Coconut Phospholipid Species: Isolation, Characterization and Application as Drug Delivery System

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Submission date: 16-Nov-2019 03:05PM (UTC+0700)
Submission ID: 1214987856
File name: 69884-2-18.pdf (1.53M)
Word count: 5234
Character count: 29113

Chapter

Coconut Phospholipid Species: Isolation, Characterization and Application as Drug Delivery System

Dwi Hudiyanti, Nur Kamila, Febriani Kusuma Wardani and Khairul Anam

Abstract

The purpose of this study was to isolate the ethanolamine species of coconut phospholipid and to investigate their potency as drug delivery system by using it to encapsulate vitamin C. The study consisted of two stages: the first stage was isolation and characterization of coconut phosphatidylethanolamine species; and the second stage was utilization of coconut phosphatidylethanolamine liposomes to encapsulate vitamin C. A dark brown gel of coconut phosphatidylethanolamine species (CocoPEs) was isolated from dried coconut meat (9.3×10^{-3}%, w/w). At least 15 species were found in coconut phosphatidylethanolamine. The fatty acyl chains of the species were capric, linoleic, oleic, stearic and arachidic acyl chains. At least four different phases were identified on CocoPEs i.e. planar-shape gel phase, rippling phase, liquid crystal phase and hexagonal phase. The temperature (T_p) was at 25.29°C for changing from planar-shaped gel to rippling phase, 32.62°C (T_m) for major transition from gel to liquid crystal, and 65.53°C (T_h) from liquid crystal to hexagonal phase. All of CocoPEs liposomes encapsulation efficiency with cholesterol concentration up to 30% were above 80%. CocoPEs showed great potency as encapsulation material. It had high encapsulation efficiency and addition of cholesterol to the liposome membrane only slightly reduced the efficiency.

Keywords: cholesterol, *Cocos nucifera* L., coconut phosphatidylethanolamine, drug delivery, encapsulation, fatty acyl chain, liposome, phase behavior, phospholipid

1. Introduction

Phospholipids are major constituent of cellular membrane hence they have excellent biocompability. They are amphiphilic molecules which usually built by glycerol backbone with two different polarity groups attached to it. On the one hand is the hydrophilic group renowned as the head group which then becomes the basis of species classification of phospholipids, such as phosphatidylcholine (PC), phosphatidyletanolamine (PE), and phosphatidylserine (PS). On the other hand is the hydrophobic fatty acyl chains distinguished as the tails. The variation of the length and the saturation, the bonding position of fatty acyl chains to glycerol

backbone as well as the head group type become a crucial part of their application, for instance in drug delivery systems.

The development of phospholipids based drug delivery systems have been proven prominent by the emergence of many phospholipid-related drug formulation. Among of them are doxorubicin in stealth liposomes for cancer treatment, which has been on the market since 1995 [1, 2]; Verteporfin in cationic liposomes for molecular degeneration [3] and vincristine in conventional liposome for Non-Hodgkin lymphoma [2]. They have been used in clinic, and achieve good results. Many more phospholipids based liposomal preparation have been developed to find better therapeutic results [4–6]. Furthermore various sources, synthetic and natural, have been explored [2, 7].

The isolation of phospholipids from natural sources cost lower than synthesizing them hence the preference is the isolation of natural phospholipids. For natural origin, the more pure they are, the greater the value is [8]. Phospholipids from natural origin can be refined into diverse levels, comprising food and pharmaceutical grade [2, 9]. In term of natural phospholipids, different source enhance the species variety of phospholipids [7]. Egg yolk and soybean phospholipids mainly consist of phosphatidylcholine species but they have differences in the tail portions which influence their physical, chemical properties and their applications. Other natural phospholipids that currently are being explored extensively are sunflower [10–12], candlenut [13], jack bean [14], sesame [13, 15–17] and coconut [13, 15, 16, 18–22].

Coconut is one of the native plantations in tropical countries and produces mainly copra and coconut oil. Exploration of coconut by-products such as coconut phospholipids needs to be done to increase the added value of these coconut plantations. Previous studies have found that dried coconut contain phospholipids from cephaline species with their fatty acyl chains are dodecanoic and octanoic acyl chains [15]. Purification with eluent chloroform: methanol (9:1) follows by identification using thin layer chromatography (TLC) also detects the presence of phosphatidylcholine (PC), phosphatidyletanolamine (PE), and phosphatidylserine (PS) species in coconut phospholipids (CocoPLs) [20, 21].

In the matter of its application, coconut liposomes (CocoPLs liposomes) have been used in the encapsulation of hydrophilic agent namely carboxyfluoresence and vitamin C and resulted in that CocoPLs liposomes has high efficiency of encapsulation [16, 19, 22]. The addition of cholesterol improves the encapsulation efficiency and low storage temperature reduces CocoPLs liposomes leakage. The results advocated the CocoPLs potency as drug delivery material. Moreover since we have established that CocoPLs consist of many phospholipid species therefore it would be valuable to study the component of the species and their capability as drug delivery system. In this study we explore the isolation and purification of coconut phospholipid species specifically coconut phosphatidylethanolamine (CocoPEs) and utilization of their liposomes (CocoPEs liposomes) for vitamin C encapsulation with various cholesterol concentrations. To our knowledge this is the first study of such.

2. Materials and methods

2.1 Materials

Materials used in this study were ripe coconut meat purchased from local market, TLC silica gel 60 F_{254} plate, silica gel powder 60 G for thin layer chromatography, various solvents and regents for analytical grade.

2.2 Coconut phospholipid (CocoPLs) isolation

Isolation technique was carried out based on the previous method used [20, 21]. Briefly coconut meat powder was macerated in a chloroform: methanol (2:1, v/v) mixture. The filtrate obtained was washed using 0.9% NaCl. The lipid was evaporated until thick coconut lipid extract were obtained. The extract was then subjected to solvent partition using n-hexane and ethanol 87%. The lower phase was evaporated to yield brownish yellow extract of CocoPLs.

2.3 Coconut phosphatidyletanolamines (CocoPEs) separation using vacuum liquid chromatography

About 5 g of CocoPLs was mixed with 5 g of silica gel in a small amount of chloroform: methanol (9:1, v/v) solution to form a silica slurry. The slurry was then stirred until the mixture was dried and formed fine powder of CocoPLs-SG.

A total of 80 mg of silica gel was poured into chromatography column and compressed by vacuum. The column was rinsed using chloroform:methanol (9:1, v/v) eluent and vacuumed until all the eluent was eluted. The CocoPLs-SG powder was poured onto the column. Then the column was subjected to compression. Elution was performed using 10 ml of chloroform:methanol (9:1, v/v) solution. Fraction eluted from the column was collected into clean vials. The fraction was analyzed using TLC plate. The spot on the TLC plate was identified with 10% H_2SO_4 and ninhydrin. Elution was repeated every 10 ml of the eluent until the TLC plate did not show any spot when subjected to identification. The CocoPLs fractions contained ethanolamine species were gathered into an evaporating flask and evaporated at 40°C to obtain dark brownish gel of CocoPEs.

2.4 Characterization of CocoPLs and CocoPEs

Both CocoPLs and CocoPEs obtained were characterized using FT-IR (Prestige 21 Shimadzu), GC-MS (Shimadzu QP2010S), and LCMSMS (Waters Xevo TQD) and DSC (Shimadzu DSC-60A). The FTIR was employed to probe the phospholipids functional groups. The GC-MS was used to determine the phospholipids fatty acyl chains. The LC-MS/MS was for identifying the chemical component of CocoPEs and the DSC analysis was carried out to explore the CocoPEs phase behavior.

2.5 Vitamin C encapsulation in coconut liposomes

In this research, vitamin C (VC) was used as a model for hydrophilic drug to be encapsulated in coconut liposome [13, 16, 17, 22]. Stock solution of 500 ppm CocoPEs with cholesterol concentration (0%, 10%, 20%, 30%, 40% w/w) were made. A total of 2 mL of each stock solution was diluted with chloroform to 10 mL and poured into a test tube. The liquid solution was evaporated using N_2 gas flow to form a thin layer. After that hydration process was carried out. Around 10 mL of phosphate buffer solution was added to the thin film. The mixture was subjected to freeze-thawing process until the thin film was dispersed completely. The dispersions contained empty coconut liposome and was used as control. Other set of dispersions were prepared by adding 8 ppm (C_0) VC solution in phosphate buffer pH 7.4 to each 2 mL stock solution and followed by similar process to obtained encapsulated VC in coconut liposome dispersion. The VC concentration in the filtrate obtained after all coconut liposome dispersions were centrifuged were analyzed using UV-Vis spectrophotometer at 265 nm. The concentration of VC was calculated from the filtrate absorbance and represented as $C_{\text{liposome+VC}}$ and $C_{\text{empty liposome}}$ in equation 2.

In addition we used CocoPLs as comparison. The encapsulation efficiency of VC in coconut liposome was determined based on Eqs. (1) and (2):

$$EE = \frac{C_0 - C_t}{C_0} \times 100\% \quad (1)$$

$$C_t = C_{liposome+VC} - C_{empty\ liposome} \quad (2)$$

where *EE* is the encapsulation efficiency; C_0 is the initial concentration of VC; and C_t is the unencapsulated VC concentration.

3. Result and discussion

3.1 Isolation and separation

A brownish yellow gel of CocoPLs was obtained from dried coconut meat (6.86×10^{-2}%, w/w) (**Figure 1**). The result was confirmed by the appearance similarity of the CocoPLs from the previous research [20, 21]. The CocoPLs was then subjected to separation to obtain CocoPEs.

In the separation process using vacuum column chromatography, CocoPLs was eluted continuously using chloroform:methanol (9:1, v/v). Each fraction of 10 mL eluent was collected and subjected to identification. As much as 520 fractions were obtained to elute CocoPEs from the CocoPLs samples completely. Identification by

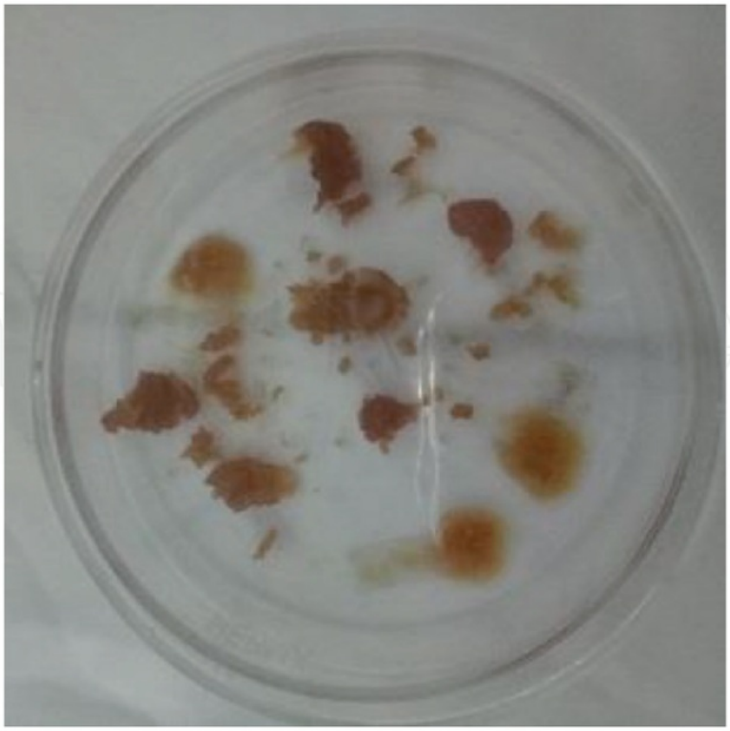

Figure 1.
CocoPLs.

TLC using 10% H_2SO_4 and ninhydrin spotting agent [23] resulted in that CocoPEs were present in the 105th to the 520th fraction.

The fraction contained CocoPEs were then combined and evaporated to remove the eluent that resulted in dark brown CocoPEs gel (9.8×10^{-3}%, w/w of dried coconut meat) (**Figure 2**).

3.2 Functional groups identification

The functional groups identification of CocoPLs and CocoPEs was conducted by FTIR spectra analysis. The FTIR spectra of both CocoPLs and CocoPEs were displayed on **Figure 3**. To analyze further the spectra were scrutinized using a deconvolution program [21, 24], at wavenumbers 3500–2800 cm^{-1} and 1800–700 cm^{-1} as presented in **Figure 4**.

The absorption data obtained from both FTIR spectra and deconvolution analysis were compared (see **Table 1**) to the specific infrared absorption area for phospholipids proposed by Stuart [25] and Hudiyanti et al. [20, 21]. The presence of a typical spectrum of phospholipids was clearly revealed. Significant differences between CocoPLs and CocoPEs spectra was disclosed by the typical absorption of choline and ethanolamine groups on both spectra of CocoPLs and CocoPEs. The choline group absorptions; $(CH_3)_3N^+$ asymmetric bending and $(CH_3)_3N^+$ asymmetry stretching; were not present on the CocoPEs spectra. The typical absorption that indicate the presence of ethanolamine species by N-H vibration absorptions was displayed on CocoPLs and CocoPEs spectra. This evident indicated that CocoPLs contained choline and ethanolamine species while CocoPEs did not contain choline

Figure 2.
CocoPEs.

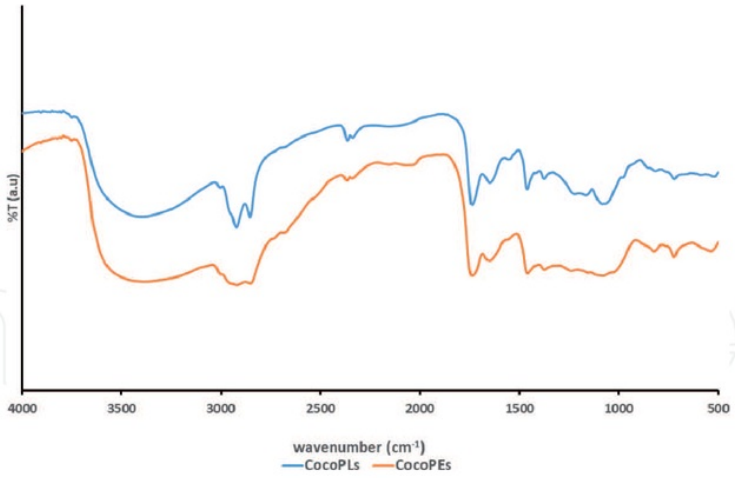

Figure 3.
CocoPLs and CocoPEs absorption spectra.

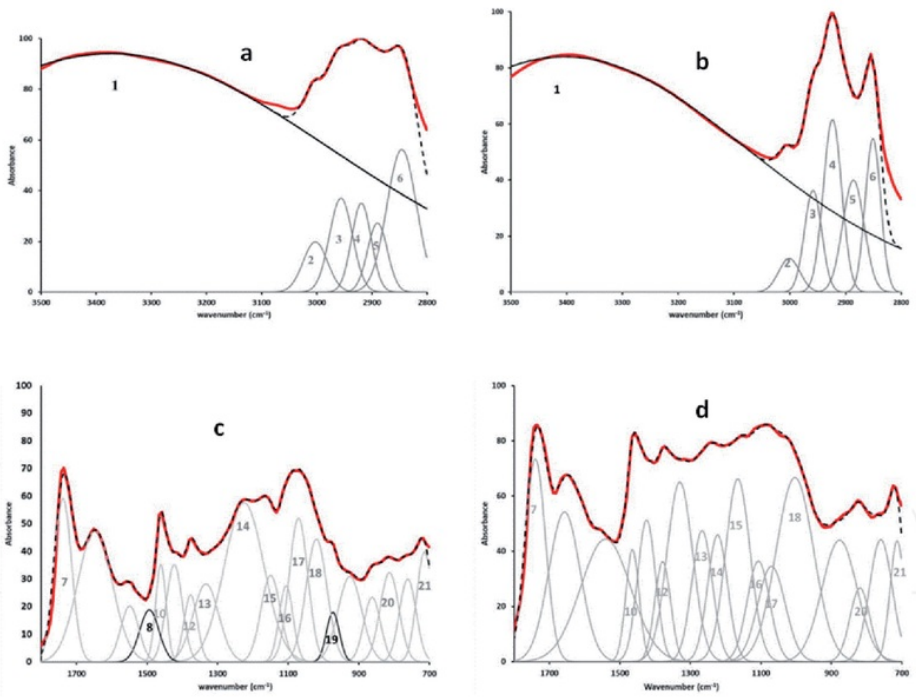

Figure 4.
Deconvolution results: (a) CocoPLs at wavenumbers 1800–700 cm^{-1}; (b) CocoPLs at wavenumbers 3500–2800 cm^{-1}; (c) CocoPEs at wavenumbers 1800–700 cm^{-1}; (d) CocoPEs at wavenumbers 3500–2800 cm^{-1}.

species. From The FTIR spectra point of view this data disclosed that the CocoPEs separation from CocoPLs was successful.

3.3 Characterization of fatty acyl chains

The fatty acyl chains content of CocoPLs and CocoPEs was analyzed by GC-MS. The CocoPLs chromatogram was presented on **Figure 5**. A total of nine

No.	Absorption type	References [15, 20, 21, 25] (cm^{-1})	CocoPLs (cm^{-1})	CocoPEs (cm^{-1})	CocoPLs Deconvolution (cm^{-1})	CocoPEs Deconvolution (cm^{-1})
1.	**N-H vibration**	**3471**	**3394**	**3379**	**3403**	**3373**
2.	=C-H stretching	3010	—	—	3001	3002
3.	CH_3 asymmetric stretching	2956	—	—	2958	2956
4.	CH_2 asymmetric stretching	2920	2924	2924	2923	2919
5.	CH_3 symmetric stretching	2870	—	—	2885	2890
6.	CH_2 symmetric stretching	2850	2854	2854	2850	2848
7.	C=O stretching, sn-1 chain trans-conformation	1730	1735	1735	1738	1739
8.	**(CH3)3N+ asymmetric bending**	**1485**	—	—	**1493**	—
9.	CH_2 scissoring	1473, 1472, 1468, 1463	—	—	—	—
10.	CH_3 asymmetric bending	1460	1458	1458	1461	1464
11.	$(CH_3)_3N$, symmetric bending	1405	—	—	—	—
12.	CH_3 symmetric bending	1378	1373	1373	1376	1378
13.	CH_3 rocking ribbon progression	1400–1200	—	—	1333	1266
14.	PO_2^- asymmetric stretching	1228	1226	1242	1225	1222
15.	CO-O-C asymmetric stretching	1170	1165	—	1150	1165
16.	PO_2^- symmetric stretching	1085	—	1080	1106	1107
17.	CO-O-C symmetric stretching	1070	1072	—	1071	1070
18.	C-O-P stretching	1047	—	—	1020	1003
19.	**(CH3)3N+ asymmetric stretching**	**972**	—	—	**973**	—
20.	P-O asymmetric stretching	820	817	—	813	819
21.	CH_2 rocking	730, 720, 718	717	725	714	713

Bold entries represented the typical absorption of choline and ethanolamine groups on both spectra of CocoPLs and CocoPEs.

Table 1.
Typical Absorption of CocoPLs and CocoPEs functional groups.

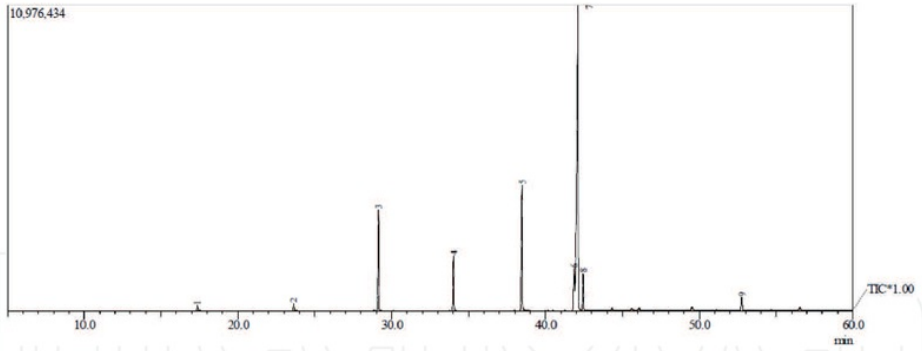

Figure 5.
CocoPLs chromatogram.

Peak number	t_R (min)	Fatty acyl chains	Area (%)
3.	29.164	Lauric acid, C12:0 (dodecanoic acid)	11.31
4.	34.037	Myristic acid, C14:0 (tetradecanoic acid)	5.71
5.	38.497	Palmitic acid, C16:0 (hexadecanoic acid)	15.26
6.	41.872	Linoleic acid, C18:2 (9(Z),12(Z)-octadecadienoic acid)	6.00
7.	42.117	Oleic acid, C18:1 (9(Z)-octadecenoic acid)	55.18
8.	42.482	Stearic acid, C18:0 (octadecanoic acid)	3.97
9.	52.794	Lignoceric acid, C24:0 (tetracosanoic acid)	1.49

Table 2.
The fatty acyl chains of CocoPLs.

peaks was recognized. Seven peaks were with abundance above 1%. The chromatogram suggested that there were at least 9 types of fatty acyl chains present on the CocoPLs. The MS reading revealed the identity of these fatty acyl chains. Three fatty acyl chains worth mentioning with the abundance more than 10%, i.e., lauric acid, palmitic acid and oleic acid which were indicated by peak number 3 (abundance of 11.31%); peak number 5 (15.26%); and peak number 7 (55.18%). The result was in agreement with previous research [15, 20, 21]. The seven fatty acyl chains recognized in CocoPLs was displayed on **Table 2**.

The chromatogram of CocoPEs was disclosed on **Figure 6**. The resulting chromatogram exposed the presence of five peaks with abundance above 1% which suggested the presence of five types of fatty acyl chains in the CocoPEs. Three of them had great abundance i.e. capric, linoleic and oleic acids as indicated by peak number 2, 3 and 4 and with abundance of 17.09%, 43.17% and 31.88% respectively. The MS reading of fatty acyl chains content in the CocoPEs was tabulated on **Table 3**.

Tables 2 and **3** revealed differences to some extent in fatty acyl chains composition between CocoPLs and CocoPEs. CocoPLs had more variation in fatty acyl chains type compared to CocoPEs. This fact plausible considering that CocoPEs was obtained from the separation of CocoPLs. The separation was mainly based on the common head group namely ethanolamine that reflected on the polarity of the separated CocoPEs molecules hence the choice of the separation eluent. More over fatty acyl chains profile were closely related to the position of phospholipid species in the bio-membrane bilayer [26–28]. Phosphatidylethanolamine (PE) species

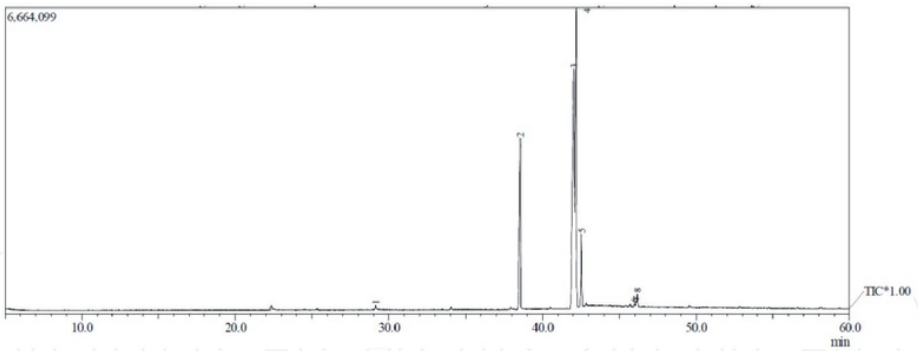

Figure 6.
CocoPEs chromatogram.

Peak number	t_R (min)	Fatty acyl chains	Area (%)
2.	38.566	Capric acid, C10:0 (decanoic acid)	17.09
3.	42.041	Linoleic acid, C18:2 (9(Z),12(Z)-octadecadienoic acid)	43.17
4.	42.198	Oleic acid, C18:1 (9(Z)-octadecenoic acid)	31.88
5.	42.555	Stearic acid, C18: 0 (octadecanoic acid)	5.93
8.	46.186	Arachidic acid, C20:0 (eicosanoic acid)	1.04

Table 3.
The Fatty acyl chains of CocoPEs.

generally would be positioned in the inner leaflet of bilayer due to their molecular geometry, i cylinder [2]. The PE species molecular shape was supported by more abundance composition of unsaturated fatty acyl chains in the CocoPEs extract, **Table 3**.

3.4 Parent ion screening

Based on the fatty acyl chains of the CocoPEs we conducted parent ion screening using LCMSMS. The CocoPEs parent ion spectrogram was presented on **Figure 7**. The spectrogram gave us a representation of the molecular species composing CocoPEs extract. At least 11 molecular species of CocoPEs were found. The CocoPEs molecular species was tabulated on **Table 4**. The molecular species was predicted based on the head group and combination of two fatty acyl chains for the nonpolar part of CocoPEs species. These similar species would govern the CocoPEs phase behavior and other properties as well.

3.5 Phase behavior

Every phospholipid species has unique phase behavior that related to their molecular structure and phase behavior. The phase behavior of CocoPLs and CocoPEs were investigated by thermal analysis using DSC. The thermogram for CocoPLs, **Figure 8**, exhibited a small peak at 28.85°C and larger peak at 83.95°C. These peaks indicated that CocoPLs underwent phase changes as temperature changes. A pre-transition process from planar-shaped gel (L_b') to the rippling phase (P_b') was at a temperature of 28.85°C (T_p), then proceed with the

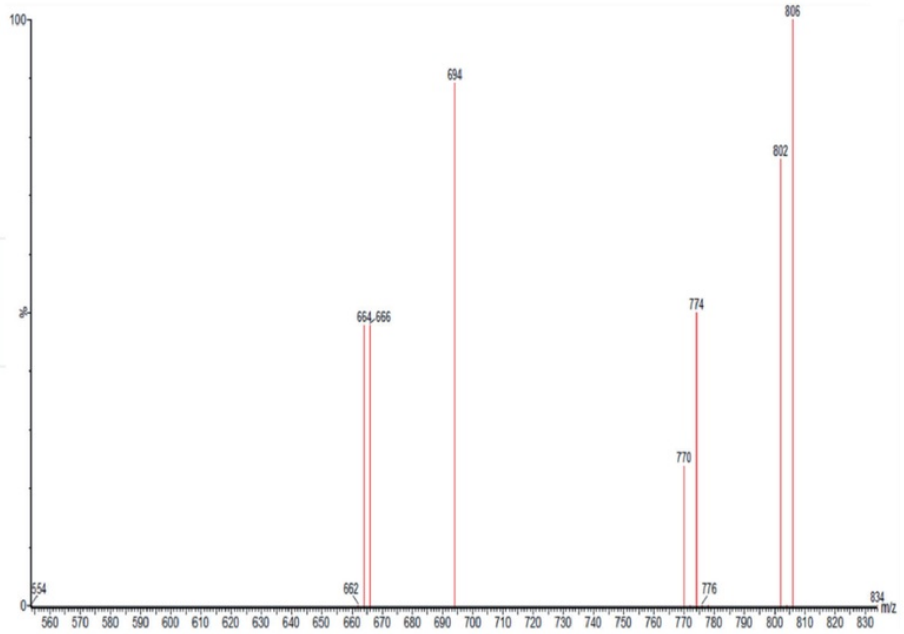

Figure 7.
CocoPEs spectrogram.

No.	m/z (M-H)	Molecular weight	CocoPEs molecular species	
			Head group	Fatty acyl chains
1.	554	555	Ethanolamine	Capric acid Capric acid
2.	662	663	Ethanolamine	Capric acid Linoleic acid
3.	664	665	Ethanolamine	Capric acid Oleic acid
4.	666	667	Ethanolamine	Capric acid Stearic acid
5.	694	695	Ethanolamine	Capric acid Arachidic acid
6.	770	771	Ethanolamine	Linoleic acid Linoleic acid
7.	774	775	Ethanolamine	Oleic acid Oleic acid
8.	776	777	Ethanolamine	Oleic acid Stearic acid
9.	802	803	Ethanolamine	Linoleic acid Arachidic acid
10.	806	807	Ethanolamine	Stearic acid Arachidic acid
11.	834	835	Ethanolamine	Arachidic acid Arachidic acid

Table 4.
CocoPEs molecular species prediction.

main transition from gel (L_b') to the liquid crystal phase (L_a) at a temperature of 83.95°C (T_m) [29–31]. T_p and T_m were the pre-transition and melting temperature correspondingly.

Different phase behavior of CocoPEs was exhibited in **Figure 9**. The thermogram for CocoPEs was more complex than CocoPLs indicated that CocoPEs had more complex phase transition than CocoPLs. CocoPEs displayed pre-transition process from planar-shaped gel (L_b') to a rippling phase (P_b') at a temperature of 25.29°C (T_p), followed by a major transition from gel (L_b') to liquid crystal phase (L_a) at a temperature of 32.62°C (T_m), and then a transition from the liquid crystal phase (L_a) to hexagonal phase (H) at a temperature of 65.53°C (T_h) [32]. The

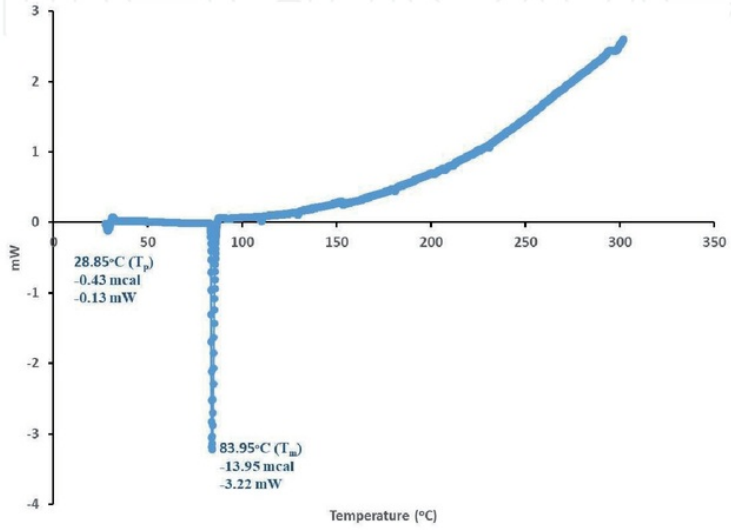

Figure 8.
Thermal analysis of CocoPLs.

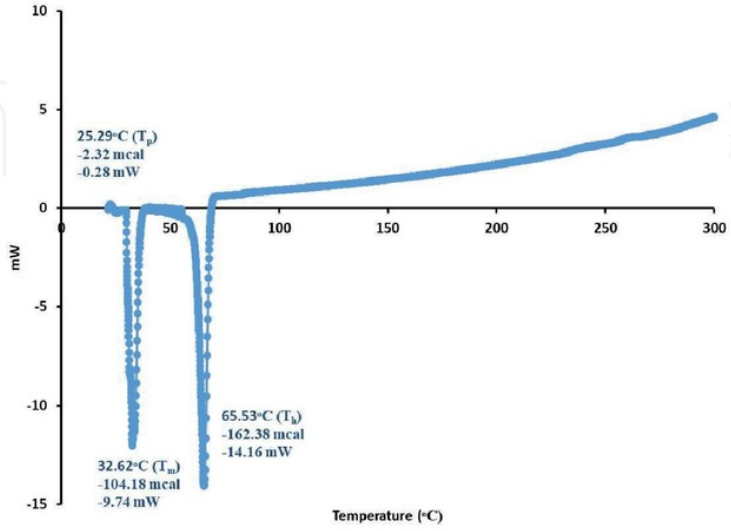

Figure 9.
Thermal analysis of CocoPEs.

hexagonal phase formation was consistent to cylindrical molecular shape attributed to CocoPEs. The CocoPEs gradual change of phase was estimated because of the similar molecular species composing CocoPEs.

The phase behavior of CocoPEs dan CocoPLs above indicated that they were both had complex self-assembly structures which would be beneficial for future applications [2].

3.6 Encapsulation of vitamin C in coconut (CocoPLs and CocoPEs) liposomes

Phospholipids has long been known as drug delivery substance due to their liposome forming ability. Liposome was a spherical aggregation structure with bilayer phospholipid as its shell surrounding aqueous core. This unique structure was especially a perfect vehicle for delivering hydrophilic and hydrophobic drugs with storage and controlled release purposes. In this paper as a preliminary study for further application of coconut phospholipid as drug delivery material we used vitamin C as a hydrophilic drug model to be encapsulated in coconut liposomes. Vitamin C was a hydrophilic drug and would be encapsulated inside the aqueous core of liposome. The study lead to that encapsulation efficiency of vitamin C in CocoPEs were higher than CocoPLs i.e. 94.44% and 92.40% respectively, **Figure 10**.

In relation to their application as drug delivery, liposomes were usually made from phospholipid and a small amount of cholesterol. Cholesterol was added to the liposome membrane to control liposome rigidity and penetrability [33]. Therefore to explore the effect of cholesterol on the encapsulation efficiency of coconut liposomes we also prepared coconut liposomes with several different concentration of cholesterol namely 10%, 20%, 30% and 40%. The encapsulation efficiency of the liposomes were presented on **Figure 10**. The results suggested that addition of cholesterol up to 40% in the liposome's membrane reduced the encapsulation efficiency of CocoPEs and CocoPLs liposomes. Furthermore CocoPEs liposomes demonstrated slighter reduction than CocoPLs liposomes. The encapsulation efficiency of CocoPEs diminished gradually as the cholesterol concentration increased while for CocoPLs liposomes the decline was arbitrary. Addition up to 30% of cholesterol only reduced the CocoPEs encapsulation efficiency to around 80% while CocoPLs was as low as 52%. Cholesterol effect on the encapsulation efficiency of CocoPEs

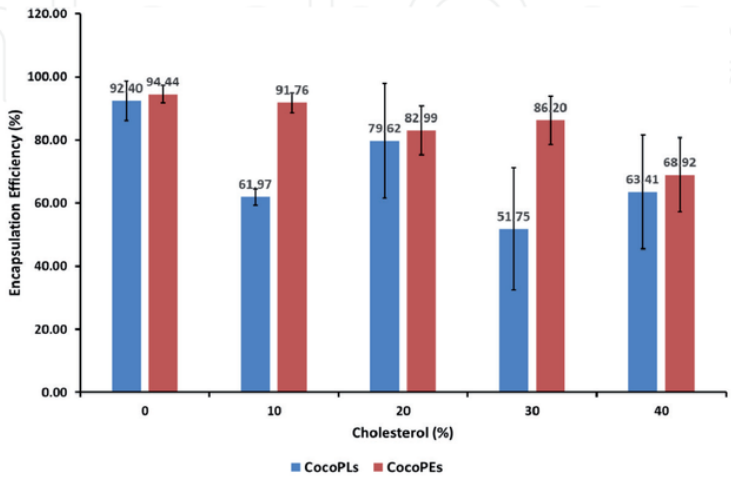

Figure 10.
Encapsulation efficiency of CocoPLs and CocoPEs liposomes with cholesterol composition variation.

liposomes more consistent than CocoPLs. We suspected it was due to the molecular composition of the phospholipid in the membrane. The molecular composition was represented by the composition of functional group and fatty acyl chains in the CocoPEs and CocoPLs, **Tables 1–3**. In the liposome membrane cholesterol interacted with CocoPEs and CocoPLs through their functional groups and fatty acyl chains. Cholesterol with small hydrophilic head group i.e., –OH and big and rigid hydrophobic steroid ring would interact better with small head group phospholipid species like CocoPEs than CocoPLs which had big spherical choline group and possibly other head groups as well. The composition of fatty acyl with double bonds also suspected would give more room for cholesterol hydrophobic moiety. The fatty acyl chains would assume "kink" structure at the double bond position [34, 35] and allocate more space hence more comfortable for cholesterol to integrate. With smaller number of fatty acyl chains type and higher concentration of double bond made cholesterol effect became more systematic in the CocoPEs liposome membrane. The data gave an insight about the application of CocoPEs as encapsulation material. CocoPEs was a good candidate for encapsulation hydrophilic material.

4. Conclusion

A total of (9.8×10^{-3}%, w/w) of coconut phosphatidylethanolamine species (CocoPEs) was isolated from dried coconut meat. The CocoPEs were obtained in the form of a dark brownish gel. Parent ion screening by LCMSMS revealed that 15 species were found in CocoPEs. Characterization of fatty acyl chains by GCMS resulted in that the hydrophobic part of the species were comprised of capric, linoleic, oleic, stearic and arachidic acyl chains. Phase behavior analysis using DSC obtained at least four different phases on CocoPEs i.e. planar-shape gel phase, rippling phase, liquid crystal phase and hexagonal phase. Each phase change occurred at a particular temperature. The pre-transition temperature (T_p) was from planar-shaped gel to rippling phase at 25.29°C, the melting temperature (T_m) for major transition from gel to liquid crystal at 32.62°C, and the hexagonal phase formation from liquid crystal (T_h) at 65.53°C. CocoPEs liposome had high encapsulation efficiency. The presence of cholesterol in the membrane liposome up to 30% did not change much of their encapsulation efficiency. The encapsulation efficiencies were above 80%. Meanwhile coconut phospholipids (CocoPLs) had them above 90% but then decrease irregularly to 52% at 0% and 30% cholesterol respectively.

Acknowledgements

DH and KA would like to express their gratitude of financial support by DIPA Selain APBN FSM UNDIP Riset Madya, 2018.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this chapter.

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