ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2020, *65*(10), 955-962
DOI: 10.33224/rrch.2020.65.10.10

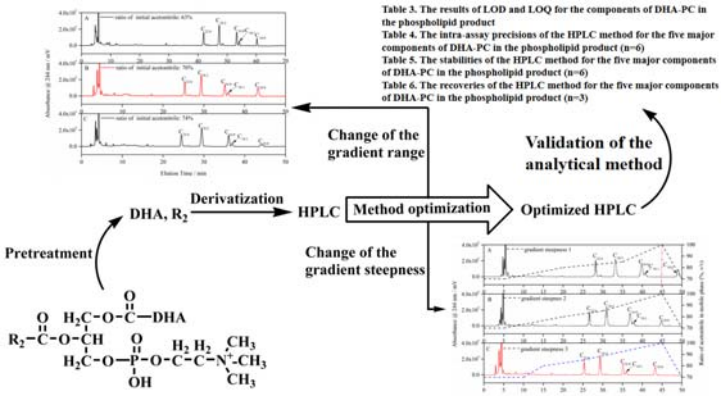
AN EFFICIENT METHOD FOR DETERMINATION OF COMPONENTS IN DOCOSAHEXAENOIC ACID-PHOSPHATIDYLCHOLINE USING PRE-COLUMN DERIVATIZATION HPLC

Li LIU,[a] Tiantian ZHANG,[b] Xiaoli ZHANG,[*b] Jiao WANG,[a] Wenfang SHU,[a] Xinyu QI,[b] Binxia ZHAO[a] and Binglin LI[*b]

[a]School of Chemical Engineering, Northwest University, 229 North Taibai Road, Xi'an, 710069, Shaanxi, China
[b]College of Food Science and Technology, Northwest University, 229 North Taibai Road, Xi'an, 710000, Shaanxi, China

Received September 21, 2020

The content of docosahexaenoic acid-phosphatidylcholine (DHA-PC) in the phospholipid product is closely related to its nutritional value and health function. An efficient high performance liquid chromatography (HPLC) was firstly proposed and successfully applied to the determination of DHA-PC. DHA-PC was pretreated by alkaline hydrolysis, acidification and then the obtained components underwent derivatization using α-bromoacetophenone as a derivatization reagent, triethylamine as a catalyst, to make them have ultraviolet (UV) absorption. Major components of DHA-PC could reach baseline separation within a short time (50 min). A gradient elution method using acetonitrile and methanol/water (1:1, volume ratio) at 0.55 mL/min, UV detection at 244 nm and a column temperature of 50 °C was found to optimally separate the components of DHA-PC. The intra-assay precisions (n = 6) and stabilities (n = 6) relative standard deviations (RSDs) for the major components were 2.5%–3.2% and 1.9%–4.0%, respectively. The average recoveries were 97.8%–106%. The results indicated that this method was rapid, accurate and reliable to analyze the content of DHA-PC in the phospholipid product. This method could be used not only to detect the content of DHA-PC in the phospholipid product, but to analyze the content of various fatty acids in mixed PC.



INTRODUCTION

Docosahexaenoic acid (DHA) is an essential omega-3 long-chain polyunsaturated fatty acid for normal brain growth and disease regulation. It can promote the maturation of photoreceptor cells to avail the development of the retina in infants and can potentiate nerve growth factor-induced neurite outgrowth to improve brain function through effectively conveying information. Therefore, it has been used as an additive in infant formula since the 1990s. [1–4] Clinical trials have shown that high DHA intake is linked to decreased risk of neurological diseases, such as Parkinson's, Alzheimer's, and depression, by alleviating cognitive deficits and protecting against synaptic degeneration.[5–8] However, it couldn't be synthesized in the body and must be ingested from outside.[9]

So far, most of the DHA supplements, such as algal oil and fish oil, couldn't be effectively

* Corresponding author: xlzhang@nwu.edu.cn or libinglin@nwu.edu.cn

absorbed by the brain, because they would be converted into free DHA by the pancreatic enzymes in vivo. The later couldn't be transported through the blood-brain barrier by the transporter (Mfsd2a). [6,10] Fortunately, cell-based studies have indicated that Mfsd2a showed the unique performance to transport DHA-phospholipids (DHA-PLs),[10] which had the same structure with the cell membrane and could smoothly cross the blood-brain barrier. Next, phospholipase A1 in the brain would hydrolyze DHA-PLs and released DHA.

Therefore, the transesterification of the main dietary sources of DHA, algal oil and fish oil, has an important commercial value. DHA-PC is prepared by transesterification of DHA and phosphatidylcholine (PC), the main component of cellular membrane phospholipids, to improve fluidity of DHA on the cell membrane. The widely used strategy is candida antarctica lipase B (CalB)-mediated transesterification. [11] The obtained DHA-PC is widely applied as an additive in infant formula and health products. Thus, the analysis of DHA-PC in the phospholipid product is critically important. Gas chromatography (GC) or thin layer chromatography coupled with GC (TLC-GC) was used as the traditional method for the analysis of DHA-PC.[12–16] It is known that the use of the capillary column coupled to a flame ionization detector (FID) provided the high resolution for the analysis, but the high column temperature limited its further application. Methylation was used to reduce the high column temperature, but the detection temperature of GC still reached 250 °C, which would have a negative effect on the accurate quantification due to the thermal sensitivity of DHA-PC.[17–20] For TLC-GC, the process of TLC was time-consuming and the connected GC also needed the high temperature for analysis. Thus, the ideal choice of detection method for DHA-PC in the phospholipid product should be measured under the mild temperature with an accurate and rapid response.

In this paper, an efficient HPLC analysis method was first proposed to measure the concentration of DHA-PC obtained by CalB-mediated transesterification. DHA-PC was synthesized by PC and algal oil containing triglyceride of DHA (DHA-TAG). After the transesterification, obtained DHA-PC was pre-treated by alkaline hydrolysis and acidification. The obtained components underwent derivatization using α-bromoacetophenone as a derivatization reagent, triethylamine as a catalyst, to make them have UV absorption. Then, the mixture would be analyzed by HPLC. The gradient elution program was employed and two important parameters (the gradient range and steepness) were systematically investigated to reduce the analysis time and improve efficiency. Compared with traditional methods, the detection temperature of HPLC was merely 50 °C, which could protect the structure of the compounds to realize the accurate detection of DHA-PC. This method could be used not only to detect the content of DHA-PC in the phospholipid product but to analyze the content of various fatty acids in mixed PC.

RESULTS AND DISCUSSION

1. Exploration of chromatographic conditions

According to the UV spectrum seized by the SPD-20A, the detection wavelength was set at 244 nm, under which the derivatives of fatty acids showed the maximum absorbance. The gradient elution method was used to improve the resolution, shorten the detection time and achieve the effective separation of the target compounds. The gradient elution program was optimized mainly by the gradient range and steepness.

1.1. Exploration of the gradient range

The mobile phases were performed with solution A (acetonitrile) and solution B (methanol/water, 1:1, volume ratio). The mixture obtained from the transesterification was complicated and had many components with similar physical and chemical properties, making separation very difficult. For the reversed-phase column, the low ratio of the organic phase in mobile phases could improve the separation effect of the target compounds but the detection time was increased at the same time. Inversely, the enhanced ratio of the organic phase could deteriorate the separation effect but the detection time was shortened. Stearic acid ($C_{18:0}$) was a component with the weakest polarity and longest retention time among the five main components of DHA-PC in the phospholipid product. Therefore, it was necessary to greatly increase the ratio of the low-polarity organic solvent to reduce the polarity of the mobile phases and, then, stearic acid ($C_{18:0}$) would be eluted sufficiently. In addition, the increased ratio of the organic phase could also effectively reduce the detection time. Therefore, with time extending, the ratio of acetonitrile needed to be increased to 100% gradually in this method. The retention factor indicated the ratio of the total amount of solute molecules in the stationary phase and the total solute molecules in the mobile phase. It was an important parameter in HPLC and should be in the range of 0.5 to 20. The

retention factor of each major component with a different initial ratio of acetonitrile met the requirement, as all of them were between 9-13.

The main components of DHA-PC were separated under different gradient ranges, as shown in Figure 2. When the initial acetonitrile ratio was 63%, the separation of each component was good and the first peak was observed at about 40min, as shown in Figure 2A. The detection time was up to 60 min and it indicated this was a time-consuming process. It was known that the increase in the initial ratio of acetonitrile was an efficient way to shorten the detection time. As shown in Figure 2B, the first peak elution time was decreased from 40 min to 25 min and the detection time was less than 50 min when the initial acetonitrile ratio was increased to 70%. The resolution of each component was good under this condition. Then the initial ratio of acetonitrile was continued increasing to 74% to shorten the detection time. It could be shown in Figure 2C that when the initial ratio of acetonitrile was 74%, there was no significant improvement in the detection time but the width of each peak broaden, particularly for the peak of the stearic acid ($C_{18:0}$). This might be explained that the ratio of water decreased and the ratio of acetonitrile increased in the mobile phases. It inhibited the ionization of fatty acids and the Si-OH groups in reversed-phase column, leading to a significant increase in hydrophobicity. Then, the interaction force between fatty acids and Si-OH groups became much larger, causing peak tailing. Therefore, the initial ratio of acetonitrile in the mobile phases was determined as 70%. Under this condition, the main components of DHA-PC had the high resolutions and the sharp symmetrical peak shapes. Moreover, the detection time was the shortest and less than 50 min, as shown in Figure 2B.

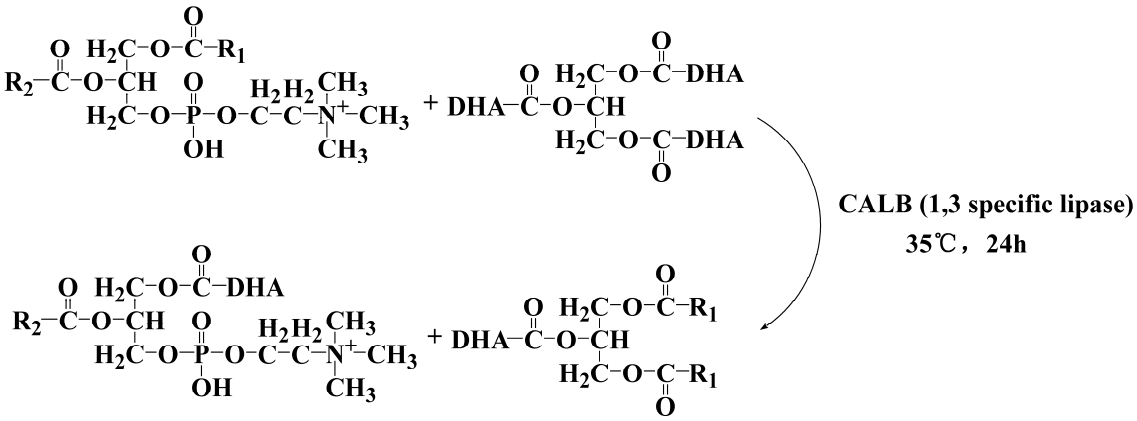


Fig. 1 – The synthesis route of DHA-PC.

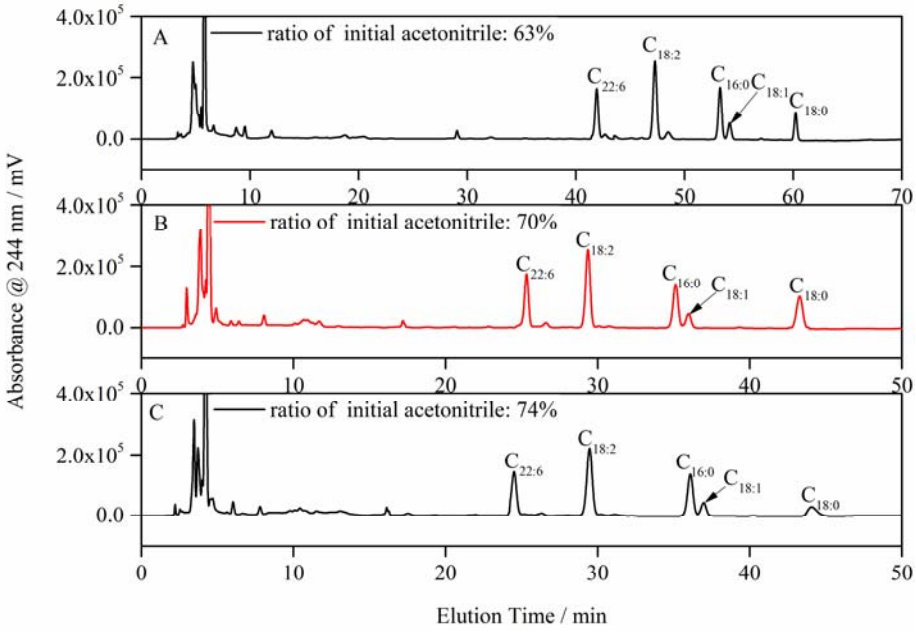


Fig. 2 – HPLC analysis of DHA-PC with different initial ratio of acetonitrile.

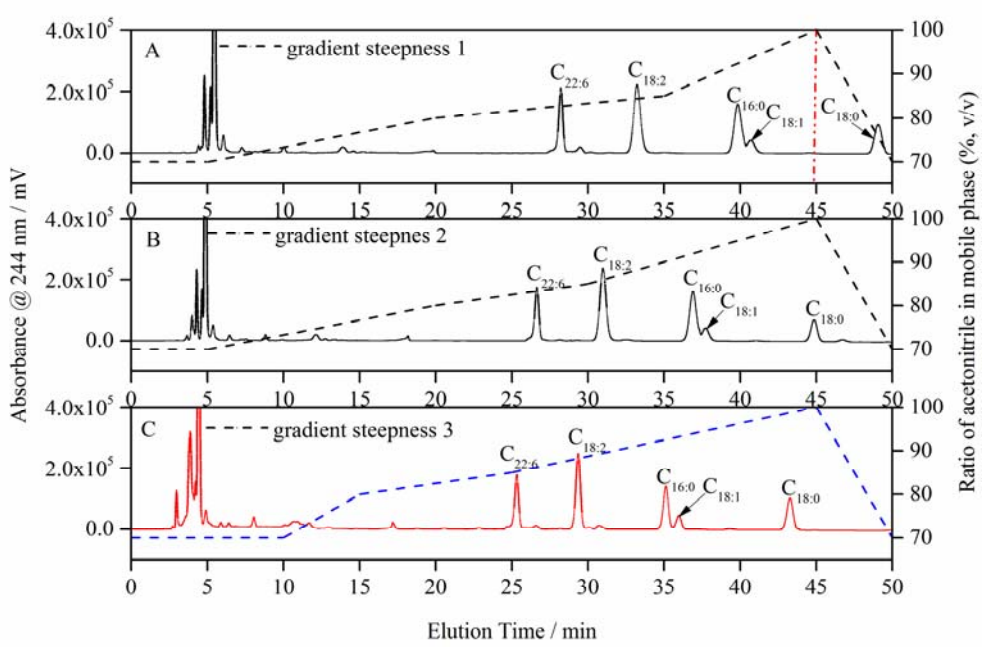


Fig. 3 – HPLC analysis of DHA-PC with different gradient steepness. Gradient elution profifile 1: 0 min = 70% A, 5 min = 70% A, 20 min = 80% A, 35 min = 85% A, 45 min = 100% A, 50 min =70% A; Gradient elution profifile 2: 0 min = 70% A, 5 min = 70% A, 20 min = 80% A, 30 min = 85% A, 45 min = 100% A, 50 min = 70% A; Gradient elution profifile 3: 0 min = 70% A, 10 min = 70% A, 15min = 80% A, 25 min = 85% A, 45 min = 100% A, and 50 min = 70% A.

1.2. Exploration of the gradient steepness

Gradient elution schemes were further optimized systematically by the linear gradient elution. The resolution was a measure by the separate effect between two adjacent peaks. The gradient steepness with a retention factor range (0.5-20) was the most convenient way to adjust the resolution. The initial ratio of acetonitrile was 70% for all different gradient steepness. As the gradient steepness increased, the detection time and the resolution were reduced at the same time. Therefore, the tradeoff between the detection time and the resolution was crucial for the selection of the suitable gradient steepness. The chromatograms of major components of PC-DHA in the phospholipid product with different gradient steepness were shown in Figure 3. It could be shown in Figure 3A that the stearic acid ($C_{18:0}$) was not eluted during the gradient program time in gradient steepness 1. As shown in Figure 3B, if the gradient steepness was increased, the peak of the stearic acid ($C_{18:0}$) would be observed before 45 min. The retention factor of each major component in gradient steepness 2 was between 11–15. It was showed in Figure 3A and Figure 3B that palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$) could not be completely separated both in gradient steepness 1 and gradient steepness 2. The gradient steepness in the location of peaks between palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$) was high, leading to insufficient elution. Therefore, it was imperative to reduce the gradient steepness to separate them drastically. All main components of DHA-PC reached baseline separation in gradient steepness 3, as shown in Figure 3C. The retention factor of each component in gradient steepness 3 was between 11–13. The gradient steepness 3 was finally determined as an optimal gradient elution program to analyze major components of DHA-PC. Meanwhile, the total each run time, including the equilibration time was 50 min.

2. HPLC analysis

The fatty acid compositions of DHA-TAG, PC and the modified PC (DHA-PC) were identified by comparing their retention times with those of fatty acid standards derivatives. The compositions of fatty acids from the reactants (DHA-TAG and PC) were presented in Table 1, together with the compositions of the product (the modified PC, DHA-PC). Compared with PC, the modified PC product (DHA-PC) contained 12.9% DHA ($C_{22:6}$), which confirmed that DHA ($C_{22:6}$) has been successfully exchanged to the PC.

Table 1

Fatty acid composition (%) from the mixture of fatty acids obtained by DHA-TAG, the original unmodified PC, and the modified PC (DHA-PC)

Fatty acids	Fatty acid from DHA-TAG	PC	
		Unmodified	Modified
Myristic acid ($C_{14:0}$)	4.6	-	-
Docosahexaenoic acid ($C_{22:6}$)	47.8	-	12.9
Linoleic acid ($C_{18:2}$)	-	55.7	30.1
Palmitic acid ($C_{16:0}$)	43.4	21.2	42.4
Oleic acid ($C_{18:1}$)	3.2	12.7	5.9
Stearic acid ($C_{18:0}$)	1.0	10.4	8.7

Table 2

The results of calibration curves, R^2 and linear ranges for the components of DHA-PC in the phospholipid product

Fatty acids	Linear equation	R^2	Linear range (mg/mL)
Docosahexaenoic acid ($C_{22:6}$)	$y = 17000400x + 319076$	0.997	0.01~1
Linoleic acid ($C_{18:2}$)	$y = 10222100x - 1158$	0.998	0.5~2.5
Palmitic acid ($C_{16:0}$)	$y = 9088570x + 27427$	0.999	0.05~1.5
Oleic acid ($C_{18:1}$)	$y = 10489000x - 19167$	0.999	0.01~1
Stearic acid ($C_{18:0}$)	$y = 9520010x + 216060$	0.997	0.05~1

(remark: *y*-the peak area of each fatty acid; *x*-the concentration of each fatty acid, mg/mL)

Table 3

The results of LOD and LOQ for the components of DHA-PC in the phospholipid product

Fatty acids	LOD (mg/mL)	LOQ (mg/mL)
Docosahexaenoic acid ($C_{22:6}$)	0.034	0.103
Linoleic acid ($C_{18:2}$)	0.118	0.358
Palmitic acid ($C_{16:0}$)	0.049	0.149
Oleic acid ($C_{18:1}$)	0.033	0.100
Stearic acid ($C_{18:0}$)	0.038	0.115

Table 4

The intra-assay precisions of the HPLC method for the five major components of DHA-PC in the phospholipid product (n=6)

Fatty acids	Actual concentration (mg/mL)	Assay result (mg/mL)	Intra-assay precision RSD (%)
Docosahexaenoic acid ($C_{22:6}$)	0.232	0.230±0.011	3.1
Linoleic acid ($C_{18:2}$)	0.542	0.543±0.022	2.6
Palmitic acid ($C_{16:0}$)	0.762	0.768±0.030	2.8
Oleic acid ($C_{18:1}$)	0.107	0.109±0.004	2.5
Stearic acid ($C_{18:0}$)	0.156	0.155±0.007	3.2

Table 5

The stabilities of the HPLC method for the five major components of DHA-PC in the phospholipid product (n=6)

Fatty acids	Actual concentration (mg/mL)	Assay result (mg/mL)	Stability RSD (%)
Docosahexaenoic acid ($C_{22:6}$)	0.232	0.233±0.014	4.0
Linoleic acid ($C_{18:2}$)	0.542	0.542±0.024	2.8
Palmitic acid ($C_{16:0}$)	0.762	0.757±0.025	2.1
Oleic acid ($C_{18:1}$)	0.107	0.106±0.005	2.5
Stearic acid ($C_{18:0}$)	0.156	0.157±0.004	1.9

Table 6

The recoveries of the HPLC method for the five major components of DHA-PC in the phospholipid product (n=3)

Fatty acids	Actual concentration (mg/mL)	Added mixed fatty acid concentration (mg/mL)	Assay result of total concentration (mg/mL)	Average recovery (%)
Docosahexaenoic acid ($C_{22:6}$)	0.232	0.125	0.365±0.009	106.0
Linoleic acid ($C_{18:2}$)	0.542	0.625	1.163±0.006	99.4
Palmitic acid ($C_{16:0}$)	0.762	1.000	1.763±0.021	99.6
Oleic acid ($C_{18:1}$)	0.107	0.200	0.303±0.001	98.3
Stearic acid ($C_{18:0}$)	0.156	0.200	0.351±0.002	97.8

3. The calibration curves of fatty acids

Linear regression analysis was used to calculate the slope, intercept, R^2 of each calibration curve. The results of calibration curves, R^2 and linear ranges were as shown in Table 2. The values of R^2 of all calibration curves were greater than 0.99, which met the quantitative requirements. The results of LOD and LOQ were shown in Table 3.

4. Determination of the components of DHA-PC

The concentrations of the components contained in DHA-PC could be calculated from the linear equations. The concentration of DHA ($C_{22:6}$) was 0.232 mg/mL. The concentration of linoleic acid ($C_{18:2}$) was 0.542 mg/mL. The concentration of palmitic acid ($C_{16:0}$) was 0.762 mg/mL, oleic acid ($C_{18:1}$) was 0.107 mg/mL and stearic acid ($C_{18:0}$) was 0.156 mg/mL and the incorporation of DHA into PC was 12.9%.

5. Validation of the analytical method

The intra-assay precisions in the measurement of compositions of fatty acid chains of DHA-PC were shown in Table 4. The intra-assay precision of each component was expressed as RSD in the range of 2.5% to 3.2% so this method has reasonably high precision. The stability of each component quantitation using this HPLC method was between 1.9% and 4.0%, as shown in Table 5. It indicated that the main components were stable within 24 h at room temperature, and the concentration of each main component could be accurately determined within 24 h. Average recoveries were shown in Table 6. The values of average recoveries ranged between 97.8% and 106%. All tested data showed this method complied with the relevant international regulations [21] and it indicated this method was precise and reliable.

EXPERIMENTAL

1. Materials

Phosphatidylcholine (PC, purity ≥ 99%) was purchased from Sigma-Aldrich Co.(St.Louis,MO,USA). Algal oil containing triglyceride of DHA (DHA-TAG, content > 53%) was purchased from Shandong Yuexiang Biological Co. Ltd. Candida antarctica lipase B (5000 LU/g) was a gift from Novozymes Biotechnology Co.Ltd.(China).

Myristic acid ($C_{14:0}$, purity ≥ 99%) was purchased from Tianjin Ruijinte Chemical Co.Ltd. Docosahexaenoic acid ($C_{22:6}$, purity ≥ 99%) was purchased from Nu-Chek Prep,Inc.USA. Linoleic acid ($C_{18:2}$, purity ≥ 99%) and oleic acid ($C_{18:1}$, purity ≥ 99%) were purchased from Sinopharm Chemical Reagent Co.Ltd. Palmitic acid ($C_{16:0}$, purity ≥ 99%) was purchased from Tianjin Ruijinte Chemical Co.Ltd. Stearic acid ($C_{18:0}$, analytical grade) was purchased from Tianjin Tianda Purification Material Fine Chemical Plant.

2. CalB-mediated transesterification to produce DHA-PC

Transesterification was carried out in a round-bottom flask (50 mL capacity) containing 300 mg of DHA-TAG, 100 mg of PC, 1 mL of CALB, and 5 mL of n-hexane. The mixture was incubated for 24 h at 35 ^{o}C under 250 rpm in a water bath. The synthesis route of DHA-PC was illustrated in Figure 1.

After the reaction, anhydrous sodium sulfate was added to remove the trace amounts of water, and chloroform was added to wash the mixture. Then anhydrous sodium sulfate was removed by filtration and the organic solvent was removed by decompression-evaporation. The obtained material was precipitated several times with acetone. White precipitates were collected by centrifugation (8000 rpm, 4 ^{o}C) and dried by the nitrogen purge.

3. Alkaline hydrolysis and acidification

Alkaline hydrolysis reaction was carried out in a microreactor (40 mL) containing 10 mg of sample (DHA-TAG or PC or DHA-PC), 4 mL of ethyl alcohol and 1 mL of 10% (w/v) ascorbic acid solution. The mixture was mixed in an ultrasonic bath. Then, 1 mL of 30% (w/v) KOH aqueous solution was added to react at room temperature for 50 min. After the reaction, the mixture was extracted three times with n-hexane. The inorganic phase was acidified by 1 M HCl and the value of pH was adjusted to 3.5. The mixture was extracted three times with n-hexane. The n-hexane phase was collected, pooled, dried by the nitrogen purge and stored at 4 ^{o}C.

4. Preparation of fatty acids reagents

4.1. Preparation of mono-fatty acid stock solutions

Individual fatty acids were diluted with n-hexane, respectively. The concentration of myristic acid ($C_{14:0}$) stock solution was 1 mg/mL. The concentration of DHA ($C_{22:6}$) stock solution was 1 mg/mL. The concentration of linoleic acid ($C_{18:2}$) stock solution was 5 mg/mL. The concentration of palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$) and stearic acid ($C_{18:0}$) stock solution were all 2 mg/mL. All of the mono-fatty acid stock solutions were shaken well and stored at 4 ^{o}C.

4.2. Preparation of standard solutions for calibration

The mixed fatty acid standards used for the calculation of average recoveries was prepared by the mono-fatty acid stock solutions including 1.25 mL of DHA ($C_{22:6}$), 1.25 mL of linoleic acid ($C_{18:2}$), 5 mL of palmitic acid ($C_{16:0}$), 1 mL of oleic acid ($C_{18:1}$) and 1 mL of stearic acid ($C_{18:0}$). Then all of them were transferred into a volumetric flask (10 mL) and brought to volume with n-hexane to make the mixed fatty acid standards. Then it was shaken well and stored at 4 ^{o}C.

Various concentrations of fatty acid standards were used to calculate the corresponding calibration curves. All fatty acid solutions were derived from mono-fatty acid stock solutions and diluted with n-hexane, respectively. The different concentrations of DHA ($C_{22:6}$), palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$), stearic acid ($C_{18:0}$) standards were as follows: 0.001 mg/mL, 0.01mg/mL, 0.1 mg/mL, 0.5 mg/mL and 1 mg/mL. Similarly, the concentrations of linoleic acid ($C_{18:2}$) standards were as follows: 0.5 mg/mL, 1 mg/mL, 1.5 mg/mL, 2 mg/mL and 2.5 mg/mL. All solutions were stored at 4 ^{o}C.

5. Derivatization

The fatty acids (mono-fatty acid stock solutions, mixed fatty acid standard, and the fatty acids obtained from DHA-TAG or PC or DHA-PC) after dried by the nitrogen purge were dissolved in 300 μL of acetone. Then 10 g/L of 2-bromoacetophenone in acetone (50 μL) and 10 g/L of triethylamine in acetone (60 μL) were added. The mixture was shaken for 1 min and reacted at 95 ^{o}C for 15 min, cooled, then 2 g/L acetic acid in acetone (75 μL) was added. The mixture was shaken for 1 min and incubated for 5 min at 95 ^{o}C. Then it was cooled, dried by the nitrogen purge and dissolved in HPLC grade methanol (0.5 mL). The solution was passed through the 0.22 μm filter membrane and 10 μL was injected over the column. The incorporation of DHA into PC was calculated by following formula (1):

$$Incorporation\ of\ DHA\ into\ PC\ (\%) = \frac{the\ incorporation\ of\ DHA\ into\ PC}{the\ incorporation\ of\ total\ fatty\ acid\ in\ PC} \times 100\% \quad (1)$$

6. HPLC apparatus and conditions

The derivatized fatty acids had chromophoric groups, which could be detected by UV detectors. UV detectors covered the wavelength range of 90 nm-400 nm, which could meet the requirement of the determination of fatty acids. Furthermore, they were the cheapest and the most widely used ones among all the detectors. The use of UV detectors was conducive to the promotion of the method proposed in this paper. The equipment used was composed of a UV-visible recording spectrophotometer (Model SPD-20A) and HPLC system from Shimadzu. The HPLC system consisted of two solvent delivery pumps were divided into A pump and B pump (Model LC-20AT), degassers (Model DGU-20A3), a manual injector (Model 7725i) and a column heater (Model CTO-20A). The reversed phase analytical HPLC column was an Inert Sustain C_{18} (150 mm×4.6 mm, 5 μm). Data was handled with LC Solution (Model Version.1.2) from Shimadzu (Japan). Sample (10 μL) was injected into the HPLC system.

The detection wavelength of a UV-visible recording spectrophotometer was 244 nm. The column temperature was maintained at 50 ^{o}C. The flow rate of mobile phases was 0.55 mL/min. The mobile phases consisting of solution A (acetonitrile) and solution B (methanol/water, 1:1, volume ratio), that formed the following linear gradient: 0 min = 70% A, 10 min = 70% A, 15min = 80% A, 25 min = 85% A, 45 min = 100% A, and 50 min = 70% A. The total each run time including the equilibration time was 50 min. The data were all obtained under this condition unless special noted.

7. Validation of the analytical method

7.1. Determination of calibration curves, the detection limits and quantification limits

The calibration curves of five major components were developed by plotting the peak areas against the concentration of the standards, respectively. Linear regression analysis was used to calculate the slope, intercept and correlation coefficient (R^2) for each calibration curve. The limit of detection (LOD) and limit of quantification (LOQ) were estimated at a signal-to-noise ratio (S/N) of 3.3:1 and 10:1, respectively. The S/N was calculated by dividing the "standard error (STEYX)" by the "slope".

7.2. The intra-assay precision of the method

The intra-assay precision of the method was estimated from processed DHA-PC (10 mg) in the phospholipid product by the mentioned methods (Alkaline hydrolysis and acidification, Derivatization, HPLC apparatus and conditions). This process was performed for six times. The concentration of each fatty acid was calculated from the calibration curve equation and, then, the relative standard deviation (RSD) was calculated.

7.3. The stability of the method

The stability of the method was assessed by processed DHA-PC (10 mg) in the phospholipid product by the mentioned methods (Alkaline hydrolysis and acidification, Derivatization, HPLC apparatus and conditions). The processed sample was placed for a certain time period (0, 3, 6, 9, 12, and 24 hours) at room temperature to analyze. The concentration of each fatty acid was calculated from the calibration curve equation and, then, the RSD was calculated.

7.4. The recovery of the method

DHA-PC (10 mg) in the phospholipid product was processed by the mentioned methods (Alkaline hydrolysis and acidification). Then 100 μL of mixed fatty acid standard (obtained by Preparation of standard solutions for calibration, the concentration of each fatty acid was as follows: the concentration of DHA ($C_{22:6}$) was 0.125 mg/mL, the concentration of linoleic acid ($C_{18:2}$) was 0.625 mg/mL, the concentration of palmitic acid ($C_{16:0}$) was 1.000 mg/mL, the

concentration of oleic acid ($C_{18:1}$) was 0.200 mg/mL and the concentration of stearic acid ($C_{18:0}$) was 0.200 mg/mL) was added, mixed thoroughly and dried by the nitrogen purge. The mixture was processed by the mentioned methods (Derivatization, HPLC apparatus and conditions). This process was performed for three times to obtain the average recoveries of the method.

CONCLUSIONS

An efficient HPLC method was proposed for the analysis of DHA-PC during CalB-mediated transesterification. Five major fatty acid chains of DHA-PC could be completely separated within a short time (50 min). Meanwhile, this method was also validated to prove its accuracy. Thus, this method could be used not only to detect the content of DHA-PC in the phospholipid product, but to analyze the content of various fatty acids in mixed PC.

Acknowledgements. This work was supported by Key Research and Invention Program in Shaanxi Province of China (Program No. 2020NY-127 and 2018NY-131).

REFERENCES

1. M. Singh, *Indian J. Pediatr.*, **2005**, *72*, 239-242.
2. W. Min, J. Xu, L. Ding, L.Y. Zhang, L. Du, J. F. Wang, Y. M. Wang and C. H. Xue, *J. Funct. Foods*., **2016**, *24*, 537-548.
3. F. Takenaga, S. Itoh and H. Tsuyuki, Food. Preserv. *Sci*., **1998**, *24*, 173-181.
4. A. H. Lloyd and K. Y. Young, *Pharmacol. Res.*, **1999**, *40*, 211-225.
5. D. D. Scott, K. Sriram and B. S. Carani, *Curr. Diabetes. Rev*., **2007**, *3*, 198-203.
6. D. Sugasini, R. Thomas, P. C. R. Yalagala, L. M. Tai and P. V. Subbaiah, *Sci. Rep*., **2017**, *7*, 11263.
7. H. X. Che, M. M. Zhou, T. T. Zhang, L. Y. Zhang, L. Ding, T. Yanagita, J. Xu, C. H. Xue and Y. M. Wang, *Food. Funct*., **2017**, *9*, 1.
8. R. K. Mcnamara, R. Jandacek, P. Tso, Y. Dwived, X. G. Ren and G. N. J. Pandey, *Psychiatr. Res*., **2013**, *47*, 1187-1191.
9. K. Green, H. Martinez-Coria, H. Eileen, K. Yurko-Mauro, L. Ellis and F. LaFerla, *Alzheimers. Dement*., **2007**, *3*, S159.
10. L. N. Nguyen, D. L. Ma, G. H. Shui, P. Y. Wong, A. Cazenave-Gassiot, X. D. Zhang, M. R. Wenk, E. L. K. Goh and D. L. Silver, *Nature*, **2014**, *509*, 503-506.
11. C. D. James, K. Z. Ma, L. Chang, J. M. Bell and S. I. Rapoport, *J. Neurochem*., **2005**, *94*, 1063-1076.
12. A. Mustranta, T. Suortti and K. Poutanen, *J. Am. Oil. Chem. Soc*., **1994**, *71*, 1415-1419.
13. W. X. Chen, W. Guo, F. Gao, L. M. Chen, S. L. Chen and D. M. Li, *Appl. Biochem. Biotech.*, **2017**, *182*, 1037-1052.
14. X. Li, J. F. Chen, B. Yang, D. M. Li, Y. H. Wang and W. F. Wang, *Int. J. Mol. Sci*., **2014**, *15*, 15244-58.
15. A. P. Kitson, A. H. Metherel, C. T. Chen, A. F. Domenichiello, M. O. Trépanier, A. Berger and R. P. J. Bazinet, *Nutr. Biochem*., **2016**, *33*, 91-102.
16. D. M. Li, X. L. Qin, W. F. Wang, Z. G. Li, B. Yang and Y. H. Wang, *Bioproc. Biosyst. Eng*., **2016**, *39*, 1305-1314.
17. M. Nabil, N. Karim, B. John, R. Aly and L. Serge, *Appl. Biochem. Biotech*., **2015**, *176*, 938-946.
18. R. W. Browne and A. Donald, *Clin. Chem*., **2000**, *46*, 6.
19. L. Bacchetta, M. Aramini, A. Zini, V. D. Giammatteo, D. Spera, P. Drogoudi, M. Rovira, A. P. Silva, A. Solar and R. Botta, *Euphytica*., **2013**, *191*, 57-73.
20. M. J. Špika, M. Žanetić, K. Kraljić, B. Soldo, I. Ljubenkov, O. Politeo and D. J. Škevin, *J. Food. Sci.,* **2019**, *84*, 877-885.
21. V. P. Shah, K. K. Midha, J. W. A. Findlay, H. M. Hill, J. D. Hulse, I. J. McGilveray, G. McKay, K. J. McGilveray, R. N. Patnaik, M. L. Powell, A. Tonelli, C. T. Viswanathan and A. Yacobi, *Pharm. Res.*, **2001**, *17*, 1551-1557.