography

Experiments	Fraction	Solvent[ml]	Concentration (ppm)	Recovery (%)
A	1	MeOH [50]	T	T
	2	MeOH:$CHCl_3$ (98:2 v/v) [100]	553,406	56.02
	3	$CHCl_3$ [50]	114,277	15.29
B	1	EtOH:H_2O (90:10 v/v) [50]	T	T
	2	EtOH:H_2O (95:5 v/v) [100]	333,668	13.46
	3	Hex [100]	387,670	53.39
C	1	EtOH:H_2O (90:10 v/v) [100]	T	T
	2	EtOH:H_2O (95:5 v/v) [100]	294,117	39.04
	3	Hex [50]	99,298	7.76
D	1	EtOH: H_2O (90:10 v/v) [50]	T	T
	2	EtOH: H_2O (90:10 v/v) [50]	T	T
	3	EtOH: H_2O (90:10 v/v) [50]	307,500	9.73
	4	Hex [50]	79,590	12.85
E	1	EtOH: H_2O (90:10 v/v) [100]	T	T
	2	EtOH: H_2O (95:5 v/v) [100]	167,141	16.74
	3	Hex [50]	3,594	0.46
F	1	EtOH: H_2O (85:15 v/v) [100]	T	T
	2	EtOH: H_2O (95:5 v/v) [100]	528,415	42.18
	3	Hex [50]	131,760	15.34
G	1	EtOH: H_2O (80:20 v/v) [50]	T	T
	2	EtOH: H_2O (95:5 v/v) [100]	113,981	11.91
	3	Hex [50]	193,682	20.53

MeOH - Methanol
EtOH - 95% ethanol
$CHCl_3$ - Chloroform
Hex - Hexane
v/v - volume to volume
T - Trace amount

Table 3.8: Phospholipids Composition of Selected Fraction from Octadecyl Open Column Chromatography

Fraction	Concentration of phospholipids /ppm			
	HPLC-ELCD[a]			UV[b] Spectrophotometry
	PC	PE	Total	
A2	503,824	55,770	559,594	553,406
B3	305,405	34,967	340,372	387,670
D3	288,531	26,937	315,468	307,500
F2	486,090	48,255	534,345	528,415

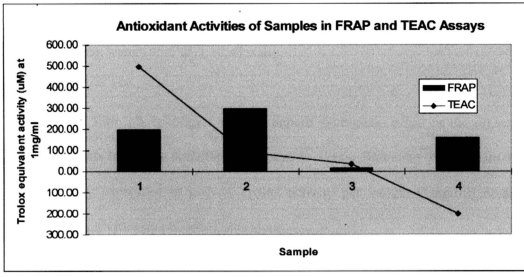
[a] Amount quantified by HPLC- ELSD
[b] Amount quantified by UV spectrophotometry
PC phosphatidylcholine
PE phosphatidylethanolamine

3.3.3 Synergistic Effect of Phospholipids

Phospholipids are known to have synergistic effect with antioxidants (Pokorny, 1991 and Segawa *et al*, 1995). The study on antioxidant activity of palm-based phospholipids together with carotenes and vitamin E have been carried out with the mixture consisted of 18 wt. % palm phospholipids and 15 wt. % carotenes (sample 2); 18 wt. % palm phospholipids and 30 wt. % of vitamin E (sample 4) respectively. The results are depicted in Figure 3.3. The phospholipids showed excellent synergistic effect with vitamin E with ten folds activities increment in Ferric Reducing Antioxidant Power (FRAP) assay, which was expressed in μM of Trolox activity per weight (1mg/ml) of sample compared to activities exhibited by phospholipids alone. However, opposite characteristic was shown in Trolox Equivalent Antioxidant Capacity (TEAC) assays. The antioxidant activities of phospholipids and carotenes mixture has been increased by

1.5 folds activities in FRAP assay expressed in μM of Trolox activity per weight (1mg/ml) of sample.

Figure 3.3: Antioxidant Activities of Reconstituted Mixture of Phospholipids, Carotene and Vitamin E



Sample 1 - 30 wt. % carotenes
Sample 2 - 18 wt. % phospholipids + 15 wt. % carotenes
Sample 3 - 50 wt. % vitamin E
Sample 4 - 18 wt. % phospholipids + 30 wt. % vitamin E
FRAP ferric reducing antioxidant power
TEAC trolox equivalent antioxidant capacity

3.3.4 Summary of Present Findings

Palm-pressed fibre which contains *ca.* 37,000 ppm has been found to be a good source for the recovery of phospholipids. Fractionation of total lipids in palm-pressed fibre was achieved by stepwise solvents extractions at room temperature, which yielded FOES with 13wt. % of phospholipids. The major components were phosphatidylcholine, phosphatidylethamine and phosphatidylinositol.

Purification of crude lecithin extract, *viz.* FOES through octadecyl open column chromatography seems to be equivalent to phosphatidylcholine enrichment. High purity phospholipids fractions with more than 90% of phosphatidylcholine were obtained. The preferable solvent systems were depicted in Experiment (F): i. Ethanol: water (85:15 v/v); ii. ethanol: water (95:5 v/v); iii. Hexane.

Palm-pressed fibre phospholipids showed superb synergistic effect in the presence of vitamin E with ten folds activities increment in Ferric Reducing Antioxidant Power (FRAP) assay expressed in μM of Trolox activity per weight (1mg/ml) of sample compared to activities by phospholipids alone.