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Research Article

Enzymatic Synthesis of Arachidonic Acid-Rich Phosphatidylcholine from Arachidonic Acid Ethyl Esters

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Abstract: Arachidonic acid (ARA) is an essential fatty acid with numerous biological activities that benefits human health. However, ARA-rich phosphatidylcholine (PtdCho), which has a higher bioavailability than ARA-rich triacylglycerols, is scarce in the natural source. In this study, we developed an enzymatic modification approach for the synthesis of ARA-rich PtdCho from ARA-rich ethyl esters (EE). The maximum incorporation of ARA into PtdCho (24.02%) was achieved from the optimized conditions, including ARA-rich EE/PtdCho mass ratio of 2:1, hexane, lipase Novozym 435 as a biocatalyst (15% of enzyme load) and reaction time of 24 h. The ^{31}P nuclear magnetic resonance (NMR) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) results revealed that the PtdCho content decreased to 17.53% and the ARA-containing PtdCho species was primarily identified as PtdCho (18:2/20:4). Taken together, this investigation offers a new reference for the efficient production of ARA-rich PtdCho via enzymatic modification and paves a theoretical groundwork of industrial production practice.

***Keywords*:** arachidonic acid, phosphatidylcholine, lipases, structured lipids, synthesis

Abbreviations

ARA	arachidonic acid
C-Gly	choline chloride-glycerin
C-U	choline chloride-urea
DHA	docosahexaenoic acid
EE	ethyl esters
EPA	eicosapentaenoic acid
FAME	fatty acid methyl esters
HPLC	high performance liquid chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LysoPtdCho	lysophosphatidylcholine
NMR	nuclear magnetic resonance
PtdCho	phosphatidylcholine

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PtdGro	n-glycero-3-phosphatidylcholine
PUFA	polyunsaturated fatty acid(s)
UHPLC	ultra-high performance liquid chromatography

1. Introduction

Arachidonic acid (ARA) is an n-6 polyunsaturated fatty acid (PUFA) that has been extensively studied because of its importance in maintaining a healthy neurological system, preventing cardiovascular disease, and promoting immunity [1]. ARA supplementation has been shown to modulate lipid metabolism and vascular elasticity, providing some cardiovascular protection [2-3]. ARA-derived metabolites can affect leukocyte activity by inhibiting neutrophil infiltration and promoting macrophage clearance [4]. More importantly, ARA has neuroprotective properties at all stages of human life [5]. In early life, the brain rapidly accumulates ARA for neuronal development. ARA, together with docosahexaenoic acid (DHA), accounts for approximately 25% of the total fatty acid content and is predominantly found in the brain as a phospholipid. It is a primary structural component of the nerve cell membrane and is essential for cell division and signaling [6]. ARA has been shown to control the lengthening of neuronal protuberances by acting directly on the protein Syntaxin-3 [7]. Numerous studies in both humans and animals have demonstrated that ARA supplementation has profound positive effects on the neural development and cognitive function of infants and toddlers [8-11]. Furthermore, cognitive function degrades with normal aging, and ARA supplementation can improve cognitive function by retaining hippocampus' plasticity, lowering amyloid-protein deposition, and modulating synaptic transmission [12-13]. The excellent bioactivity of ARA has contributed to its widespread use as a health supplement and infant formula.

ARA is available in trace amounts and cannot be used for large-scale production, although it can be found in meat, fish, eggs, and dairy products. In recent years, single-cell triglyceride oil produced by *Mortierella alpina* has become a reliable source of ARA [14]. However, the bioavailability and oxidative stability of ARA-rich triacylglycerols are deficient [15].

Phosphatidylcholine (PtdCho) is the most abundant phospholipid in mammalian cells, typically constituting 40-50% of total cellular phospholipids. It serves as a crucial component in the formation of biological membranes [16]. Furthermore, PtdCho plays a crucial role in metabolic health as a significant supplier of choline, which is a crucial dietary nutrient necessary for the synthesizing neurotransmitters and methyl donors [17]. Hence, PtdCho is an appealing product extensively employed in the food and pharmaceutical industries as an emulsifier, nutritional supplements, and a constituent in pharmaceutical formulations, among other applications.

Studies have shown that ARA-rich PtdCho demonstrated higher incorporation efficiency in tissues than ARA-rich triacylglycerols [18]. Additionally, fatty acids in the PtdCho form are more readily absorbed in vivo compared to their respective triacylglycerol or ester forms [19]. This indicates that ARA-rich PtdCho can perform the functions of phospholipids and ARA functions more effectively. Furthermore, ARA-rich PtdCho has been reported to possess beneficial biological properties such as enhancing cognitive function, promoting growth and development, and acting as an anti-inflammatory agent [20-21]. These advantages make PtdCho a more competitive form of the fatty acid carrier to improve the absorption and activity of ARA. On the one hand, the composition of natural phospholipids is constrained; on the other hand, it is particularly difficult to isolate specific phospholipids [22]. Hence, it is necessary to prepare high content ARA-rich PtdCho for further applications.

Structured phospholipids are synthesized using chemical and enzymatic methods. In the chemical method, the functional groups of phospholipids, such as polar head groups and carbon-carbon double bonds, react with chemicals [23]. However, some chemical reagents have poor safety which restricts their application in the field of food. The enzymatic production of structured phospholipids has various benefits over chemical methods, including gentle reaction conditions, high catalytic efficiency and good position selectivity [24]. However, enzymatic synthesis is still plagued by high process costs, low substrate conversion rates, and challenges in scaling-up production. Selecting appropriate substrates with optimal lipase activity is the foundation for improving the yield of the target products. Meanwhile, the yield and purity of the eventual products are significantly affected by process variables such as the reaction medium and acyl migration [23]. Therefore, experimental designs have been proposed to optimize the critical parameters in a given system.

Although there have been multiple studies on the enzymatic production of phospholipids rich in n-3 PUFA, the synthesis of ARA-rich phospholipids has rarely been reported. Depending on the type of acyl donor, PUFA-rich PtdCho is synthesized mainly through the acidolysis of unesterified fatty acids with PtdCho and transesterification of fatty acid ethyl esters (EE) with PtdCho [25]. Generally, acidolysis is carried out under milder conditions because of the chemical instability of unesterified fatty acids [26]. Therefore, ARA-rich EE, as the primary product of ARA on the market, is a suitable raw material for transesterification to synthesize ARA-rich PtdCho. Moreover, lipases and phospholipases of diverse origins can be used as catalysts. They modified the specific fatty acid sites on the lipid glycerol backbone [27]. Immobilized lipases have recently gained popularity because of their excellent economy, durability, and selectivity [28]. Considering that the synthesis of ARA-rich PtdCho has received little attention, it is essential to identify lipases with extraordinary catalytic performance to produce ARA-rich PtdCho.

The purpose of this research was to evaluate the impact of catalytic conditions on the incorporation of ARA into PtdCho, including types of enzymes, the mass ratio of substrates, reaction medium, and reaction time. Moreover, we identified the composition and molecular species of synthesized phospholipids by ^{31}P nuclear magnetic resonance (NMR) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). We offer a new reference for the synthesis of ARA-rich phospholipids that serve crucial nutritional functions.

2. Materials and methods

2.1 *Materials*

ARA-rich ethyl ester (ARA-rich EE, purity > 90%, used as raw material) was obtained by Macklin Biochemical Co., Ltd (Shanghai, China). Phosphatidylcholine (PtdCho, purity > 90%, used as raw material) was provided by Yuanye Bio-Technology Co., Ltd (Shanghai, China). Phospholipase A1, immobilized lipozyme RM IM, immobilized lipozyme TL IM, and immobilized Novozym 435 were obtained from Novozymes (China) Investment Co., Ltd (Shanghai, China). Standards of 37 methylated fatty acids mix, sn-glycero-3-phosphatidylcholine (PtdGro), lysophosphatidylcholine (LysoPtdCho), and phosphatidylcholine (PtdCho) were purchased from Sigma-Aldrich (St. Louis, USA). HPLC-grade n-Hexane and isopropanol were obtained from Thermo Fisher Scientific (Auckland, New Zealand). The analytical grade was used for all other solvents and chemicals.

2.2 *Transesterification of PtdCho with ARA-EE*

Transesterification reactions catalyzed by enzymes were conducted to investigate the impact of conditions on the incorporation of ARA into PtdCho, including types of enzymes, the mass ratio of ARA-rich EE and PtdCho, reaction medium, and reaction time. First, four different lipases (phospholipase A1, immobilized Lipozyme RM IM, immobilized Lipozyme TL IM, and immobilized Novozym 435 at 15% by weight of total substrates) were selected for catalyzing the reaction. ARA-rich EE and PtdCho were mixed at a mass ratio of 3:1 in 2 mL hexane solvent and reacted for 24 h at 55 °C under N_2. Subsequently, under the catalysis of the optimized lipase (15% by weight of total substrates), ARA-rich EE and PtdCho were mixed at different mass ratios (1:1, 2:1, 3:1, 4:1, and 5:1) in 2 mL hexane solvent and reacted for 24 h at 55 °C under N_2 conditions. Following that, under the catalysis of the optimized lipase (15% by weight of total substrates), ARA-rich EE and PtdCho were mixed at the optimal mass ratio in 2 mL different solvents (hexane, toluene, heptane, choline chloride-urea (C-U), and choline chloride-glycerol (C-Gly)) and reacted for 24 h at 55 °C under N_2 conditions. Finally, under the catalysis of the optimized lipase (15% by weight of total substrates), ARA-rich EE and PtdCho were mixed at the optimal mass ratio in 2 mL suitable reaction media and reacted for 3, 6, 12, 24, and 36 h at 55 °C under N_2 conditions. Each condition described above was repeated three times. The reaction was incubated in a magnetic stirrer at 200 rpm and terminated by separating the enzymes from the reactants. The phospholipid precipitate was obtained as before and then stored at -20 °C [29].

2.3 *Analysis of fatty acid composition*

Before analysis, the solvent in the mixed product was evaporated under vacuum. The phospholipids in the reaction mixture were then separated on thin layer chromatography plates with chloroform, methanol, and water (65:25:4, v/v/

v) as the developing agent [30]. After color development with iodine powder, the PtdCho strips were scraped off and resolubilized using chloroform/methanol (2:1, v/v). The reaction product PtdCho was obtained by desolvation following centrifugal separation.

The Nexis GC-2030 gas chromatography system (Shimadzu, Japan) was employed to analyze fatty acid, which was equipped with a TR-FAME column (60 m × 0.25 mm, i.d. × 0.25 μm). The analysis protocols were described according to the literature [31]. And fatty acids were derivatized according to previous report [32]. Briefly, 2 mL of methanol-potassium hydroxide was added to saponify the lipids at 65 °C, followed by methylation of the fatty acids with 2 mL of potassium hydroxide-boron trifluoride at 70 °C. The resulting fatty acid methyl esters (FAME) were introduced into the gas chromatography apparatus after HPLC-grade hexane extraction. By contrasting their retention times of fatty acids, we identified them using FAME standards.

2.4 *Analysis of PtdCho compositions and molecular species in the reaction products*

The phospholipid compositions of the products were analyzed by ^{31}P NMR. The resulting precipitated phase (50 mg) was mixed with 0.6 mL chloroform/methanol (2:1, v/v) and then transferred to the NMR tube. The probe temperature was set at 25 °C, 90° pulse, and the number of sampling points was 32 k. The sampling time and relaxation time were set to 3.22 s and 12.25 s, respectively. The spectrometer frequency and the pulse width were selected as 161.98 Hz and 11.66 μs. The area percentages of PtdCho, LysoPtdCho, and PtdGro were determined using the relative integrated signal responses of the NMR spectra. The internal standard was triphenylphosphate. The data were analyzed using MestReNova instrument software.

A ultra-high performance liquid chromatography (UHPLC) equipment plus Triple TOF 5600 (Q-TOF; AB Sciex, Framingham, MA) was used for LC-MS/MS analysis. The chromatographic column was the C18 column (2.1 × 100 mm, 2.6 μm, Phenomenex). The specific analysis conditions were based on the previous article [33]. The molecular species of phospholipids were examined in the negative ion mode. The UHPLC data were opened in the data processing software SCIEX OS 2.0.1 and MS1 spectra were extracted to observe the peak widths of the compounds. The molecular species of phospholipids containing ARA were determined using the decomposition laws deduced by combining the mass/charge ratio and secondary fragment information of each compound.

2.5 *Statistical analysis*

We replicated each experiment three times Then, the significance of the discrepancies among the measured values was determined using the analysis of variance (ANOVA) procedure. The data were showed in the form of means ± standard deviations (SD).

3. Results

3.1 *Screening of enzymes*

The lipase-catalyzed transesterification reaction used to produce ARA-rich PtdCho is shown in Figure 1.

The potential of four commercial enzymes to catalyze the ARA-rich EE and PtdCho transesterification reactions was investigated. We compared Novozym 435, Lipozyme RM IM, Lipozyme TL IM, and phospholipase A1, all of which are widely employed in the production of structured phospholipids. Although the enzymes displayed varying activities, we chose to utilize them in an identical weight proportion to lower the overall cost. The initial conditions were based on previous studies.

The following parameters were used for the enzyme screening: lipase as a biocatalyst (15% by weight of total substrates), ARA-rich EE/PtdCho mass ratio of 3:1, hexane solvent, and reaction time of 24 h at 55 °C. The incorporation of ARA into PtdCho catalyzed by these four enzymes was investigated using gas chromatography. The results are shown in Figure 2a. The enzyme screening revealed that Novozym 435 had the best catalytic performance, with ARA incorporated into PtdCho by more than 15% after 24 h. Lipases appear to be more efficient than phospholipases for the synthesis of structured phospholipids. The amount of ARA incorporated into PtdCho was in the following order after 24 h: Lipozyme Novozym 435 > Lipozyme RM IM > phospholipase A1 > Lipozyme TL

IM. Among the lipases, Lipozyme TL IM exhibited the poorest catalytic performance for incorporating ARA into PtdCho, which follows the results of Chojnacka et al. [34]. Therefore, immobilized Novozym 435 was selected for the subsequent experiments to produce ARA-rich PtdCho.

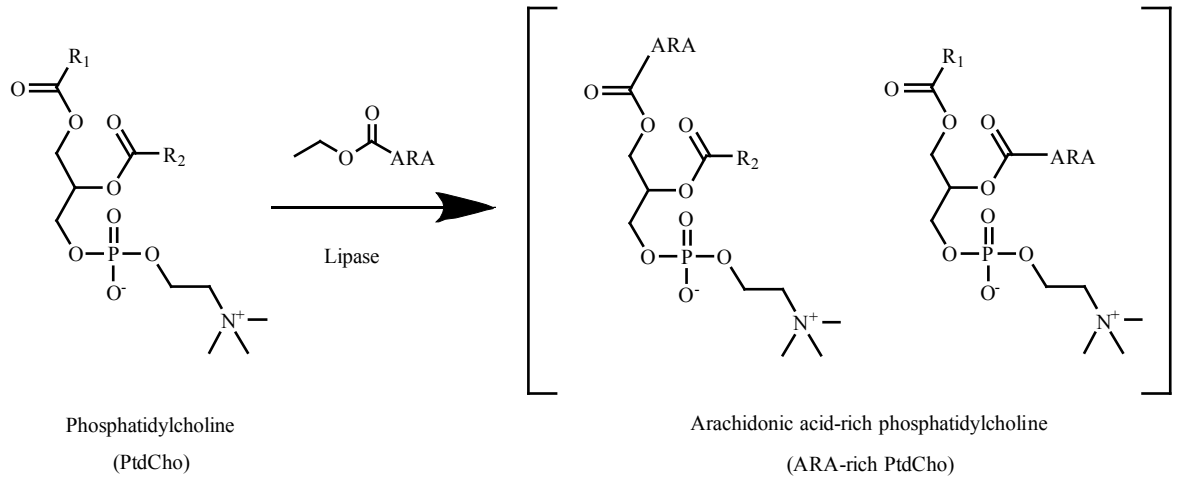

Figure 1. Immobilized lipase-catalyzed transesterification of ARA-rich EE with PtdCho

3.2 *Effect of substrate mass ratio*

The acyl donor concentration is one of the critical parameters affecting transesterification according to the research. We examined the effects of the ARA-rich EE/PtdCho mass ratios ranging from 1:1 to 5:1 on ARA incorporation into PtdCho. The results are given in Figure 2b.

The incorporation of ARA into PtdCho was enhanced when the mass ratio of ARA-rich EE/PtdCho was changed from 1:1 to 2:1. The maximum incorporation of ARA reached 24.02% with a substrate mass ratio of 2:1. Subsequently, the mass ratio of ARA-rich EE/PtdCho was further increased, which resulted in a significant decrease in the incorporation of ARA. An excessively mass ratio of substrates could block the diffusion of substrates, making it challenging to separate the reaction products. Moreover, excess acyl donors may partially inhibit lipase activity. Considering the economy of the process, 2:1 was determined to be the optimal ARA-rich EE/PtdCho mass ratio for subsequent investigations.

3.3 *Effect of solvents*

The effects of hydrophobic solvents, C-U, and C-Gly on the transesterification reaction were evaluated. The results are shown in Figure 2c.

The results indicated that we could obtain the maximum ARA incorporation using hexane as the solvent, which is similar to the results of Chojnacka et al. [35]. The incorporation of ARA was almost half of that of hexane when toluene was used as the solvent. Among the three hydrophobic organic solvents selected, heptane was found to disfavor ARA the most. We observed that the high viscosity of the low-eutectic solvent was not conducive to the homogeneous stirring of the reactants during the reaction. Therefore, hexane was used as the ideal reaction solvent.

3.4 *Effect of reaction time*

Figure 2d shows the results of the Novozym 435 lipase-catalyzed transesterification reaction over time. The amount of ARA incorporated into PtdCho steadily increased as the reaction continued. From 12 h to 24 h, ARA incorporation increased dramatically, and the highest incorporation was obtained at 24 h. However, the incorporation of ARA

showed a decreasing trend over the next 12 h. We speculate that with the prolongation of the reaction time, the PtdCho incorporated with ARA underwent hydrolysis again. The reaction time was set to 24 h to achieve the highest ARA incorporation. The fatty acid composition of the raw material ARA-rich EE, PtdCho and PtdCho synthesized under optimal conditions was determined by gas chromatography, as shown in Table 1.

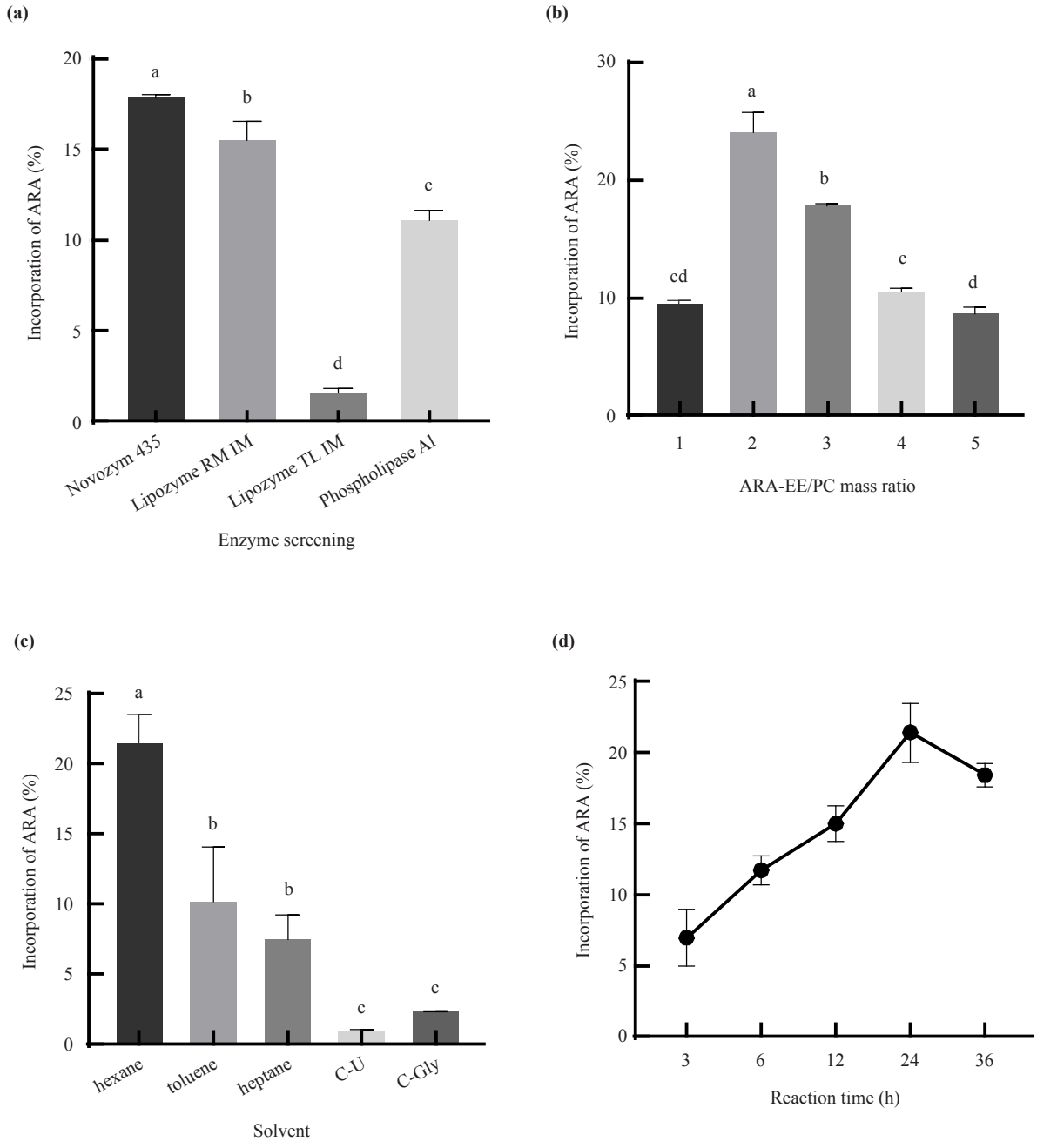

Figure 2. The effects of catalytic conditions on the incorporation of ARA into PtdCho were evaluated. (a) Effect of enzyme types on the transesterification reactions. (b) Effect of ARA-rich EE/PtdCho mass ratio on the transesterification reactions. (c) Effect of solvents on the transesterification reactions. (d) The time course of Novozym 435-catalyzed transesterification of PtdCho with ARA-rich EE. Different lowercase letters indicate significant differences ($P < 0.05$)

Table 1. Fatty acid composition (%) of material PtdCho, ARA-rich EE and produced PtdCho

Fatty Acid (%)	PtdCho	ARA-rich EE	produced PtdCho
16:0	14.58 ± 0.01	0.14 ± 0.02	7.74 ± 1.29
18:0	4.35 ± 0.01	-	4.07 ± 0.86
18:1n-9	9.80 ± 0.06	0.45 ± 0.01	7.63 ± 0.25
18:2n-6	65.37 ± 0.08	0.60 ± 0.02	51.77 ± 0.91
18:3n-3	5.90 ± 0.02	-	3.23 ± 0.04
20:0	-	4.37 ± 0.02	1.54 ± 0.09
20:4n-6	-	94.44 ± 0.02	24.02 ± 1.40

3.5 *Identification of reaction products*

The products were obtained under the optimized conditions, including Novozym 435 as a biocatalyst, an ARA-rich EE/PtdCho mass ratio of 2:1, hexane as the solvent, and a reaction time of 24 h under N_2. Their compositions and molecular species were analyzed using ^{31}P NMR and LC-MS/MS.

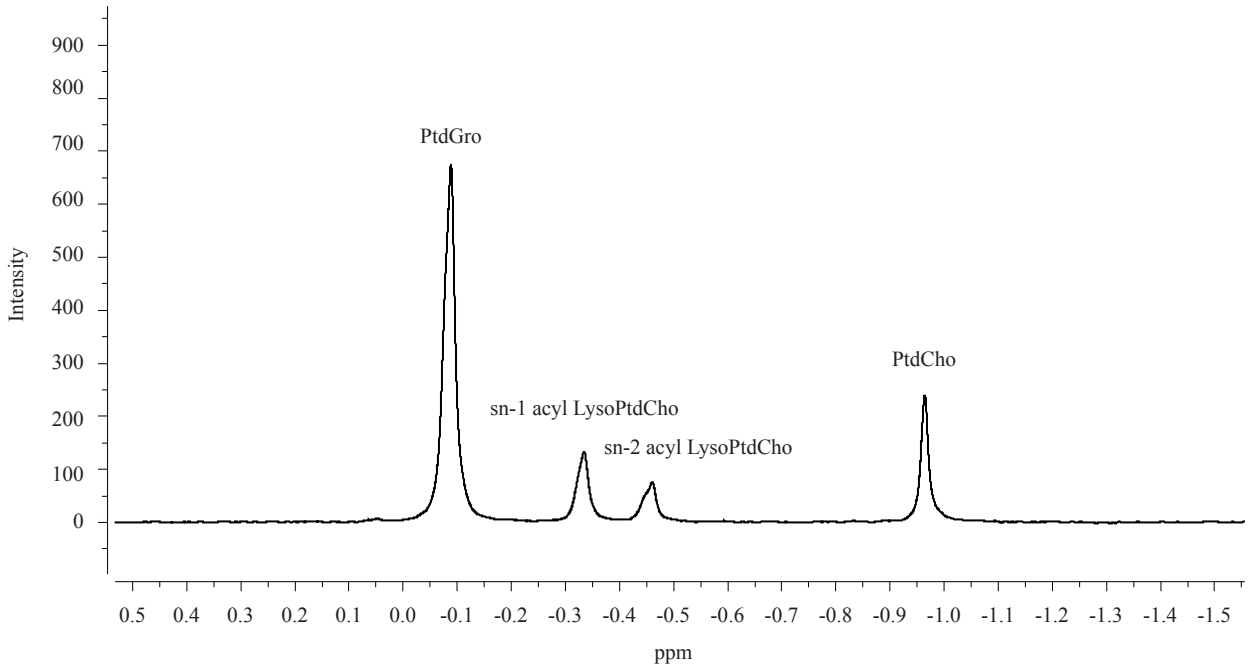

Figure 3. ^{31}P NMR results of PtdGro, sn-1 acyl LysoPtdCho, sn-2 acyl LysoPtdCho and PtdCho in reaction products

The ^{31}P NMR spectra of the reaction products are shown in Figure 3. As shown in Table 2, the PtdCho content decreased to 17.53% after 24 h of reaction, while the PtdGro, sn-1 acyl LysoPtdCho, and sn-2 acyl LysoPtdCho contents increased to 62.43%, 12.47%, and 7.57%, respectively. The lipase used in our experiments is highly selective for the sn-1 position of phospholipids, and preferentially hydrolyze the sn-1 position to generate sn-2 acyl LysoPtdCho [30, 34]. According to the current reaction mechanism of enzymatic synthesis, weak acyl migration occurs during the transesterification [19]. The sn-1 acyl LysoPtdCho generated by acyl migration, followed by further hydrolysis to create PtdGro, likely caused the observed phospholipid composition.

Table 2. Phospholipid composition of the reaction product

Phospholipids	Contents (%)
PtdGro	62.43 ± 2.75
sn-1 acyl LysoPtdCho	12.47 ± 1.23
sn-2 acyl LysoPtdCho	7.57 ± 1.56
PtdCho	17.53 ± 2.56

LC-MS/MS was used to identify the ARA-containing PtdCho species in the produced products. We identified six ARA-containing PtdCho species, and the detailed information is provided in Table 3. The PtdCho molecules containing ARA were mainly identified as PtdCho (18:2/20:4) and PtdCho (18:1/20:4) based on the corresponding peak areas. The specific secondary mass spectrum information is shown in Figure 4.

Table 3. Identification of ARA-rich PtdCho species in reaction products

Lipid class	Molecular species	Adducts	m/z	Retention time (min)
PtdCho	PtdCho (16:0/20:4)	$[M + OAc]^-$	840.5760	7.813
	PtdCho (18:0/20:4)	$[M + OAc]^-$	868.6073	8.292
	PtdCho (18:1/20:4)	$[M + OAc]^-$	866.5917	8.310
	PtdCho (18:2/20:4)	$[M + OAc]^-$	864.5760	7.205
	PtdCho (18:3/20:4)	$[M + OAc]^-$	862.5604	6.354
	PtdCho (20:4/20:4)	$[M + OAc]^-$	888.5760	6.742
LysoPtdCho	LysoPtdCho (20:4)	$[M + OAc]^-$	602.3464	1.821

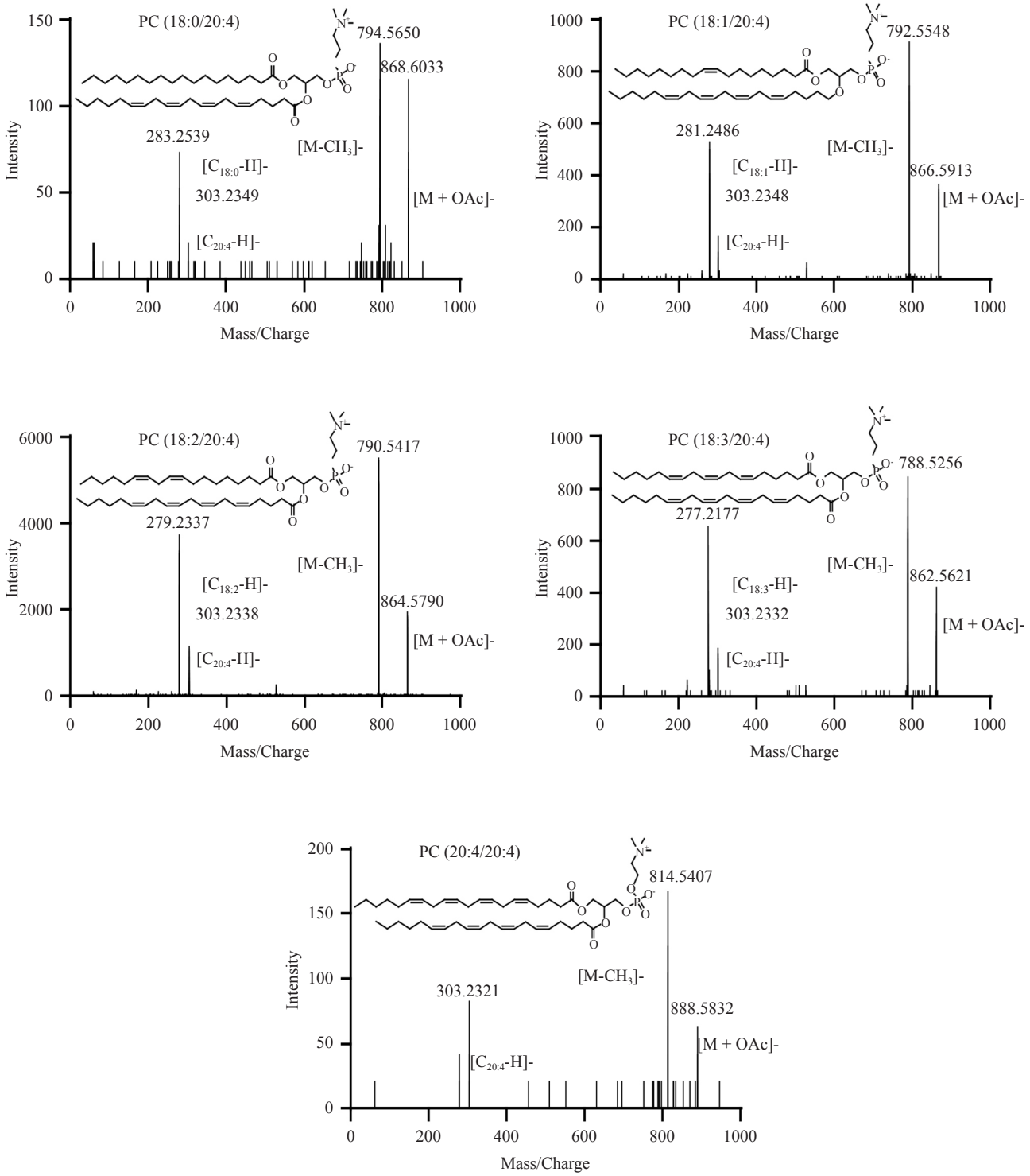

Figure 4. Secondary mass spectra of PtdCho (18:0/20:4), PtdCho (18:1/20:4), PtdCho (18:2/20:4), PtdCho (18:3/20:4) and PtdCho (20:4/20:4)

4. Discussion

The ARA-rich PtdCho obtained using Novozym 435 lipase reached 24.02% at 24 h under the optimized conditions.

At present, researchers prefer to incorporate medium-long chain fatty acids such as caprylic and decanoic acids and long-chain fatty acids such as conjugated linoleic acid and eicosapentaenoic acid (EPA) into phospholipids [25, 36-38]. Few studies have focused on the enzymatic transesterification of ARA incorporated into phospholipids, and the functional advantages of ARA-rich PtdCho have been overlooked. Our study broadens the synthesis and application of structured phospholipids rich in n-6 PUFA.

Production of ARA-rich PtdCho is based on the utilization of appropriate arachidonic donors. Unesterified ARA or ARA in the ester form can be subjected to acidolysis or transesterification reactions to obtain the desired product. However, transesterification may be more appropriate than acidolysis for generating ARA-rich phospholipids. In a study, Chojnacka et al. [30] examined the effect of transesterification and acidolysis on the incorporation of myristic acid into PtdCho. They found that the incorporation of myristic acid in the hexane system was up to 44% when the substrate molar ratio (PtdCho/trimyristin) was 1:5, and the enzyme loading was 30% after 48 h of reaction. Compared with the 26% incorporation obtained from the acidolysis reaction, the interest in trimyristin on PtdCho resulted in a higher incorporation of myristic acid. In a previous study, the reaction of PtdCho with PUFA-rich EE resulted in structured phospholipids with high EPA and DHA incorporation [39]. To achieve a high rate of ARA incorporation, ARA-rich EE were employed as an acyl donors in this study.

The high substrate ratio between the ARA-rich EE and PtdCho promoted the incorporation of ARA into PtdCho. Excessive acyl donors contribute to the phospholipid solubility, which strengthens the mass transfer surrounding the enzyme and causes a higher incorporation of ARA [40]. However, the incorporation of ARA may decrease once the optimal ratio is reached and the amount of ARA-rich EE continues to increase. The additional ARA-rich EE makes the reaction system more viscous, which impedes the diffusion of substrates and reduces the possibility of molecular collisions [23]. A higher stirring intensity allows the substrate to be mixed uniformly, and the enzyme effect is unrestricted by substrate diffusion [41]. However, the use of a higher substrate mass ratio is not economically beneficial. This study achieved the optimal incorporation effect by using a substrate ratio of 2:1 for ARA-rich EE/PtdCho in the selected hexane system.

Four commercial enzymes were used to manufacture the ARA-rich PtdCho. Immobilized lipases are frequently used in the synthesis of structured phospholipids because of their excellent catalytic activity, high stability in the reaction system and simplicity of recycling [42]. Lipozyme RM IM and lipozyme TL IM are specific to the sn-1,3 region, whereas the Novozym 435 is classified as non-specific [43]. Phospholipase A1 primarily acts on phospholipid bonds at the sn-1 position and demonstrated superior catalytic properties in incorporating medium and long-chain fatty acids into phospholipids [23]. Although phospholipases are capable of catalyzing the production of structured phospholipids, their low productivity and high cost preclude their widespread application [22]. Our screening results revealed that the effect of lipase on the incorporation of ARA into PtdCho was equal to or even better than that of phospholipase. Similar to our results, Chojnacka et al. [35] achieved a higher n-3 PUFA incorporation when employing immobilized lipase as the enzyme catalyst compared to immobilized phospholipase A1. Of particular note, Novozym 435 exhibited the highest ARA incorporation rate among the three immobilized lipases. Several studies have shown that Novozym 435 has the highest efficiency in catalyzing the incorporation of citronellic acid, punicic acid, and n-3 PUFA into phospholipids [34-35, 42]. This suggests that Novozym 435 holds potential for the synthesis of various structural phospholipids.

The majority of the reaction systems are either solvent-free or organic solvent systems. And ARA-rich EE, PtdCho and solvent interactions determine the incorporation efficiency of ARA in this reaction. Due to non-uniform stirring, solvent-free systems may suffer from low reaction rates and difficult product separation [44]. Therefore, solvent-free systems may require more ARA-rich EE to increase the solubility of phospholipids. In a research, Wang et al. [25] found that the maximum incorporation of n-3 PUFA reached 33.5% in the solvent-free system when the mass ratio of n-3 PUFA-rich EE/PtdCho was 6:1, which was similar to the research of Li et al. [45]. In these investigations, the amount of acyl donors employed was substantially more than the amount of ARA-rich EE in the current study. The existence of solvent will enhance the mixing of the reaction substrates and facilitate the removal of subsequent enzymes [46]. The maximum ARA incorporation rate was found in the hexane system, where hexane, as a hydrophobic solvent, could hold an essential layer of water around the enzymes. According to published reports, the most appropriate solvents to utilize in lipase-catalyzed systems are those with log P values from 2 to 4 [44]. The log P value refers to the solvent-water partition coefficient for organic solvents, serving as a quantitative measure of solvent polarity. For solvents with water solubility exceeding 0.4%, their log P are below 2, as strong interactions exist between water and biocatalysts,

rendering such solvents unsuitable for biocatalysis. Solvents with log P greater than 4 are highly hydrophobic and do not disrupt the crucial water layer, preserving the biocatalyst's activity. Solvents with log P values between 2 and 4, where water solubility falls between 0.04 and 0.4%, exhibit higher activity in systems with relatively low substrate polarity [47]. However, employing organic solvents has several areas for improvement, including the requirement of subsequent processing, potential environmental issues, and the high costs of the large-scale production [27]. Therefore, the application of low eutectic solvents in the transesterification of structured phospholipids required further research and exploration.

The molecular species of ARA-rich PtdCho were identified by LC-MS/MS. Due to the great selectivity of lipase for acyl sites in phospholipids, the ARA-rich PtdCho is mostly one ARA molecule bound to one structural phospholipid in contrast to the chemical approach. Furthermore, hydrolysis is an inevitable side effect of the enzyme-catalyzed phospholipid transesterification. When the composition of phospholipids was identified by ^{31}P NMR, we observed that the PtdCho was converted into significant amounts of LysoPtdCho and PtdGro. Similar results were reported in many studies, where the PtdCho content decreased substantially while the by-product content increased considerably when the reaction was finished [25, 36]. The water used for phospholipid hydrolysis in our method is mainly derived from the substrate. Therefore, adjusting the water to a suitable level in the reaction may be critical for phospholipid production. Subsequent studies can regulate water content by drying substrates or equilibrating systems with saturated salt solution [41, 45, 48].

In summary, this study successfully synthesized ARA-rich structured phospholipids using ARA-rich EE and PtdCho catalyzed by Novozym 435 lipase. Under the optimized condition, the maximum amount of ARA incorporated into PtdCho was 24.02%. The results demonstrated that Novozym 435 lipase serves as an advantageous biocatalyst in the production of structured phospholipids. Based on the ^{31}P NMR results, we found that the hydrolysis of PtdCho in our system is relatively high. In future research, enzyme engineering can be employed to modify enzyme molecular structures or screen novel enzymes more suitable for specific reactions. Designing and screening appropriate carriers for enzyme immobilization could achieve highly efficient and specific catalysis on substrates. The enzymatic synthesis process can be combined with other physical or chemical auxiliary methods to enhance production efficiency. In addition, future studies need to further explain the mechanism of acyl migration and aim to explore more effective methods to regulate substrate hydrolysis. In conclusion, ARA-rich PtdCho has a promising production application as a more competitive form of fatty acid carrier.

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Author contributions

Jingnan Lei: Methodology, Software, Formal analysis, Data curation, Writing-original draft; Jiajia Gong: Software, Validation, Writing-review & editing; Yuan He and Shuang Zhu: Methodology, Software, Data curation; Chin-Pin Tan: Writing-review & editing; Yuanfa Liu and Yong-Jiang Xu: Conceptualization, Project administration, Funding acquisition, Validation, Writing-review & editing. All authors contributed to the final draft of the manuscript.

Conflict of interest

The authors declare no competing financial interest.

References

[1] Tallima H, El Ridi R. Arachidonic acid: Physiological roles and potential health benefits-A review. *Journal of Advanced Research*. 2018; 11: 33-41. Available from: doi:10.1016/j.jare.2017.11.004.

[2] Burns JL, Nakamura MT, Ma DWL. Differentiating the biological effects of linoleic acid from arachidonic acid in health and disease. *Prostaglandins Leukotrienes and Essential Fatty Acids*. 2018; 135: 1-4. Available from: doi:10.1016/j.plefa.2018.05.004.

[3] Wang B, Wu L, Chen J, Dong L, Chen C, Wen Z, et al. Metabolism pathways of arachidonic acids: mechanisms and potential therapeutic targets. *Signal Transduct Target Ther*. 2021; 6(1): 94. Available from: doi:10.1038/s41392-020-00443-w.

[4] Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. *Nature*. 2014; 510: 92-101. Available from: doi:10.1038/nature13479.

[5] Sambra V, Echeverria F, Valenzuela A, Chouinard-Watkins R, Valenzuela R. Docosahexaenoic and arachidonic acids as neuroprotective nutrients throughout the life cycle. *Nutrients*. 2021; 13(3): 986. Available from: doi:10.3390/nu13030986.

[6] Hadley KB, Ryan AS, Forsyth S, Gautier S, Jr Salem N. The essentiality of arachidonic acid in infant development. *Nutrients*. 2016; 8(4): 216. Available from: doi:10.3390/nu8040216.

[7] Darios F, Davletov B. Omega-3 and omega-6 fatty acids stimulate cell membrane expansion by acting on syntaxin 3. *Nature*. 2006; 440: 813-817. Available from: doi:10.1038/nature04598.

[8] Basak S, Mallick R, Banerjee A, Pathak S, Duttaroy AK. Maternal supply of both arachidonic and docosahexaenoic acids is required for optimal neurodevelopment. *Nutrients*. 2021; 13(6): 2061. Available from: doi:10.3390/nu13062061.

[9] Carlson SJ, O'Loughlin AA, Anez-Bustillos L, Baker MA, Andrews NA, Gunner G, et al. A diet with docosahexaenoic and arachidonic acids as the sole source of polyunsaturated fatty acids is sufficient to support visual, cognitive, motor, and social development in mice. *Frontiers in Neuroscience*. 2019; 13: 72. Available from: doi:10.3389/fnins.2019.00072.

[10] Alshweki A, Munuzuri AP, Bana AM, de Castro MJ, Andrade F, Aldamiz-Echevarria L, et al. Effects of different arachidonic acid supplementation on psychomotor development in very preterm infants; A randomized controlled trial. *Nutrition Journal*. 2015; 14: 101. Available from: doi:10.1186/s12937-015-0091-3.

[11] Hoffman DR, Boettcher JA, Diersen-Schade DA. Toward optimizing vision and cognition in term infants by dietary docosahexaenoic and arachidonic acid supplementation: A review of randomized controlled trials. *Prostaglandins Leukotrienes and Essential Fatty Acids*. 2009; 81(2-3): 151-158. Available from: doi:10.1016/j.plefa.2009.05.003.

[12] Hosono T, Mouri A, Nishitsuji K, Jung C-G, Kontani M, Tokuda H, et al. Arachidonic or docosahexaenoic acid diet prevents memory impairment in Tg2576 mice. *Journal of Alzheimer's Disease*. 2015; 48(1): 149-162. Available from: doi:10.3233/jad-150341.

[13] Sanchez-Mejia RO, Mucke L. Phospholipase A(2) and arachidonic acid in Alzheimer's disease. *Biochimica et Biophysica Acta, Molecular and Cell Biology of Lipids*. 2010; 1801(8): 784-790. Available from: doi:10.1016/j.bbalip.2010.05.013.

[14] Ji XJ, Ren LJ, Nie ZK, Huang H, Ouyang PK. Fungal arachidonic acid-rich oil: Research, development and industrialization. *Critical Reviews in Biotechnology*. 2014; 34(3): 197-214. Available from: doi:10.3109/07388551.2013.778229.

[15] Thomas MH, Pelleieux S, Vitale N, Olivier JL. Dietary arachidonic acid as a risk factor for age-associated neurodegenerative diseases: Potential mechanisms. *Biochimie*. 2016; 130: 168-177. Available from: doi:10.1016/j.biochi.2016.07.013.

[16] van der Veen JN, Kennelly JP, Wan S, Vance JE, Vance DE, Jacobs RL. The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. *Biochimica et Biophysica Acta, Biomembranes*. 2017; 1859(9): 1558-1572. Available from: doi:10.1016/j.bbamem.2017.04.006.

[17] Wiedeman AM, Barr SI, Green TJ, Xu Z, Innis SM, Kitts DD. Dietary choline intake: Current state of knowledge across the life cycle. *Nutrients*. 2018; 10(10): 1513. Available from: doi:10.3390/nu10101513.

[18] Wijendran V, Huang MC, Diau GY, Boehm G, Nathanielsz PW, Brenna JT. Efficacy of dietary arachidonic acid provided as triglyceride or phospholipid as substrates for brain arachidonic acid accretion in baboon neonates. *Pediatric Research*. 2002; 51(3): 265-272. Available from: doi:10.1203/00006450-200203000-00002.

[19] Wang X, Qin X, Li X, Zhao Z, Yang B, Wang Y. An efficient synthesis of lysophosphatidylcholine enriched with n-3 polyunsaturated fatty acids by immobilized MAS1 lipase. *Journal of Agricultural and Food Chemistry*. 2020; 68(1): 242-249. Available from: doi:10.1021/acs.jafc.9b05177.

[20] Carlson SE, Montalto MB, Ponder DL, Werkman SH, Korones SB. Lower incidence of necrotizing enterocolitis in infants fed a preterm formula with egg phospholipids. *Pediatric Research*. 1998; 44(4): 491-498. Available from: doi:10.1203/00006450-199810000-00005.
[21] Willatts P, Forsyth S, Agostoni C, Casaer P, Riva E, Boehm G. Effects of long-chain PUFA supplementation in infant formula on cognitive function in later childhood. *American Journal of Clinical Nutrition*. 2013; 98(2): 536S-542S. Available from: doi:10.3945/ajcn.112.038612.
[22] Hama S, Ogino C, Kondo A. Enzymatic synthesis and modification of structured phospholipids: recent advances in enzyme preparation and biocatalytic processes. *Applied Microbiology and Biotechnology*. 2015; 99(19): 7879-7891. Available from: doi:10.1007/s00253-015-6845-1.
[23] Li Y, Dai L, Liu D, Du W. Progress & prospect of enzyme-mediated structured phospholipids preparation. *Catalysts*. 2022; 12(7): 795. Available from: doi:10.3390/catal12070795.
[24] Akanbi TO, Adcock JL, Barrow CJ. Selective concentration of EPA and DHA using Thermomyces lanuginosus lipase is due to fatty acid selectivity and not regioselectivity. *Food Chemistry*. 2013; 138(1): 615-620. Available from: doi:10.1016/j.foodchem.2012.11.007.
[25] Wang X, Qin X, Li X, Zhao Z, Yang B, Wang Y. Insight into the modification of phosphatidylcholine with n-3 polyunsaturated aatty acids-rich ethyl esters by immobilized MAS1 lipase. *Molecules*. 2019; 24(19): 3528. Available from: doi:10.3390/molecules24193528.
[26] Hama S, Miura K, Yoshida A, Noda H, Fukuda H, Kondo A. Transesterification of phosphatidylcholine in sn-1 position through direct use of lipase-producing Rhizopus oryzae cells as whole-cell biocatalyst. *Applied Microbiology and Biotechnology*. 2011; 90(5): 1731-1738. Available from: doi:10.1007/s00253-011-3234-2.
[27] Ang X, Chen H, Xiang J-Q, Wei F, Quek SY. Preparation and functionality of lipase-catalysed structured phospholipid-A review. *Trends in Food Science & Technology*. 2019; 88: 373-383. Available from: doi:10.1016/j.tifs.2019.04.005.
[28] Kim BH, Akoh CC. Recent research trends on the enzymatic synthesis of structured lipids. *Journal of Food Science*. 2015; 80(8): C1713-1724. Available from: doi:10.1111/1750-3841.12953.
[29] Marsaoui N, Laplante S, Raies A, Naghmouchi K. Incorporation of omega-3 polyunsaturated fatty acids into soybean lecithin: Effect of amines and divalent cations on transesterification by lipases. *World Journal of Microbiology & Biotechnology*. 2013; 29(12): 2233-2238. Available from: doi:10.1007/s11274-013-1388-z.
[30] Chojnacka A, Gładkowski W. Production of structured phosphatidylcholine with high content of myristic acid by lipase-catalyzed acidolysis and interesterification. *Catalysts*. 2018; 8(7): 281. Available from: doi:10.3390/catal8070281.
[31] Ye Z, Li R, Cao C, Xu YJ, Cao P, Li Q, et al. Fatty acid profiles of typical dietary lipids after gastrointestinal digestion and absorbtion: A combination study between in-vitro and in-vivo. *Food Chemistry*. 2019; 280: 34-44. Available from: doi:10.1016/j.foodchem.2018.12.032.
[32] Zhang Y, Zheng Z, Liu C, Tan CP, Xie K, Liu Y. A comparative study between freeze-dried and spray-dried goat milk on lipid profiling and digestibility. *Food Chemistry*. 2022; 387: 132844. Available from: doi:10.1016/j.foodchem.2022.132844.
[33] Chen X, Peng X, Sun X, Pan L, Shi J, Gao Y, et al. Development and application of feature-based molecular networking for phospholipidomics analysis. *Journal of Agricultural and Food Chemistry*. 2022; 70(25): 7815-7825. Available from: doi:10.1021/acs.jafc.2c01770.
[34] Chojnacka A, Gładkowski W, Gliszczyńska A, Niezgoda N, Kiełbowicz G, Wawrzeńczyk C. Synthesis of structured phosphatidylcholine containing punicic acid by the lipase-catalyzed transesterification with pomegranate seed oil. *Catalysis Communications*. 2016; 75: 60-64. Available from: doi:10.1016/j.catcom.2015.11.019.
[35] Chojnacka A, Gladkowski W, Grudniewska A. Lipase-catalyzed transesterification of egg-yolk phophatidylcholine with concentrate of n-3 polyunsaturated fatty acids from cod liver oil. *Molecules*. 2017; 22(10): 1771. Available from: doi:10.3390/molecules22101771.
[36] Vikbjerg AF, Mu H, Xu X. Parameters affecting incorporation and by-product formation during the production of structured phospholipids by lipase-catalyzed acidolysis in solvent-free system. *Journal of Molecular Catalysis B: Enzymatic*. 2005; 36(1-6): 14-21. Available from: doi:10.1016/j.molcatb.2005.07.002.
[37] Ochoa-Flores AA, Hernandez-Becerra JA, Cavazos-Garduno A, Vernon-Carter EJ, Garcia HS. Optimization of the synthesis of structured phosphatidylcholine with medium chain fatty acid. *Journal of Oleo Science*. 2017; 66(11): 1207-1215. Available from: doi:10.5650/jos.ess17087.
[38] Baeza-Jiménez R, Noriega-Rodríguez JA, García HS, Otero C. Structured phosphatidylcholine with elevated content of conjugated linoleic acid: Optimization by response surface methodology. *European Journal of Lipid Science and Technology*. 2012; 114(11): 1261-1267. Available from: doi:10.1002/ejlt.201200038.

[39] Li X, Chen JF, Yang B, Li DM, Wang YH, Wang WF. Production of structured phosphatidylcholine with high content of DHA/EPA by immobilized phospholipase A(1)-catalyzed transesterification. *International Journal of Molecular Sciences*. 2014; 15(9): 15244-15258. Available from: doi:10.3390/ijms150915244.
[40] Xi X, Feng X, Shi N, Ma X, Lin H, Han Y. Immobilized phospholipase A1-catalyzed acidolysis of phosphatidylcholine from Antarctic krill (Euphausia superba) for docosahexaenoic acid enrichment under supercritical conditions. *Journal of Molecular Catalysis B: Enzymatic*. 2016; 126: 46-55. Available from: doi:10.1016/j.molcatb.2016.01.011.
[41] Niezgoda N, Gliszczyńska A. Lipase catalyzed acidolysis for efficient synthesis of phospholipids enriched with isomerically pure cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid. *Catalysts*. 2019; 9(12): 1012. Available from: doi:10.3390/catal9121012.
[42] Rychlicka M, Niezgoda N, Gliszczynska A. Lipase-catalyzed acidolysis of egg-yolk phosphatidylcholine with citronellic acid. New insight into synthesis of isoprenoid-phospholipids. *Molecules*. 2018; 23(2): 314. Available from: doi:10.3390/molecules23020314.
[43] Chojnacka A, Gladkowski W, Kielbowicz G, Wawrzenczyk C. Enzymatic enrichment of egg-yolk phosphatidylcholine with alpha-linolenic acid. *Biotechnology Letters*. 2009; 31(5): 705-709. Available from: doi:10.1007/s10529-009-9915-6.
[44] Rychlicka M, Gliszczyńska A. Interesterification of egg-yolk phosphatidylcholine with p-methoxycinnamic acid catalyzed by immobilized lipase b from candida antarctica. *Catalysts*. 2020; 10(10): 1181. Available from: doi:10.3390/catal10101181.
[45] Li D, Qin X, Wang W, Li Z, Yang B, Wang Y. Synthesis of DHA/EPA-rich phosphatidylcholine by immobilized phospholipase A1: effect of water addition and vacuum condition. *Bioprocess and Biosystems Engineering*. 2016; 39(8): 1305-1314. Available from: doi:10.1007/s00449-016-1609-6.
[46] Vikbjerg AF, Rusig JY, Jonsson G, Mu H, Xu X. Strategies for lipase-catalyzed production and the purification of structured phospholipids. *European Journal of Lipid Science and Technology*. 2006; 108(10): 802-811. Available from: doi:10.1002/ejlt.200600138.
[47] Laane C, Boeren S, Vos K, Veeger C. Rules for optimization of biocatalysis in organic solvents. *Biotechnology and Bioengineering*. 1987; 30(1): 81-87. Available from: doi:10.1002/bit.260300112.
[48] Verdasco-Martin CM, Corchado-Lopo C, Fernandez-Lafuente R, Otero C. Prolongation of secondary drying step of phospholipid lyophilization greatly improves acidolysis reactions catalyzed by immobilized lecitase ultra. *Enzyme and Microbial Technology*. 2020; 132: 109388. Available from: doi:10.1016/j.enzmictec.2019.109388.