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Enzymatic modification of phosphatidylcholine with n-3 PUFA from silkworm oil fatty acids

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SUMMARY: α-Linolenic acid (ALA) containing phosphatidylcholine (PC) was prepared by an enzymatic method employing natural substrates comprising of egg and eri silkworm oil. Eri silkworm oil extracted from eri pupae was saponified to obtain the fatty acid mixture which was further subjected to urea complexation to obtain an ALA rich fraction with a purity of about 93%. Transesterification of PC with the ALA rich fraction with three immobilized lipases namely Lipozyme TL IM, Lipozyme RM IM and lipase from *Candida Antarctica* showed that only the lipase from *Candida antarctica* was successful for the incorporation of ALA into egg yolk PC. It was found that ALA was incorporated by up to 27% in the *sn*-1 position of egg PC and the positional distribution analysis of fatty acids in the modified PC showed that the *sn*-1 position was found to contain about 59% ALA.

KEYWORDS: *α-linolenic acid; Egg phosphatidylcholine; Lipase; Nutraceuticals; Silkworm oil*

RESUMEN: ***Modificación enzimática de fosfatidilcolina con n-3 PUFA a partir de ácidos grasos de aceites de gusanos de seda***. El ácido α-linolénico (ALA) contenido en fosfatidilcolina (PC) se preparó mediante un método enzimático empleando sustratos naturales que comprenden huevo y aceite de gusanos de seda. El aceite extraído de las crisálidas de gusanos de seda se saponificó para obtener la mezcla de ácidos grasos que se sometió a complejación con urea para obtener la fracción rica en ALA, con una pureza aproximadamente del 93%. La transesterificación de PC con fracción rica en ALA con tres lipasas inmovilizadas, Lipozyme TL IM, Lipozyme RM IM y lipasa de Candida antárctica, mostró que sólo la lipasa de Candida antarctica tuvo éxito en la incorporación de ALA en PC de yema de huevo. Se encontró que el ALA fue incorporado hasta 27% en la posición sn-1 de PC de huevo y el análisis de la distribución de los ácidos grasos en PC modificado mostró que la posición sn-1 que contenía aproximadamente 59% de ALA.

PALABRAS CLAVE: *Aceite de gusano de seda; Ácido α-linolénico; Fosfatidilcolina de huevo; Lipasa; Nutracéuticos*

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1. INTRODUCTION

Phospholipids (PLs) are the most ubiquitous polar lipids available in most biological membranes. PLs are well known to be the major constituents of cell membranes and play important role in the biochemistry and physiology of the cell (Guo *et al.*, 2005). Phospholipids are widely used in food industries, pharmaceutical and cosmetic products due to their emulsifying properties. Among all PLs, phosphatidyl choline or diacyl-sn-glycero-3-phosphorylcholine (PC) is the most abundant PL and plays an important role in liver and cell functions apart from being the structural constituents of biological membranes (Larsson and Quinn *et al.*, 1994; Reddy *et al.*, 2005).

PC is reported to have a great demand in the food, cosmetic and pharmaceutical industries and has gained lot of interest in phospholipid research. In food applications, PC is well known for its emulsifying properties, and as an anti-staling agent in bakery products (Kim *et al.*, 2009). It also helps to control the flow properties in chocolate products and in fat-based products as it acts as an anti-spattering agent (Kiełbowicz *et al.*, 2012). The function of PLs is reported to be dependent on the fatty acid composition of the PL and their distribution on the PL. The overall molecular structure of PLs has a significant effect on the biological functions of the PL in question. (Adlercreutz *et al.*, 2002)

It is reported that the PLs with long chain poly unsaturated fatty acids help in maintaining membrane fluidity and mobility at lower temperatures thereby contributing to membrane integrity and function when working at lower temperatures (Haraldsson and Thorarensen *et al.*, 1999). α-linolenic acid (ALA) is one of the nutritionally essential polyunsaturated fatty acids which is reported to be a precursor of other long chain n-3 PUFA such as EPA and DHA by enzymatic desaturation and the elongation pathway. ALA is reported to exert positive biological effects in the human body such as accelerating brain development in neonates, anti arrhythmic and neuroprotective functions (Barcelo-Coblijn and Murphy *et al.*, 2009). Recent studies by Xu *et al.*, (2012) have shown that ALA has beneficial effects on blood lipid profiles and inflammation which can be responsible for the protection against cardiovascular diseases. It has been shown that n-3 PUFA, when incorporated into one of the positions of PC, offered more protection against oxidation which is important when preparing such molecules of nutraceutical importance (Lyberg *et al.*, 2005). Nguemeni *et al.*, (2013) reported that dietary ALA exerted a protective role against stroke and also decreased lipid peroxidation and oxidative stress associated with brain ischemia. With the beneficial effects of ALA in mind, there has been a growing interest in the production of structured lipids containing ALA (Khodadadi *et al.*, 2013). Therefore, it would be interesting to combine both ALA and phospholipids into one molecule as the product would then have the combined benefits of both phospholipids and ALA.

There are a number of reports on the incorporation of different types of fatty acids into PC where as such type of studies are very limited in the preparation of PC rich in ALA (Guo *et al.*, 2005; Chojnacka *et al.*, 2009; Baeza-Jiménez *et al.*, 2012). This has generated a lot of interest in the research community for the production of phospholipids with a nutraceutically important supplement such as ALA. A recent report on the enrichment of egg yolk PC with ALA was described where the ALA was procured from commercial suppliers (Chojnacka *et al.*, 2009). Thus it can be useful to prepare a PC rich in ALA which can have the beneficial effects of both PL and ALA from economical sources. There are very few natural sources which have a high content of ALA and flax seed is one such source. Other vegetable oils such as soybean and rapeseed oils contain very small quantities of ALA.

The eri silk worm (*Samia Cynthia ricini*) is known to be a good source of ALA and the oil extracted from eri silkworm oil is reported to possess a high content of ALA (Kaki *et al.*, 2006). Eri silkworm feeds on leaves of two major plants, castor and tapioca and it was reported that the pupae fed on tapioca leaves had a higher content of ALA compared to the pupae fed on castor leaves. A recent report suggested that the oil was toxicologically safe and nutritionally equivalent to commonly used vegetable oils with added benefits due to high ALA content (Longvah *et al.*, 2012). In the present study, we report the isolation of ALA from eri silkworm oil and its incorporation into egg PC which could be more economical than the reported methods for the preparation of nutraceutical products with ALA.

2. MATERIALS AND METHODS

2.1. Materials

Immobilized lipases from *Candida antarctica*, Lipozyme TL IM and Lipozyme RM IM were procured from Novozymes (Bagsvaerd, Denmark). Snake venom (*Naja naja Atra*, Formosan cobra) as the phospholipase A_2 source was purchased from M/s Sigma Chemicals, St Louis, USA. Urea, toluene and all other reagents were purchased from SDFCL and were of analytical grade. The solvents for HPLC and TLC plates were purchased from Merck, Darmstadt, Germany.

2.2. Methods

Isolation of PC from eggs. Initially, the PL mixture was isolated from egg yolk following a reported procedure using methanol for extraction. Later the

PL mixture was further purified by column chromatography to obtain pure PC (Kiełbowicz *et al*., 2012).

Preparation of ALA enriched free fatty acid concentrate from eri pupae. This was carried out in three steps. Initially silkworm oil was extracted from dried pupae using hexane with a Soxhlet extractor as described earlier (Kaki *et al*., 2006). Then the obtained oil was hydrolyzed to free fatty acids employing a reported method for fish fatty acids (Mbatia *et al*., 2011). Briefly, silkworm oil (100 g) was mixed with 250 mL of 90% ethanol containing 15 g of NaOH and the mixture was refluxed for 1–2 h with stirring. The reaction was monitored by TLC, and the reaction was complete within 2 h. The FFAs were recovered by lowering the pH to 2 using 12 N HCl and the recovered FFAs were washed with water to neutralize the acid and then dried over anhydrous sodium sulphate. Finally, the enrichment of ALA from the FFA mixture was accomplished by the urea complexation method as described earlier with slight modifications (Chakraborty and Raj, 2007). Briefly, a mixture of urea and methanol (1:3 wt/vol) was heated to 65–75 °C with magnetic stirring until a clear solution was observed. To this solution, the FFA mixture (20 g) was added in portions under stirring to form a homogenous solution. This mixture was cooled to room temperature under tap water and then refrigerated at 4 °C for 6 hours. The crystallized part was filtered and the filtrate containing the PUFA was recovered and the solvent was removed under reduced pressure by rotavapor. To this PUFA concentrate, water (100 mL) was added and was slightly acidified with 6M HCl (to pH 5). Hexane (150 mL) was added to recover the PUFA and the hexane layer was washed with water until neutral and dried over an anhydrous sodium sulphate and concentrated to obtain the PUFA (7.4 g), which was stored in a freezer under nitrogen until further use.

Lipase-catalyzed incorporation of ALA into egg PC by transesterification. Enzymatic transesterification of PC and ALA was carried out by 3 lipases (Scheme 1) by employing the reported conditions with slight modifications (Chojnacka *et al*., 2009). PC (76 mg; 0.1 mmol) and ALA (30 eq, 3 mmol; 834 mg) were solubilized in toluene (5 mL) and transesterification was initiated by the addition of 228 mg of lipase (30% to wt. of substrates). The reaction mixture was magnetically stirred for 12 h at 50 °C. After the reaction, the lipase was separated by filtration and the components were purified by TLC using chloroform/methanol/water (65/25/4; v/v/v) as the mobile phase. The bands corresponding to PC were scraped and converted to methyl esters using 0.5M sodium methoxide in methanol reagent for GC analysis. The reaction product containing the highest incorporation of ALA was further purified by silica gel column chromatography. The free fatty acids were eluted with chloroform and the modified PC was eluted with 80:20 (chloroform:methanol; vol/vol). After the complete elution of PC, 100% methanol was used as eluent to obtain the lyso PC.

Positional distribution of fatty acids on the modified PC. Positional distribution of fatty acids on the modified PC was carried out by phospholipase A_2 (source: snake venom) mediated regiospecific hydrolysis as described by Christie (Christie, 1982). Briefly, phosphatidyl choline (50 mg) was dissolved in 4 ml of diethyl ether and to this 300 µL of snake venom solution (prepared by dissolving 6 mg of snake venom in 1 mL of Tris buffer; pH 7.5) were added at room temperature (28 °C) and the contents were shaken vigorously for 1 hour. The reaction progress was monitored by TLC using a solvent system of chloroform/methanol/water (65/25/4; v/v/v). After completion of hydrolysis, the ether solution was evaporated to dryness under a nitrogen stream and this mixture containing liberated fatty acids and lyso phospholipids was purified on a TLC plate. The bands corresponding to FFA and lyso PC were scraped separately. The lyso PC band was converted to fatty acid methyl esters using sodium methoxide and the free fatty acid band was converted to fatty acid methyl esters using 2%-sulphuric acid in methanol reagent.

Gas Chromatographic (GC) analysis. GC was performed on an Agilent 6890 gas chromatograph equipped with a flame ionization detector. The column used was a DB-225 column having a length of 30 m, 0.25 mm i.d and 25 µm film thickness. The carrier gas was nitrogen at a flow rate of 1 $mL \cdot min^{-1}$. The oven programming was as follows: 160 °C for 2 minutes, which rose to 230 °C at a rate of 5 °C min^{-1} and held at 230 °C for 20 minutes. The injector and detector temperatures were maintained at 220 and 250 °C respectively.

High Performance Liquid Chromatography (HPLC) analysis. The reversed phase high-performance liquid chromatography (RP-HPLC) analysis was performed on an Agilent 1100 series HPLC chromatograph equipped with an evaporative light scattering detector (ELSD) 2000 (Alltech Associates Inc., United States). The PL was solubilized in methanol (1 $mg \cdot min^{-1}$) and about 25 µL of this solution was injected into the RP-column (Merck RP-18 (5 µm) 250-4). The component species were eluted within 25 min using methanol as the mobile phase at a flow rate of 1 $mL \cdot min^{-1}$. The PL molecular species were identified tentatively by their retention times. The operating conditions for ELSD are as follows: drift tube temperature, 50 °C; flow of nitrogen, 1.5 $L \cdot min^{-1}$ with impactor "on" mode.

3. RESULTS AND DISCUSSION

The substrates for enzymatic transesterification were obtained from natural materials such as egg and eri silkworms. PC was isolated from egg and ALA was obtained from eri silkworm oil which is a cheaper alternative than the commercial supplies. The silkworm oil is considered a by-product obtained from desilked pupae which are reported to be nutritious due to the presence of high fat and protein (Singh and Suryanarayana, 2003). The desilked pupae are generally used as fertilizer or used as constituents in chicken and fish feeds. (Rao, 1994). Therefore, the objective of the present study was to make use of the high content of ALA from eri silkworm oil by isolation and enrichment in the form of free fatty acids and employ it as one of the substrates.

Urea complexation has been a method of choice for obtaining PUFA enriched fractions from fatty acid mixtures due to its simplicity, ease of scaling and eco-friendly nature (Hayes *et al*., 1998). The fatty acid composition of the initial fatty acid mixture and the PUFA concentrate obtained after urea complexation is given in Table 1. In the present study, the best proportions for the enrichment of ALA were found to be 1:4:8 (FFA:urea:metanol, wt/wt/mL) at a temperature of 4 °C with a slight variation in methanol content compared to earlier reported conditions (Chakraborty and Raj, 2007). From 20 g of silkworm oil fatty acid mixture about 7.4 g of PUFA concentrate were obtained which accounts for 72.5% recovery corresponding to initial ALA contents in the silkworm fatty acids.

The silkworm oil from silkworms fed on tapioca leaves was rich in unsaturated fatty acids with ALA constituting about 51% followed along with oleic acid and linoleic acids in minor amounts. Among the saturated fatty acids palmitic acid was the major fatty acid (24.9%) followed by stearic acid and traces of myristic, arachidic and behenic acids.

The urea crystallization method was found to enrich the unsaturated fatty acid content with a high selectivity towards ALA. It can be observed that the enriched free fatty acid mixture was mainly composed of ALA with more than 93% purity followed by linoleic and oleic acids. Further studies on the optimization of the urea crystallization method might provide suitable conditions for an efficient recovery of the ALA.

Egg PC was a better choice than soy PC since egg PC does not contain ALA which is convenient to work with when dealing with the incorporation of ALA into PC. Three immobilized lipases were examined for the incorporation of ALA at the *sn*-1 position and the results of the incorporation of ALA into egg PC are given in table 2. The methodology for enzymatic incorporation reported by Chojnacka *et al*., 2009 was followed with a slight modification.

It can be observed from the above results that the best enzyme for the incorporation of ALA into PC was found to be *Candida antarctica* which gave 26.9% incorporation compared to the other two lipases. It has been shown that, though *Candida antarctica* is a non-specific lipase, it showed higher selectivity towards the *sn*-1 position (Virto and Adlercreutz, 2000). The other two lipases were not successful in incorporating ALA and the reactions catalyzed by those two lipases resulted in a very low incorporation of ALA. Similar results were observed earlier where authors reported that *Candida antarctica* gave the best results for the incorporation of ALA into egg

Table 1. Fatty acid composition (wt %) of silkworm oil from silkworms fed on tapioca leaves and the PUFA rich fraction after urea crystallization

	Silkworm oil fatty acids	
FA	**Before urea complexation**	**After urea complexation**
14:0	0.3±0.00	n.d.
16:0	24.9±0.05	n.d.
16:1	1.1±0.01	n.d.
18:0	4.6±0.06	n.d.
18:1	13.3±0.11	1.6±0.14
18:2	4.3±0.01	4.5±0.23
18:3	51.1±0.11	93.8±0.08
20:0	0.2±0.00	n.d.
22:0	0.1±0.01	n.d.

FA: Fatty acid; n.d.: not detected.

Table 2. Reaction conditions with different lipases

S No	PC (mg)	ALA (mg; 30 eq)	Lipase	Temp (°C)	ALA %
1	100	228	*C.antarctica*	50	26.9
2	100	228	*Lipozyme RM IM*	50	2.6
3	100	228	*Lipozyme TL IM*	50	4.2

PC: phosphatidylcholine; ALA: alpha linolenic acid.

Table 3. Fatty acid composition (wt %) of substrates and modified PC and lyso PC

FA	Egg PC	SWO-FFA	Modified-PC	Lyso PC
16:0	39.3±0.05	n.d.	15.2±0.05	4.4±0.35
16:1	2.4±0.02	n.d.	2.2±0.05	1.7±0.24
18:0	9.7±0.07	n.d.	6.0±0.05	1.4±0.03
18:1	35.2±0.06	1.6±0.14	35.8±0.07	53.6±0.16
18:2	12.1±0.03	4.5±0.23	12.5±0.1	21.1±0.11
18:3	n.d.	93.8±0.08	27.0±0.1	17.7±0.20
20:4	1.2±0.01	n.d.	1.1±0.07	n.d.

SWO-FFA: silkworm oil free fatty acids; n.d.: not detected.

TABLE 4. Positional distribution of fatty acids (wt %) of egg PC and the modified PC

	Egg PC			Modified-PC		
FA	**Total**	***Sn-1***	***Sn-2***	**Total**	***Sn-1***	***Sn-2***
16:0	39.3±0.05	67.8±0.66	11.4±0.22	15.2±0.05	16.0±0.24	15.2±0.19
16:1	2.4±0.02	2.5±0.66	1.6±0.11	2.2±0.05	2.6±0.10	0.9±0.72
18:0	9.7±0.07	21.5±0.70	4.2±0.09	6.0±0.05	6.9±0.15	4.8±0.40
18:1	35.2±0.06	7.0±0.78	56.7±0.05	35.8±0.07	11.3±0.44	64.8±0.55
18:2	12.1±0.03	1.1±0.02	21.1±0.02	12.5±0.1	3.6±0.03	10.9±0.31
18:3	n.d.	n.d.	n.d.	27.0±0.1	59.3±0.83	2.6±0.23
20:4	1.2±0.01	n.d.	4.8±0.22	1.1±0.07	n.d.	0.6±0.10

PC (Chojnacka *et al*., 2009). Therefore, further studies on the purification and characterization of PC for positional analysis were carried out for the product obtained from the *Candida antarctica* lipase catalyzed reaction. The reaction product mixture from the *Candida antarctica* lipase catalyzed reaction was

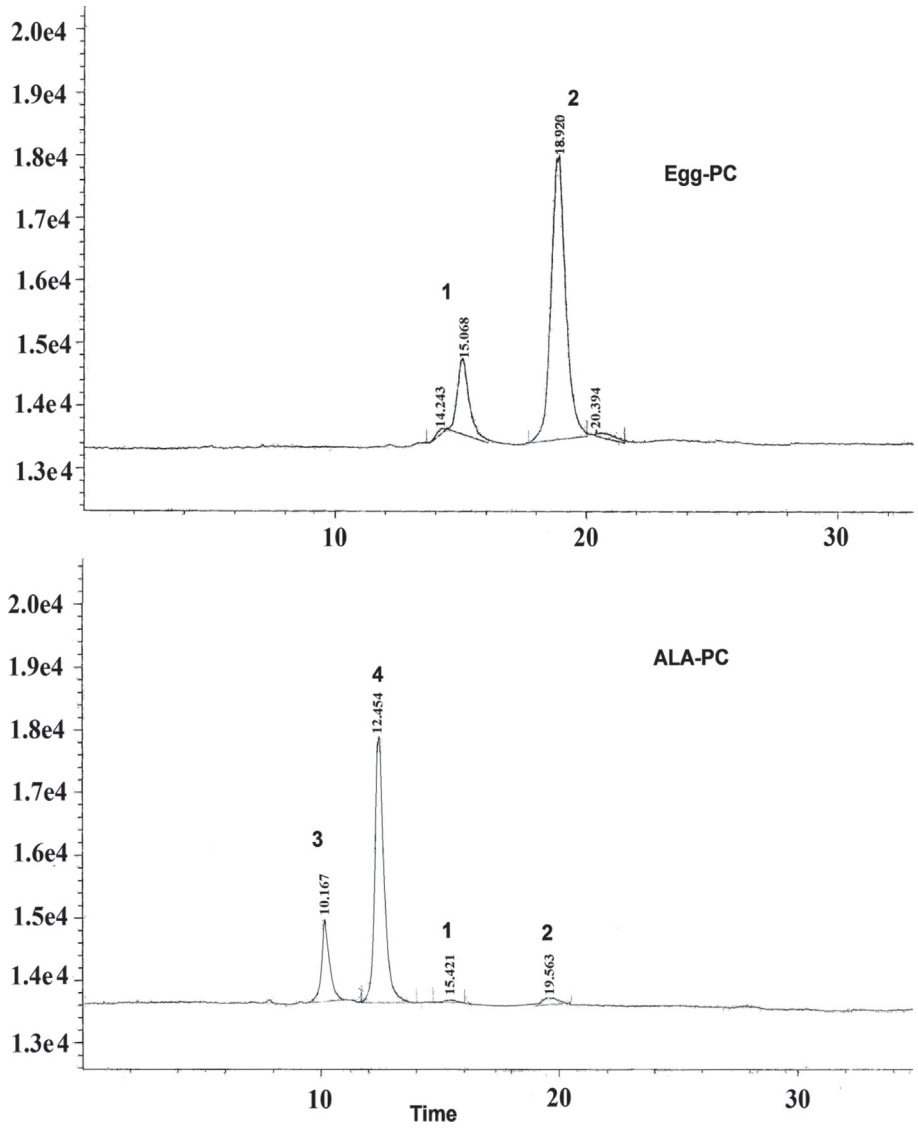


FIGURE 1. HPLC chromatograms of Egg-PC and modified/ALA-PC.

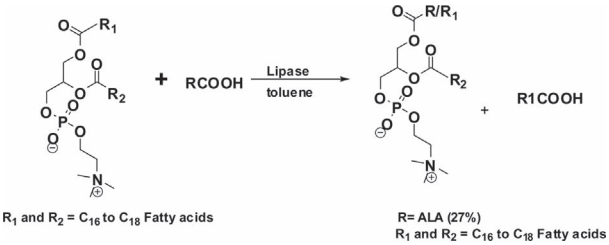
further purified by column chromatography which resulted in 67.2% modified PC and 28% lyso PC. The formation of lyso PC could be due to partial hydrolysis of the PC during the acidolysis reaction and it was found to contain 17.7% ALA. There was a decrease in the content of palmitic acid in both product PC and lyso PC compared with initial egg PC. The fatty acid composition of the modified and lyso PC purified by column chromatography along with the substrates is given in Table 3.

A high fatty acid concentration was necessary for obtaining greater incorporation since a lower concentration of ALA resulted in lower incorporation into PC. The results with lower amounts of fatty acids were not encouraging as the incorporation of ALA was not satisfactory. With a molar equivalent of 1:5 (PC:ALA) the incorporation was only 15%, even with *Candida antarctica* lipase. It was earlier reported that a high fatty acid concentration is favorable when employing an enzymatic acidolysis reaction for the modification of phosphatidylcholines (Svensson *et al*., 1993). Therefore, the excess of ALA was used for the enzymatic reaction in toluene.

The positional distribution of fatty acids over modified PC and the initial egg PC was determined by PLA2 mediated regiospecific hydrolysis. The fatty acid composition of the released free fatty acids and the lyso PC were separately determined by GC and the values are given in Table 4.

It can be observed that *sn*-1 position of egg PC contained higher amounts of saturated fatty acids (89.3%) compared to unsaturated fatty acids. Palmitic acid was the major fatty acid followed by stearic acid. The *sn*-2 position was mostly composed of unsaturated fatty acids (84.2%). Oleic acid was the major fatty acid followed by linoleic acid. The product PC was found to contain 76.8 and 79.8% of unsaturated fatty acids in *sn*-1 and *sn*-2 positions, respectively. In the modified PC, ALA was comprised of 59.3% in *sn*-1 followed by palmitic and oleic acids whereas oleic acid was the major fatty acid in the *sn*-2 position (64.8%) followed by palmitic and linoleic acids. The ALA content in the *sn*-2 position of the modified PC was found to be only 2.6% which shows the selectivity of *Candida antarctica* lipase towards the *sn*-1 position of the phosphatidylcholine.

The purified PC was also analyzed by reversed phase HPLC. Reversed phase HPLC is an effective analytical tool to qualitatively analyze the molecular species of lipid molecules. It gives us a clear picture about the transesterification of fatty acids on the phosphatidylcholine molecule. The initial egg PC was analyzed by RP-HPLC followed by the pure modified PC and the chromatograms of the both the PCs are given in Figure 1. From the figure, it can be observed that peaks 1 and 2 which are from egg PC were found to be reduced in the chromatogram of the product of ALA-PC. Peaks 1



Scheme 1. Enzymatic transesterification of ALA with egg phosphatidylcholine.

and 2 are tentatively identified as molecular species with C30 (16:0/18:2; 18:1/18:2) and C32 (16:0/16:0; 16:0/18:1; 18:1/18:1; 18:0/18:2), respectively. The product chromatogram showed two new peaks with different retention times compared to initial egg PC and are denoted as 3 and 4. Peaks 3 and 4 could probably be composed of C26 (18:3/18:2) and C28 (18:3/18:1; 18:3/16:0) molecular species, respectively. This shows that the fatty acids in the *sn*-1 position are exchanged with ALA, which is clearly evident from the shift in the retention times in the HPLC analysis and also with the fatty acid composition from the GC analysis.

4. CONCLUSIONS

Silkworm oil was employed as a source of ALA for the preparation of PC rich in ALA. The synthetic route described is a more economical and greener method for obtaining phosphatidylcholine with ALA in quantitative yields. This study shows an improvement for the application of silkworm oil which is being used for low value applications. Further studies on the optimization of urea crystallization conditions for the efficient recovery of ALA enriched fraction will be advantageous. The ALA obtained could be useful for the preparation of potential lipid-based nutraceuticals.

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