Development of a thin-layer chromatography based method for structural analysis of phosphatidylcholine (PC)

A THESIS

SUBMITTED TO THE FACULTY OF THE

UNIVERSITY OF MINNESOTA

BY

Guiwei Tan

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

Roger Ruan, Ph.D.

09/2015

Acknowledgments

I would like to thank my advisor, Dr. Roger Ruan, for his support and guidance throughout these two years. Without his support and trust, I cannot finish this project. I learnt a lot from him, including leadership, confidence, communication abilities, presentation skills, and so on. I feel so lucky to be his student since August of 2013 and am thankful for all of these I learnt from him. And I would like to thank Dr. Paul Chen, for his invaluable help and suggestions throughout my graduate studies. It has been a privilege to learn from his scientific rigor and professionalism. I do appreciate all of these! I also thank Dr. Tonya Schoenfuss. I learnt a lot from her FScN 5131 food quality and FScN 5441 introduction to new product development classes. Her knowledgeable, accessible and caring characteristics deeply depressed and inspired me during my studying.

The research described in this thesis relies heavily on the support from Dr. Yinggang Tian. He shows me the beauties of science and logic, teaches me lots of research methodologies and opens the scientific door for me! And I am thankful to Dr. Devin Peterson. From his food chemistry class, food related knowledge could logically connect with each other like stars to form a beautiful sky. “Science based on logic not emotion”, I could not agree more with his words.

I have been fortunate to work with many brilliant and helpful people in Roger’s lab. Specifically I am grateful to Dr. Yanling Cheng, Dr. Min Min, Qinglong Xie, Bo Zhang, Shiyu Liu, Dr. Yen Doan, Qian Lu, Xindi Zhu, Dr. Hongli Zheng and Joy for their generous help and insightful discussions in the lab.

Finally I thank my parents, Min Gui and Xianwei Tan, for their unconditional and unwavering love and support during my life!

Dedications

This work is dedicated to God and my family and to my studying memories in University of Minnesota during 2013-2015, as well as the people involved in this period. Thank you all so much!

Abstract

Structural analysis of phosphatidylcholine (PC) plays an important role in omega-3 fatty acids enriched products assessment, developing structured omega-3 fatty acids–containing PC, metabolic processes studies and diseases diagnosis.

In this thesis, a new positional analysis of PC is described. Its methodology is based on a smart connection between two-dimensional chromatography and *in-situ* enzymatic hydrolysis.

Most characterizations of PC composition happened on a thin layer chromatography (TLC) plate. Firstly, PC was isolated from total lipids by TLC and its purity was confirmed by high performance liquid chromatography (HPLC). Without further extraction from the TLC plate, PC was hydrolyzed by phospholipases on the plate directly. The key step of the *in-situ* enzymatic reaction was to promote interactions between PC and phospholipases, because this *in-situ* enzymatic hydrolysis occurred on a silica gel matrix. These increases can be accomplished via adding a wetting agent consisting of chloroform/ methanol/ water (65:24:4, v/v/v) onto the reaction area. Free fatty acids （FFAs） released from different positions of PC were then isolated from products mixture by second-dimensional chromatography and were chemically transesterified into fatty acids methyl ethers (FAME).

With help of gas chromatography- flame ionization detector (GC-FID), the presented method could reveal relative percentage of each fatty acid on sn-1 and sn-2 positions of PC with 91.59% and 84.80% accuracies, respectively.

Therefore, separation of PC from total lipids, enzymatic conversion of PC to FFAs and lysophosphatidylcholine (LPC), and separation of FFAs from products mixture can be performed on one TLC plate. This will remove the need to extract the separated PC from the TLC plate for the enzymatic reaction, avoid the risk of losing materials during the extraction processes; saving time, labor and cost.

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List of Abbreviations

ALA	Alpha-linolenic acid
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ethers
FFAs	Free fatty acids
GC-FID	Gas chromatography- flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
LPC	Lysophosphatidylcholine
NMR	Nuclear magnetic resonance
PC	Phosphatidylcholine
PE	Phosphatidylethanolamines
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
RF	Retention factor
TAG	Triacylglycerol
TLC	Thin layer chromatography

Introduction

The importance of structural analysis of phosphatidylcholine (PC)

Lipids are usually referred to hydrophobic compounds that are soluble in non-polar organic solvents, such as hexane. According to Christie[1], lipids are fatty acids, their derivatives and biosynthetically or functionally related compounds. These compounds can be roughly divided into three classes: (A) simple lipids like triglyceride; (B) compound lipids, such as phospholipids; (C) derived lipids, like free fatty acids.

Phospholipid is a major component of cell membranes. The one containing glycerol is glycerophospholipid, which generally has a hydrophilic head and hydrophobic tails.

Based on the differences in the hydrophilic heads, glycerophospholipid can be classified into several classes, as shown in Figure 1. The one with choline as polar head group is phosphatidylcholine (PC), commonly termed "lecithin". In the membranes of both animal tissues and plants, PC accounts for the most part of their major lipids[2]. PC has been widely used in the food industry, acting as natural emulsifier, stabilizer, wetting agent, and baking improver[3]. Within the past decades, PC has attracted wide attention because it offers great benefits for human brain. As a choline enrichment carrier, PC, as shown in Figure 2, can be used by our body to make acetylcholine, an important brain chemical, thereby preventing some brain diseases such as memory loss, manic-depressive disorders, anxiety and Alzheimer's disease. Moreover, PC is also employed for treating hepatitis, eczema, ulcerative colitis, high cholesterol, premenstrual syndrome, kidney

dialysis, and circulation problems. PC can from a variety of sources, including soybeans, egg yolks, sunflowers, livers, whole grains and legumes.

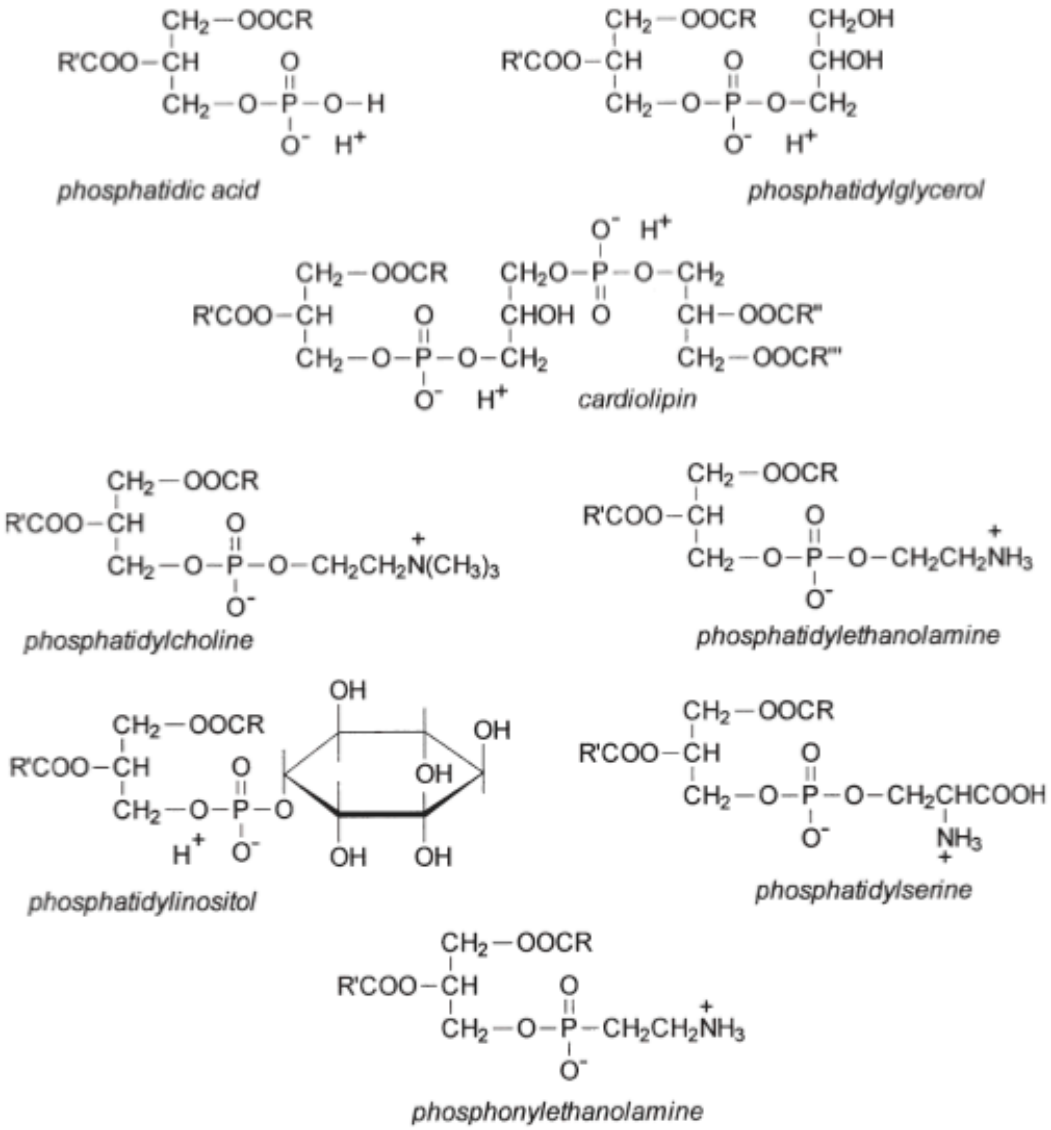


Figure 1 Main glycerophospholipids structures[2]

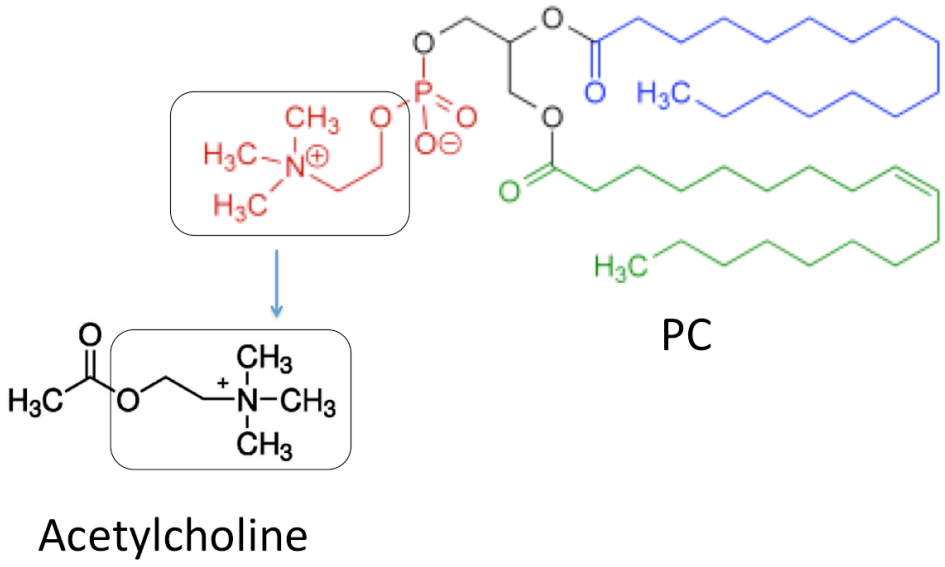


Figure 2 Relationship between PC and acetylcholine[6,7]

Hydrophobic tails of glycerophospholipid are made up with fatty acids. The fatty acids derived from plant, animal and microbiology commonly contain even numbers of carbon atoms with double bond(s) in specific positions[2]. In 1929, George Burr and Violet from the University of Minnesota Medical School first discovered omega-3 fatty acids, in which the double bonds are started from the third carbon atom from the omega end[5]. The fatty acids are very essential to normal metabolism, and have been known for several decades about their health benefits on cardiovascular disease, hypertension, as well as inflammation and thus were termed "essential fatty acids" [10, 11, 12, 13, 14, 15, 16].

Omega-3 fatty acids are mainly composed of alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as shown in Figure 3. Its concentrates are often used as food supplements[20]. Because the only way to obtain omega-3 fatty acids, for human beings, is from diet as mammals lack the ability to synthesize omega-3 fatty acids[8].

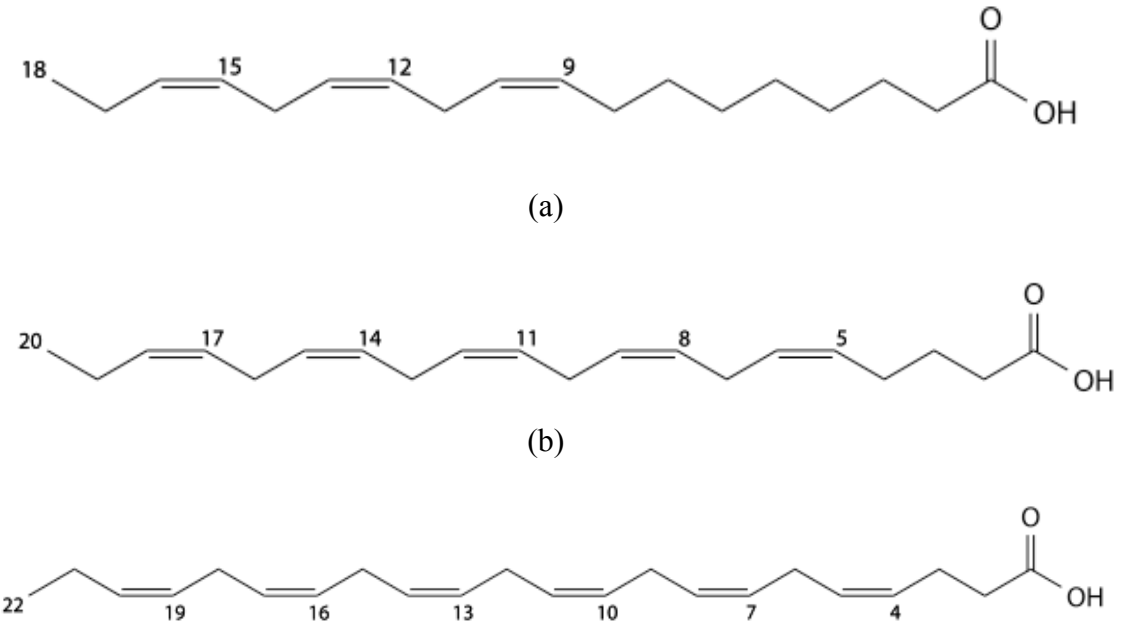


Figure 3 Main omega-3 fatty acids structures[9]

(a) alpha-linolenic acid (ALA); (b) eicosapentaenoic acid (EPA); (c) docosahexaenoic acid (DHA)

Most omega-3 fatty acids are in the forms of triacylglycerol (TAG) and phospholipids. The difference in chemical forms affects the digestion, absorption and distribution of omega-3 fatty acids[21]. A number of animal and clinical studies have revealed that omega-3 fatty acid showed better biological activities when it was esterified in a phospholipid than its carrier is a TAG [21, 22, 23, 24]. Furthermore, according to a report of Su Chen and Papasani, DHA attached to different positions of phospholipids presents varying bioavailability[25]. To our knowledge, although considerable research has been devoted to producing foods with high omega-3 fatty acids level, such as DHA enriched eggs that can be produced by feeding laying hens with flaxseed[26], rather limited attention

has been paid to omega-3 fatty acids bioavailability of these products. Therefore, besides omega-3 fatty acids composition, the detail of positional distribution of omega-3 fatty acids on the glycerol backbone of phospholipids is needed for a better assessment of omega-3 fatty acids enriched products.

Currently, marine oils, such as fish oils, are the main dietary source of DHA and EPA[20]. However it should be pointed out that the marine sources are declining and may fail to meet future nutritional and pharmaceutical requirements[20]. Moreover, most omega-3 fatty acids in fish oils are in the form of TAG with low bioavailability. On the other hand, within the human body, ALA obtained from plant oils can be transformed to DHA and EPA, for three of them sharing a similar structure; however, such natural transformation is limited[20]. Improving their bioavailability and thus the utilization efficiency in human body is a feasible solution to the shortage of DHA and EPA. In order to achieve this, much attention has been directed to the development of structured lipids. One plausible approach is to introduce omega-3 fatty acids into a specific position in the glycerol structure of PC through chemical or enzymatic transesterification[20]. When chemically or enzymatically modifying the structure of a natural PC, the understanding of the positional information of the PC is important[27].

What is more, it has long been known that lipids function as main energy reserve and play an important role in membrane reorganization, signal delivery and so on[2,17,18]. And altered lipid compositions, in tissues and/or body fluids such as blood, have been found closely related to some diseases, such as atherosclerosis, and thus some changed lipid patterns can be viewed as pathological conditions marker[4,19]. Consequently, structural analysis of specific lipids such as phospholipids is needed for some metabolic

processes studies and diseases diagnosis and has aroused great interest in diagnosis and pharmacology areas [4].

Therefore, structural or positional analysis of PC plays an important role in the synthesis of omega-3 fatty acids and PC conjugates as well as the studies of metabolic processes and diseases diagnosis.

Previous research on PC structural analysis

As mentioned above, there has been growing interest in the analysis of fatty acids compositions on specific positions of PC. However, none of the simple chemical methods could discriminate three ester bonds on a glycerol molecule of PC as they react very similar in chemical reactivity[2]. Fortunately, it was reported that some phospholipases and lipases could be employed to achieve this discrimination thanks to the region-selectivity of these enzymes.

Phospholipases can be found in many animal tissues, but for the structural studies, the main sources are snake venoms, bee venoms and mammalian pancreas[28]. Phospholipase A_2 (PLA_2) hydrolyzes the ester bond in sn-2 ester linkage of PC specifically, in which calcium ions are essential for its reaction[2]. On the other hand, phospholipase A_1 (PLA_1) targets on fatty acids coming from sn-1 position of PC[29]. Some sn-1,3 specific lipases, such as the one from Mucormiehei (Lipozyme), were also used for selective removal of fatty acids in the sn-1 position of PC[27].

With help of one of these enzymes, PC can be hydrolyzed into free fatty acids (FFAs) and LPC. Each of these components represents fatty acids coming from different positions of PC. FFAs and LPC are then transesterified into volatile compounds-fatty

acids methyl ethers (FAME), respectively. Detection was usually performed by means of gas chromatography mass spectrometry (GC-MS).

Here are some examples using one enzyme to positional analysis of PC. According to Amate, Ramirez and Gil, PLA_2 enzyme was used for regiospecific analysis of phospholipids, in which lipid (5 mg) was dissolved in diethyl ether (1 mL) and borate buffer (1 mL, 100 mM, pH 8.9). After sonication (15 min), the mixture was added with PLA_2 enzyme (8-200 units) and incubated under 37 ^oC for 30 min. The diethyl ether was then removed under N_2 and products were extracted with chloroform-methanol (2:1, v/v). Thin layer chromatography (TLC) single-dimension double development was employed to separate the FFAs and 1-acyl LPC with hexane- isopropanol-acetic acid (75:25:1.5, v/v/v) followed by chloroform-methanol-acetic acid-water (65:50:1:4, v/v/v/v). The bands were visualized with iodine vapors and then scraped off the plate for methylation. The obtained fatty acid methyl esters were separated and quantified by GC[30].

Palacios, Antequera and Muriel et. al. also use PLA_2 enzyme to hydrolyze PC. Lipids were separated into phospholipid classes by NH_2-aminopropyl minicolumns. Each phospholipid class was dissolved in diethyl ether (6 mL) with BHT (0.05%) and hydrolyzed with PLA_2 solution. The mixture, under 25^oC was incubated for 3h. Then the reaction products were washed with methanol-chloroform (2:1, v/v) and separated with NH_2-aminopropyl minicolumns again to get hydrolyzed free fatty acids and lysophospholipids separately. Both of these were turned into FAME by acidic transesterification and analyzed using gas chromatography- flame ionization detector (GC-FID)[31].

Lipozyme used by Grzegorz, Witold and Anna can completely hydrolyze PC[27]. In their study, PC was purified through column chromatography, and the products of this hydrolysis, including 2-Acyl-lysophosphatidylcholine (2-acyl LPC) and fatty acids released from sn-1 ester linkage of PC, were extracted with water-hexane (2:3, v/v). The water and hexane layers contained the 2-acyl LPC and the fatty acids, respectively. Each layer was treated with a chemical method to obtain fatty acid ethyl esters (FAEE) that was then analyzed with GC to uncover fatty acids composition in each position of the PC.

Some were focused on using two enzymes to hydrolyze fatty acids coming from different positions of PC. Through this way, acyl migration induced errors might be avoided.

Tetrahymena thermophile PLA_1 and bee venom PLA_2 enzymes were used by Florin, Narvaez and Florin et. al. for PC structure analysis[32]. Total lipids were extracted with Bligh and Dyer method[33] and were then separated on TLC with chloroform-methanol-water-acetic acid (65:35:4:2, v/v/v). Isolated sample was mixed with the PLA_2 (50 μg， 600-1800 U/mg), 50 mM Tris-HCl/100 mM sodium borate (pH 8.1) and diethyl ether (1.5 mL). For PLA_1 reaction, separated samples were dissolved in 0.1 M sodium acetate/0.1 M sodium borate (pH 4.75) and added with PLA_1 (10μg, 300 U/mg). For both of these enzymatic reactions, after incubation for 4h under 37 ^{o}C, lipids were extracted with Bligh and Dyer method again and isolated on TLC.

In a study by Witold, Grzegorz and Anna et. al., extracts from yolks by methanol were separated on silica gel column chromatography with chloroform-methanol-water (65:25:4, v/v/v). The isolated PC (0.05 g) dissolved in 95% ethanol (2.0 mL) was added

with Lipozyme (0.4 g) and reacted about 5 h. One of the products 2-acyl LPC was extracted by column chromatography again. PLA_2 solution was added for sn-1 positional analysis of PC. And its product 1-acyl LPC was also isolated via column chromatography. These two kinds of LPC were converted to FAME and detected using GC-FID[34].

Non-enzymatic methods for positional analysis of PC, such as nuclear magnetic resonance (NMR) spectroscopy, reversed-phase LC-ESIMS/MS, LTQ Orbitrap MS and reverse-phase liquid chromatography[27], are also available. For example, Ann-Marie, Dietlind and Patrick[29] used NMR to analysis PC. In their experimental runs, PC (45-50 mg) was dissolved in deuterated chloroform (0.6 mL). The samples were around 10% (wt./vol.) concentration and were analyzed in 5 mm NMR tubes. ^{13}C NMR spectra was performed at a frequency of 125.79 MHz and was recorded with 7200 scans, a 1-s acquisition time, a 5 s relaxation delay and a 307 pulse width. Its measurements were set at 300K. Unfortunately, these kinds of equipment are, in most laboratories, too expensive to be available, and thus would not be discussed in more detail.

Gaps in the previous research

For most of the procedures for positional analysis of PC, total lipids are first separated using column chromatography or preparative high performance liquid chromatography (HPLC) to obtain PC. Second, phospholipases and/or lipases are employed to enzymatic hydrolysis of a specific ester bond of the extracted PC. These reactions are carried out in solution within a test tube. Third, FFAs and LPC are separately extracted from residual lipids by a series of solvent systems on TLC or HPLC

or column chromatography. Fourth, FFAs and LPC are separately converted into FAMEs through transesterification reactions, and then analyzed using GC.

However, the total lipids isolation and products extraction via HPLC or column chromatography have the following disadvantages[35]. (1) HPLC and column chromatography are complex. Only experienced users can they well separate a complex mixture. (2) HPLC related equipment such as pump, sampler, column and detector is expensive, and thus might be inaccessible in many laboratories. (3) For each analysis, a huge amount of solvents is needed, which increases running cost, toxicity and is not environmental-friendly. (4) Both HPLC and column chromatography are known for being labor-intensive and time-consuming. (5) During running, chemical residual from previous runs may plug or even damage a column. Usually, results show poor consistency with gradual decreasing column efficiency. (6) Lastly, fatty acids loss may happen during multiple solvents extractions and long time enzymatic reaction may induce fatty acids migrations from sn-2 position to its sn-1 position of PC and vice versa.

Purpose of the present research

The objective of present work was to simplify the positional analysis procedures.

Research hypotheses

Compared with liquid chromatography, TLC is a simpler, cheaper and faster method for separations of some complex mixtures. For example, TLC consumes far smaller amounts of solvents than HPLC and column chromatography, thereby is less expensive regarding the required consumables and particularly more environment-friendly. Developing a TLC system costs much less than building an HPLC system.

TLC has wide applications in areas of food, agricultural and biological samples. Especially in the fats and oils industries, TLC is a typical method for separations, identifications and quantitative determinations of lipids[4]. Usually, a normal phase TLC system is used for the separation of a complex lipids mixture, in which the stationary phase is polar, such as silica gel, and the mobile phase is non-polar[4]. The quantification ability of TLC plate has been demonstrated in many areas, especially in pharmaceutical industry[35]. Furthermore, the versatility and reproducibility of TLC have been greatly improved because a great diversity of pre-coated TLC plates is now commercially available[4].

On a TLC plate, PC can be easily isolated from total lipids, and free fatty acids show a different retention factor (RF) value than other lipids. Both of these separation steps can be successfully performed on one TLC plate via second-dimensional chromatography, thereby the separations of samples of interest with HPLC and/or column chromatography can be totally replaced by TLC.

What is more, TLC is a non-destructive method. Separated substrates can be further analyzed and the analysis can be carried out at the same place on the TLC plate, which will be expressed *in-situ*.

Therefore, separation of PC from total lipids, enzymatic conversion of PC to FFAs and LPC, and separation of FFA from products mixture can be performed on one TLC plate. This approach would offer several advantages: (1) no need to extract the separated PC from the TLC plate for the enzymatic reaction, (2) avoiding the risk of losing materials during extraction processes, and (3) saving time, labor and cost.

Methods

Materials

Preparative TLC plate coated with a thin layer of fluorescent indicator (silica gel matrix; 20 × 20 cm; 2,000 μm thick layer; inorganic binder) was purchased from Sigma-Aldrich, PLA_1 enzyme solution was purchased from Sigma-Aldrich was used directly without further process. PLA_2 enzyme (5.5 mg) from honey bee venom (Apis mellifera) was also purchased from Sigma-Aldrich and dissolved in Tris-HCl buffer (0.495 mL; 1M; pH 8.0) containing calcium chloridedihydrate (0.055 mL; 22% (w/w)). Wild harvest's omega-3 enriched eggs were purchased from local market.

Total lipids extraction

In order to obtain the required samples with uniform mass distribution, 10 eggs were broken and the egg yolk were pooled, fully mixed and put into a freeze-drier for pretreatment at -70 ^{o}C for 24 h. Then egg yolk powder was prepared by vacuum freeze-drying. Total lipids were extracted from the powder according to modified Folch et. al.[36]. Egg yolk powder (3 g) was dissolved in chloroform-methanol (15mL, 2:1, v/v), fully mixed for 2 hours and centrifuged for 20 min. Then the supernatant containing lipids was filtered, combined and dried under nitrogen. This procedure was repeated four times until all the lipids were extracted. The 0.22 g extracted lipids was dissolved in hexane (1.11 mL) to produce a solution containing 19.82% (wt./vol.) lipids and was frozen at -20 ^{o}C until further analysis.

Total lipids separation on TLC

The diluted sample (60μL) was applied to an activated TLC plate (10 × 10cm). The plate was cut from commercially available TLC plate and had been activated at 105 ^{o}C for 1h. Then the TLC development was performed with a mixture of chloroform-methanol-water (65:24:4, v/v/v), which was prepared from a minor modification of Witold's method[34], and sample migration distance was 8 cm. After development, the plate was dried under nitrogen for 1 min, and individual lipid classes visualized under 254 nm UV light. PC band was identified by comparing its RF value with that of PC standard and was marked with a soft lead pencil.

PC qualitative and quantitative analysis via HPLC

The HPLC analysis was performed on a Varian ProStar system consisting of a ProStar 210 Solvent Delivery Module, an Autosampler Model 400 and a ProStar 325 UV-Vis Detector. The system was controlled and data acquisition was handled using Star Workstation Ver 6 software. PC analysis was carried out on a Waters Spherisorb® Amino (NH_2) column (80 Å, 5 μm, 4 mm × 125 mm) equipped with its corresponding pre-column. Lipids separations were conducted using a methodology described by Dietlind et. al.[37]. The operation parameters are as follows: mobile phase, 95% ethanol / 20 mM oxalic acid solution (92:8, v/v); flow rate, 0.75ml/min; temperature, ambient; injection volume, 100 μL, and the wavelength was set at 210 nm.

To verify the purity of the PC separated from total lipids and to determine hydrolysis rates of *in-situ* enzymatic reactions (defined as the peak areas of hydrolyzed PC to the peak areas of original PC on HPLC), PC bands were scraped off the TLC plate,

and extracted using a method adapted from Kristin et. al.[38]. The silica gel was added 2.2 ml of chloroform-methanol-0.9% aqueous NaCl (1:1:1, v.v.v), followed by sonication and vortex for 10 min and 3 min separately. The mixture was then centrifuged for 1 min. The lower phase was collected, while the supernatant was re-extracted as above for other four times until lipids were totally extracted that was confirmed by HPLC. All of those lower phases were pooled and dried by nitrogen stream. Subsequently, the residual was re-dissolved in 2 ml of ethanol and was injected into HPLC. Quantitation of PC was performed with external standard method.

In-situ enzymatic hydrolysis

Various amounts of PLA_1 enzyme loading were applied to the isolated PC band on the TLC plate. Meanwhile, to maintain a sufficiently moist reaction medium for *in-situ* enzymatic reaction, a wetting agent containing chloroform/ methanol/ water (65:24:4, v/v/v) was gradually dropped onto the PC band. After hydrolysis, TLC plate was dried under nitrogen for 5 min. FFAs were eluted from products mixtures by a second-dimensional chromatography and chloroform-acetone-acetic acids (75:25:5, v/v/v) served as its mobile phase. PLA_2 enzyme was then applied to the PC with same hydrolysis conditions of PLA_1 enzyme.

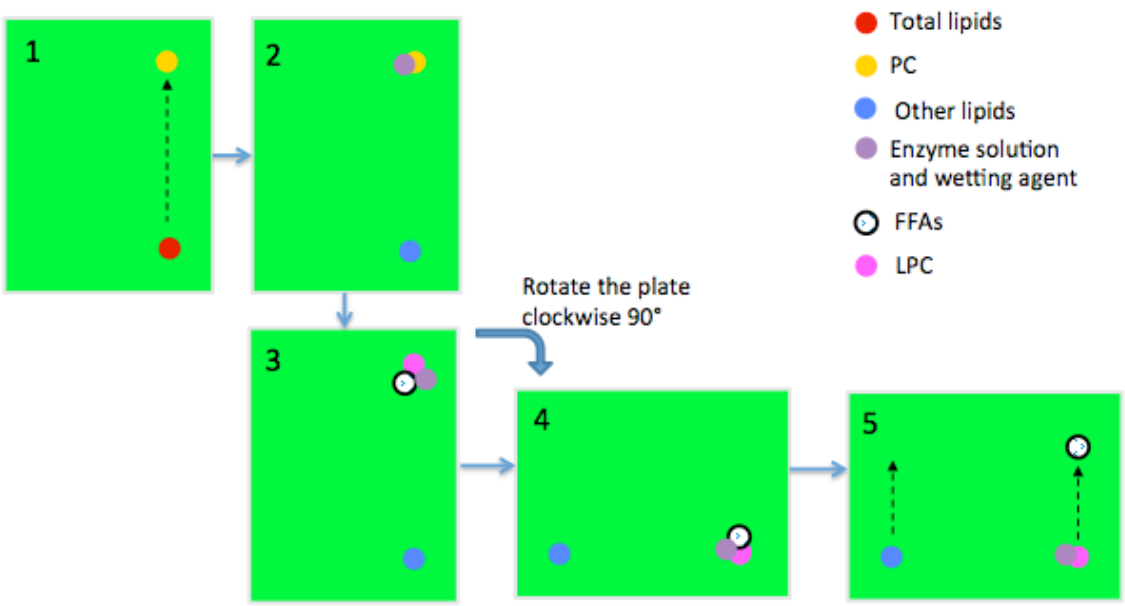


Figure 4 Flow diagram of the new structural analysis of PC on TLC plate

Determination of fatty acids composition on GC-FID

For the GC system, an Agilent 7820A GC equipped with a FID detector and an Agilent capillary column (DB-FFAP, 0.25 μm, 30m × 0.25mm) was used. Fatty acids analysis as follows: injection volume was 1 μL at a split ratio of 10:1. Injection pressure was set at 11.164 psi and the heater temperature was set at 250°C. Carrier gas was nitrogen at a flow rate of 0.6 mL/min. The column/oven temperature started at 140 °C with a 5 min hold, then raised to 240 °C at a rate of 4 °C /min and held at 240 °C for 10 min. The total run time was 40 min.

Separated PC and FFAs were transesterified into fatty acid methyl esters (FAME) as follows: silica gel containing PC or FFAs was scraped off the TLC plate and fully mixed with 6 ml methanol, 0.5g butylatedhydroxytoluene (BHT) as antioxidant and 5mL BF3-Methanol reagent as catalyst. Subsequently, the whole mixture was put in 100 °C water bath for 10 min. Then 10 ml saturated sodium chloride solution and 3 ml heptane

were added and fully mixed with FAME for extraction purpose, which was repeated for 3 times. Organic layers were collected and dried under nitrogen. Lastly, the residue was re-dissolved in 1.5 ml heptane and analyzed by GC-FID. Fatty acids identified by comparing their retention time with that of FAME standards.

Results and discussion

Total lipids extraction

The average weight of Wild harvest's omega-3 enriched eggs, was 62.99 g with an average egg yolk wet weight and dry weight at 18.32 g and 8.60 g, respectively. The lipids extraction results are presented in Figure 5. From Figure 5, we can see that the total lipid increased significantly with increasing extraction times. After 4 extractions, 0.22 g total lipids were obtained from 0.30 g dried egg yolk powder (equals to 0.64 g fresh egg yolk). On this condition, the total lipids yield (defined as the mass ratio of total lipids to fresh egg yolks) is 34.38%. As reported, lipids usually account for 27.45~37.14 wt.% of fresh egg yolks[43,44]. That means almost all the total lipids were extracted after 3 or 4 extractions in our study.

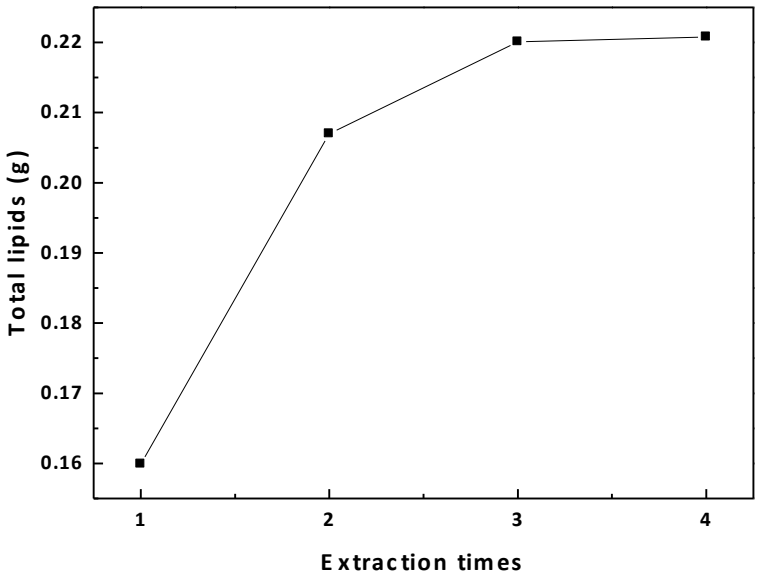


Figure 5 Total lipids extracted from egg yolks

Total lipids separation on TLC

From Figure 6, total lipids were well separated using TLC plate. PC spot was identified through comparing its retention time with that of PC standard and its purity was verified via HPLC.

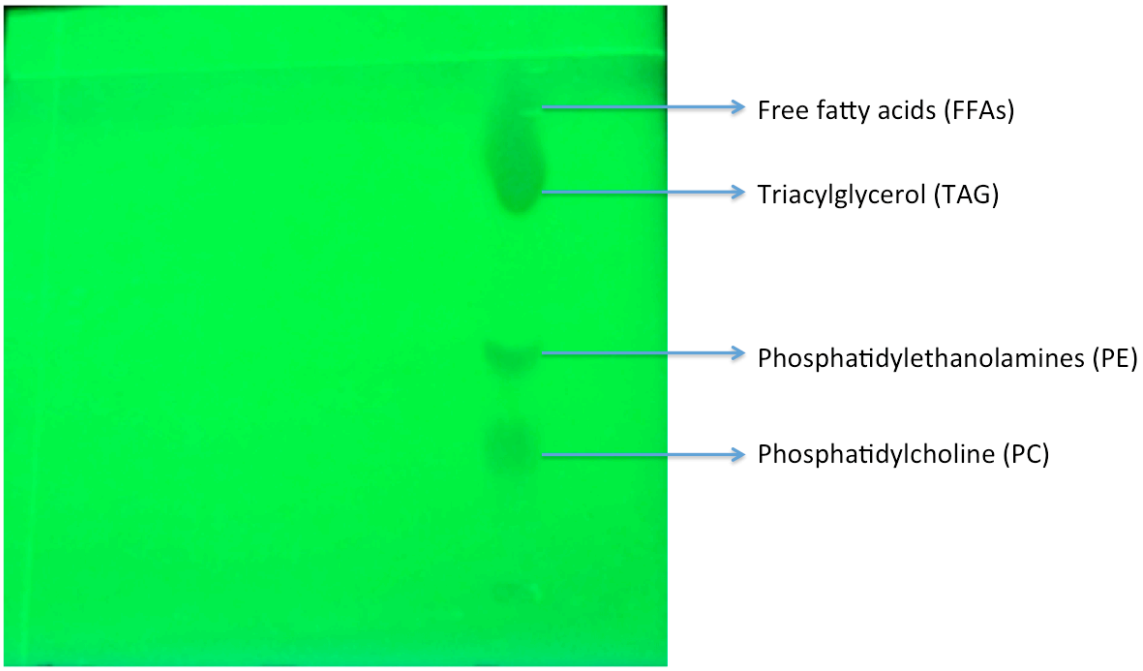


Figure 6 Total lipids separated via TLC

PC qualitative analysis using HPLC

Several HPLC chromatographs are shown in the following Figures, which include: Figure 7 PC standard. This chromatograph was obtained when PC standard was applied to an empty TLC plate and then extracted from the plate before evaluated by HPLC; Figure 8 Our purified PC, which was separated from total lipids by TLC; Figure 9 Silica gel, which was scraped off a blank TLC plate.

From these Figures, two kinds of peaks were observed. The first peak appeared around 2.0 min, and this peak could be found in all of these chromatographs, suggesting

that it probably came from silica gel matrix. Another one appeared around 3.8 min, and this peak could only be found in Figure 7 and Figure 8, revealing that it represented PC. Furthermore, we can see that Figure 8 was very similar to Figure 7, and therefore we can conclude that PC was successfully separated from total lipids via TLC with desirable purity.

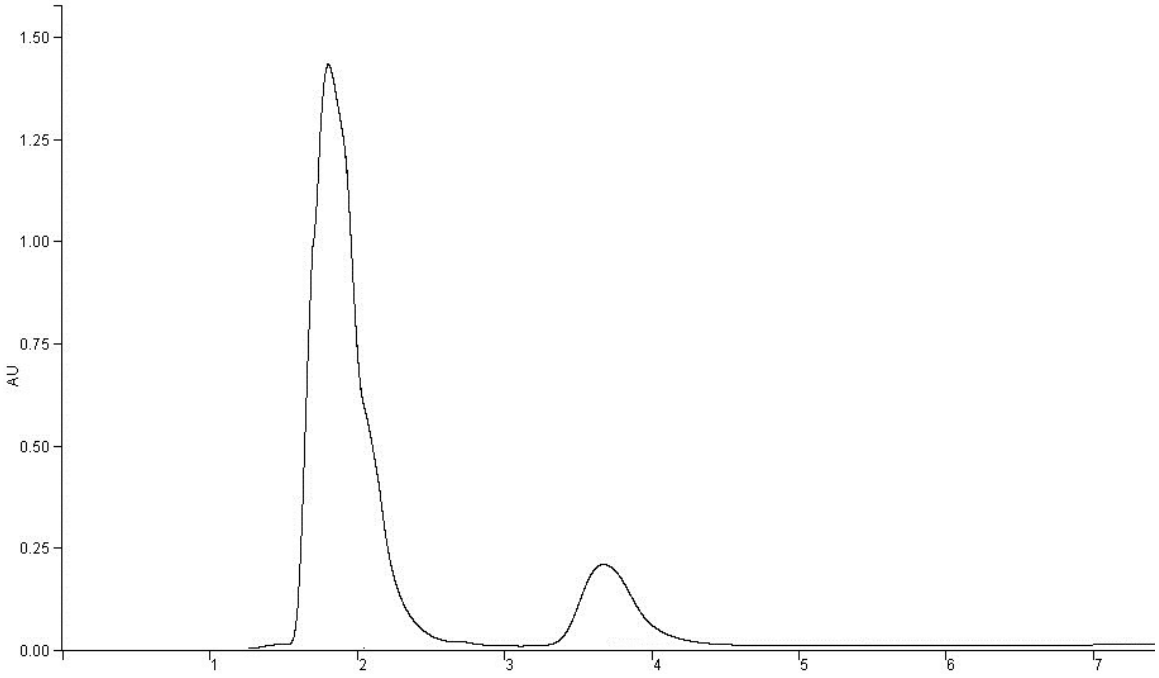


Figure 7 Chromatography of PC standard by NH_2-HPLC with UV detection

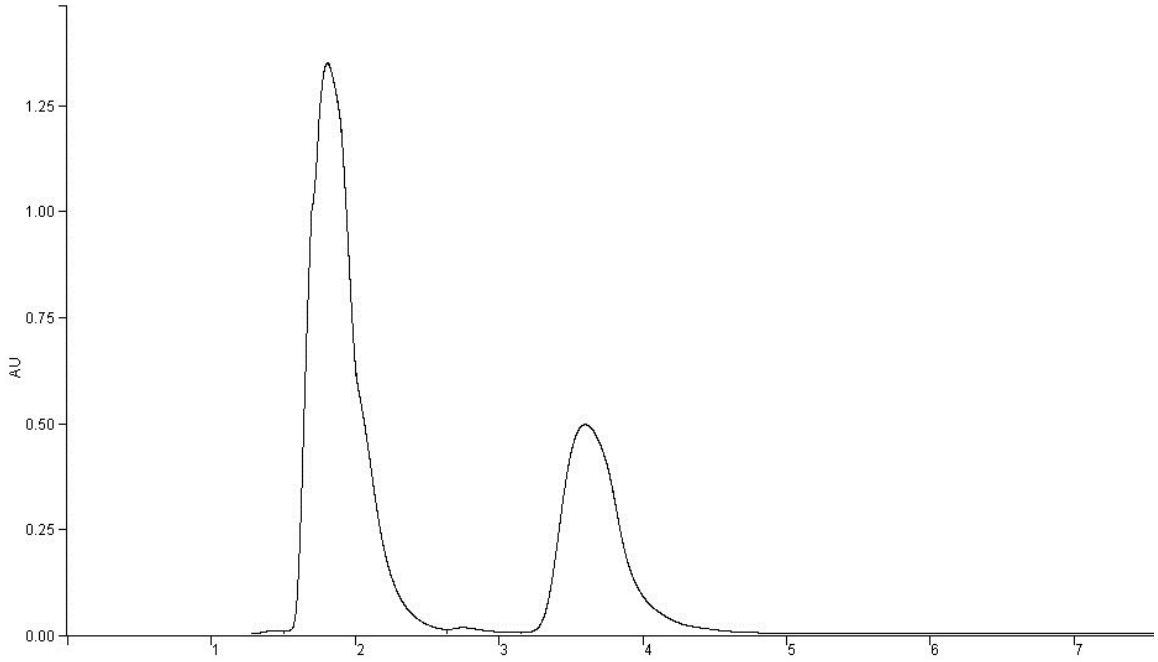


Figure 8 Chromatography of purified PC by NH_2-HPLC with UV detection

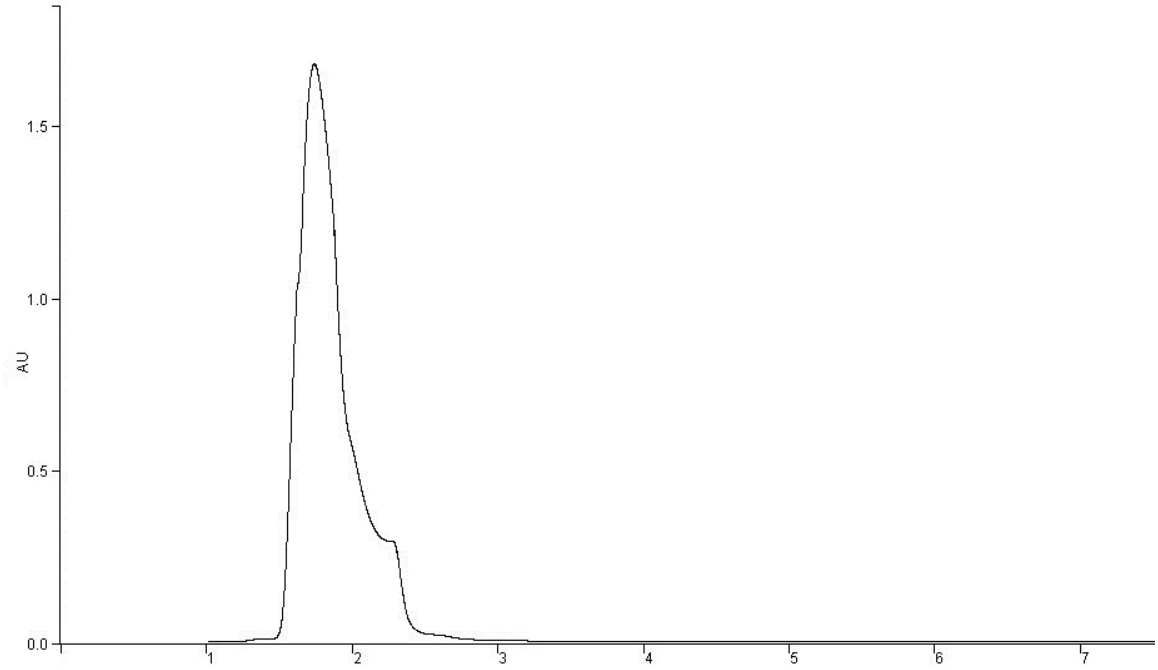
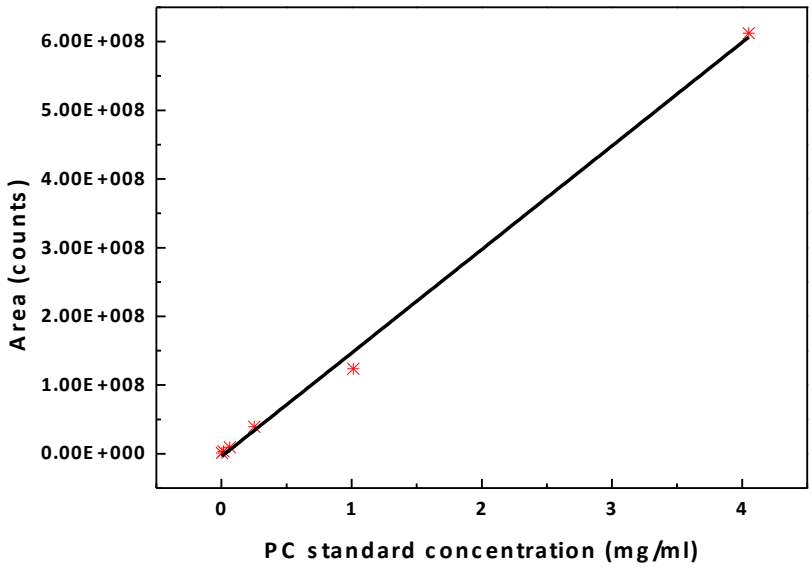


Figure 9 Chromatography of silica gel by NH_2-HPLC with UV detection

PC quantitative analysis via HPLC

Figure 10 shows a PC standard calibration curve over a range of PC standard concentrations from 0.003955 to 4.05 mg/ml. Calculated according to Figure 10, we determined that the mass of PC separated from 12 mg egg yolks total lipids was 2.02 mg, and thus the PC yield (defined as the mass ratio of PC to total lipids) was 16.83%. Previous reports indicate that PC accounts for 6~7.3% of egg yolk by weight[45], and lipids represent 27.45~37.14 wt.% of egg yolks[44]. Thus, PC accounts for 16.16~26.59 wt.% of total lipids, which was in a good agreement with our experimental value (16.83%). Therefore, TLC is an efficient method for PC separation.



$Y=1.51\times10^{8}\times X-4.15\times10^{6}$（$R^2=0.997$）

Figure 10 PC standard calibration curve

In-situ enzymatic hydrolysis

Hydrolysis rates over different PLA_1 enzyme loading are shown in Figure 11. During these experiments, initial concentration of PC was kept constant (separated from 12 mg total lipids in egg yolks). Obviously, PLA_1 enzyme loading has a great impact on PC hydrolysis rate (defined as the peak area of hydrolyzed PC to peak area of original PC). For example, when the PLA_1 enzyme loading increased from 0.06 to 0.3 ml, the PC hydrolysis rate increased greatly. However, with a further increase in the PLA_1 enzyme loading from 0.3 to 0.42 ml, the PC hydrolysis rate only showed a slight increase. PLA_1 enzyme loading of 0.3 ml appeared to be optimal for the *in-situ* enzymatic hydrolysis of PC and its hydrolysis rate has already achieved 90%.

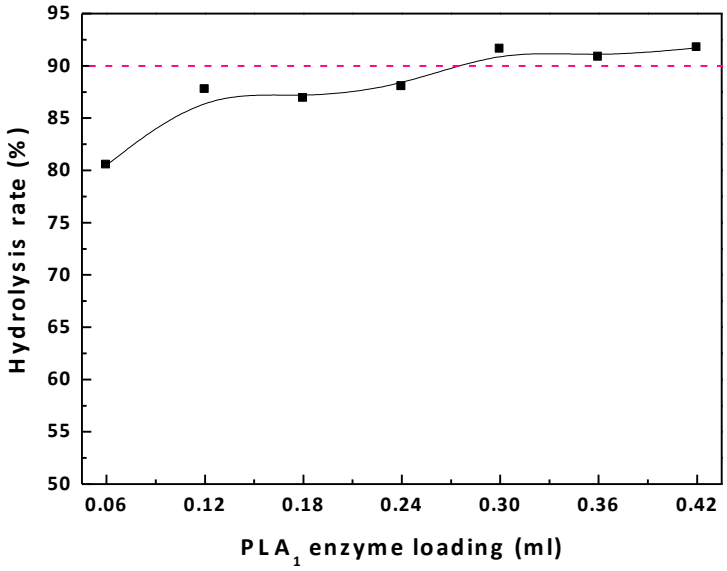


Figure 11 Hydrolysis rate over different PLA_1 enzyme loading

The horizontal broken line indicated 90% hydrolysis rate

PC hydrolyzed by phospholipases

We also conducted additional experiments to investigate the *in-situ* enzymatic hydrolysis of PC with 0.3ml PLA_2 enzyme. The HPLC chromatographs with 0.3ml PLA_1 enzyme and 0.3ml PLA_2 enzyme were shown in Figure 12 and Figure 13. As mentioned before, the first peak (at around 2 min) probably came from silica gel matrix and the other one (at around 3.8 min) represented PC. Calculated according to Figure 10 (PC standard calibration curve), we could know that PC hydrolysis rate was 91.59% with 0.3 mL PLA_1 enzyme, while 84.80% with 0.3 mL PLA_2 enzyme. Consequently, PLA_1 enzyme performed better than PLA_2 enzyme.

Our HPLC analyses were carried out according to Dietlind and Ernst[37]. Their experimental results showed that 2-acyl LPC was eluted earlier than 1-acyl LPC. Based on their study, we could find that 2-acyl LPC peak appeared around the time of 5.6 min in our Figure 12, and 1-acyl LPC peak appeared around the time of 6.6 min in Figure13. Moreover, both Figure 12 and Figure 13 had only one kind of LPC, which means that no acyl migration occurred during the *in-situ* enzymatic hydrolysis of PC.

From these Figures, we noticed that the peak areas of 1-acyl LPC and 2-acyl LPC were different (peak area: 2-acyl LPC > 1-acyl LPC). This could be explained as follows: for 1-acyl LPC hydrolyzed from egg yolks, saturated lipids accounted for the majority parts of fatty acid residuals on the LPC, whereas, for 2-acyl LPC that derived from the same source, its fatty acid residuals were largely composed of unsaturated fatty acids, which had been confirmed by our GC analysis of these two LPCs. Unsaturated fatty acids, in general, have greater UV absorption intensity than saturated fatty acids[40].

Consequently, the 2-acyl LPC shows stronger UV absorption than the 1-acyl LPC under 210 nm, and thereby the peak area of the 2-acyl LPC was larger than that of the 1-acyl LPC.

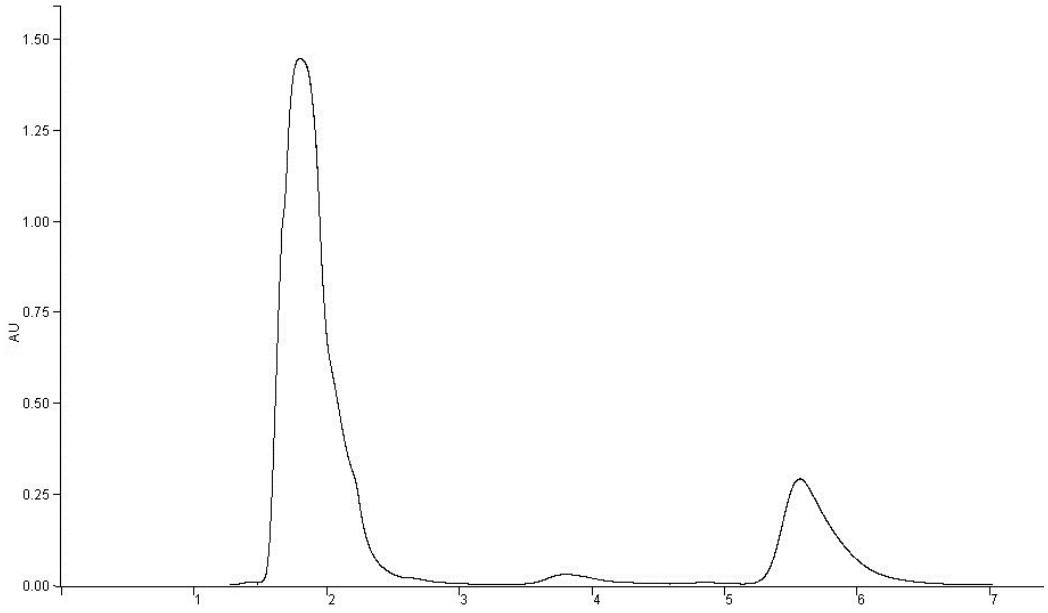


Figure 12 Chromatography of PC hydrolyzed by 0.3ml PLA_1 enzyme

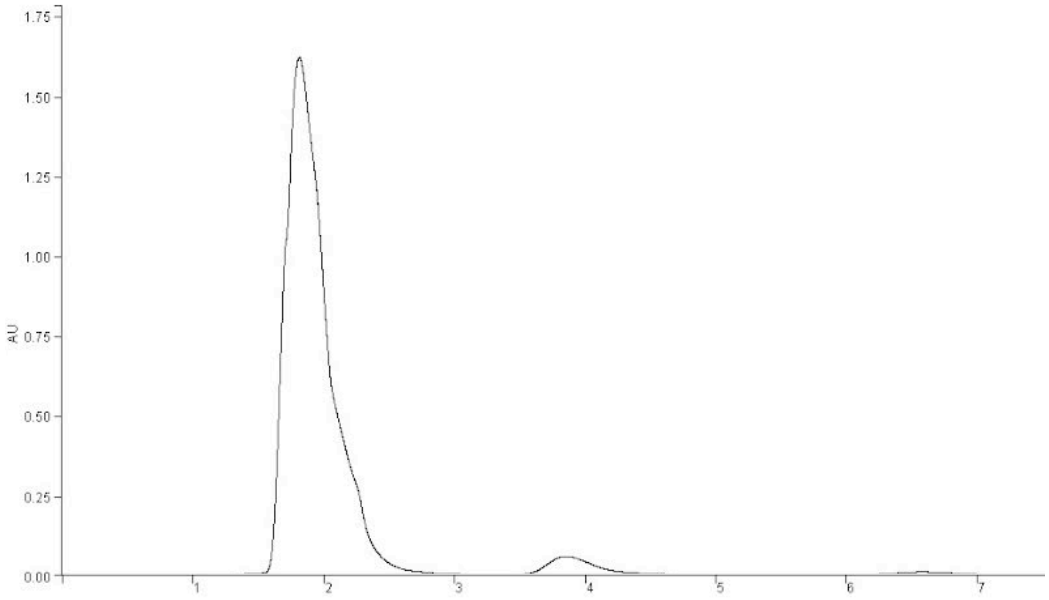


Figure 13 Chromatography of PC hydrolyzed by 0.3ml PLA_2 enzyme

Determination of fatty acids composition by GC-FID

GC chromatographs of obtained fatty acids are shown in the following figures, which includes: Figure 14 fatty acids composition of PC; Figure 15 fatty acids composition of sn-1 position of PC; Figure 16 fatty acids composition of sn-2 position of PC.

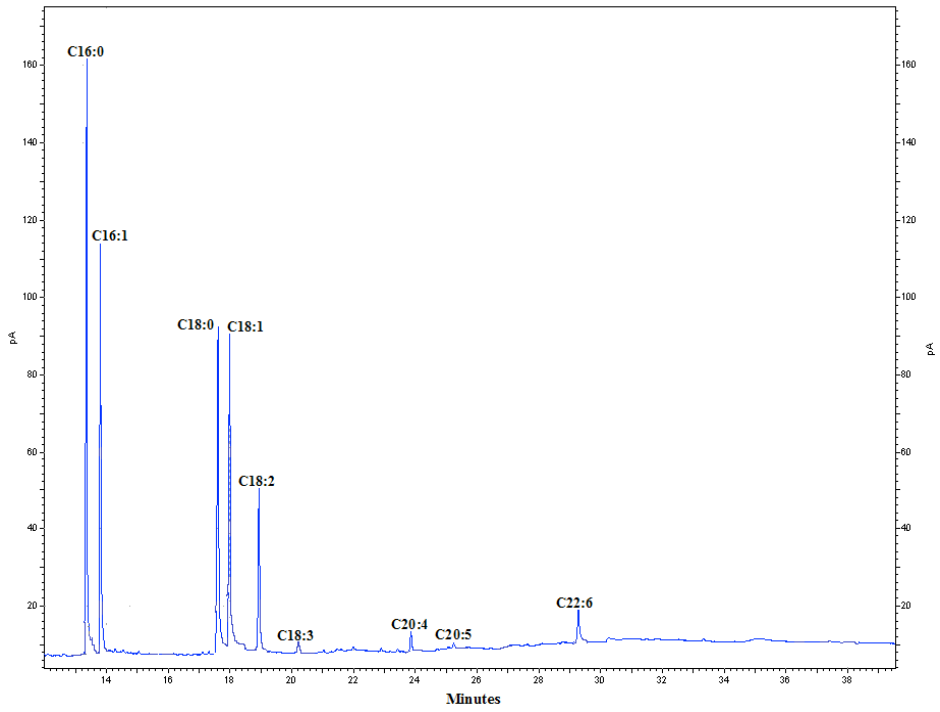


Figure 14 Fatty acids composition of PC analyzed by GC-FID

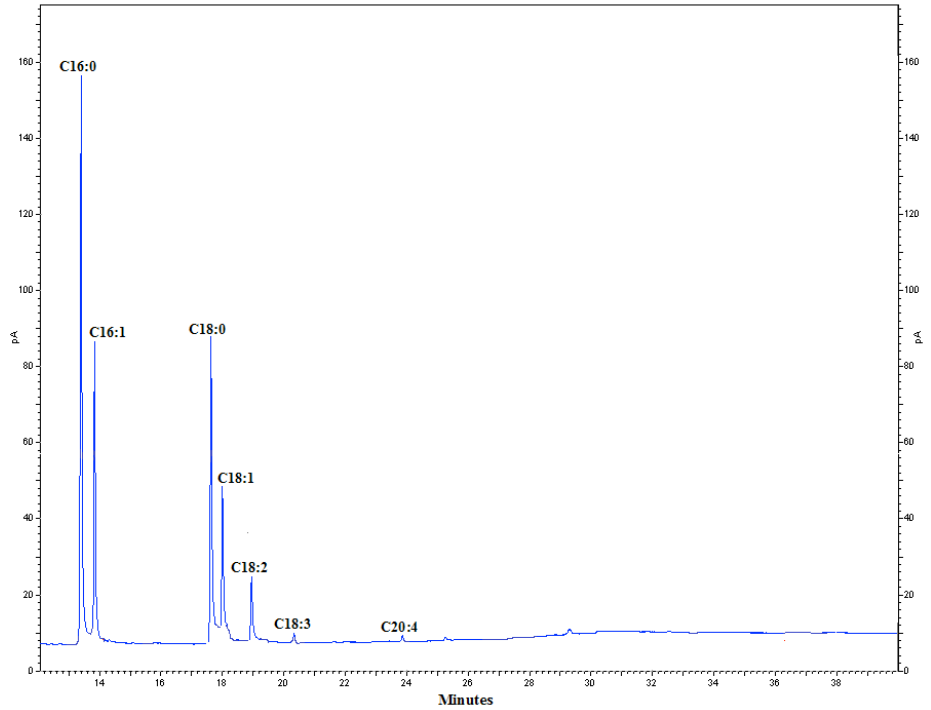


Figure 15 Fatty acids composition of sn-1 position of PC analyzed by GC-FID

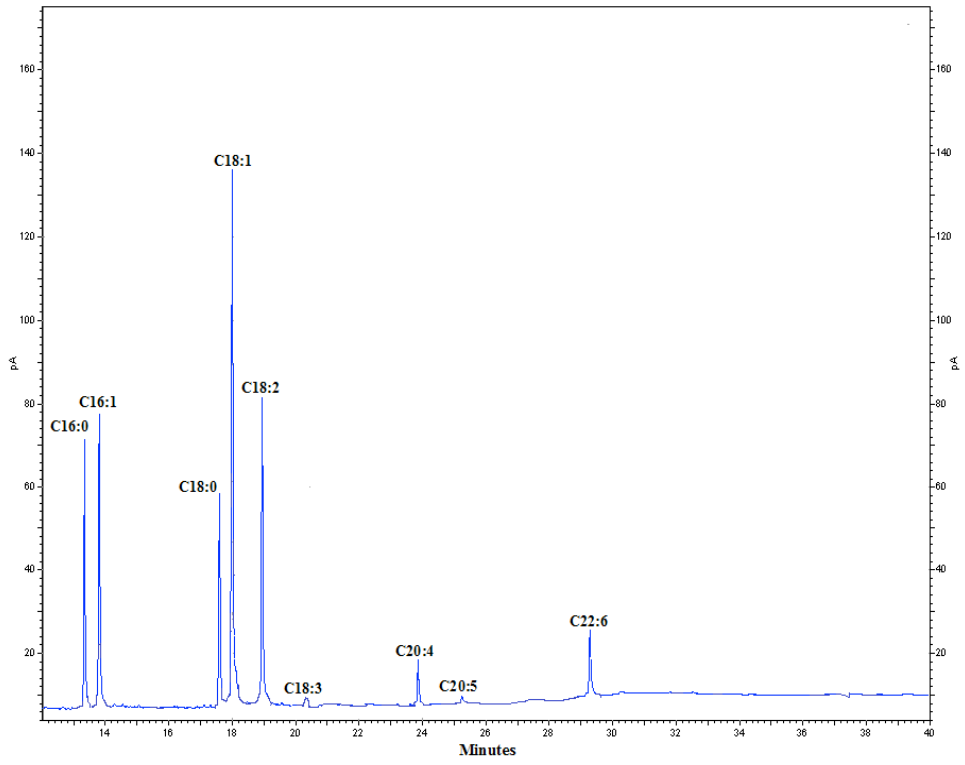


Figure 16 Fatty acids composition of sn-2 position of PC analyzed by GC-FID

Relative percentages of obtained fatty acids are shown in the following figures.

In Figure 17, the relative percentage of each fatty acid on PC was: palmitic acid (16:0, 31.28%), palmitoleic acid (16:1, 19.65%), stearic acid (18:0, 19.23%), oleic acid (18:1, 17.53%), linoleic acid (18:2, 8.49%), linolenic acid (18:3, 0.42%), ALA (20:4, 1.12%), EPA (20:5, 0.23%) and DHA (22:6, 2.04%).

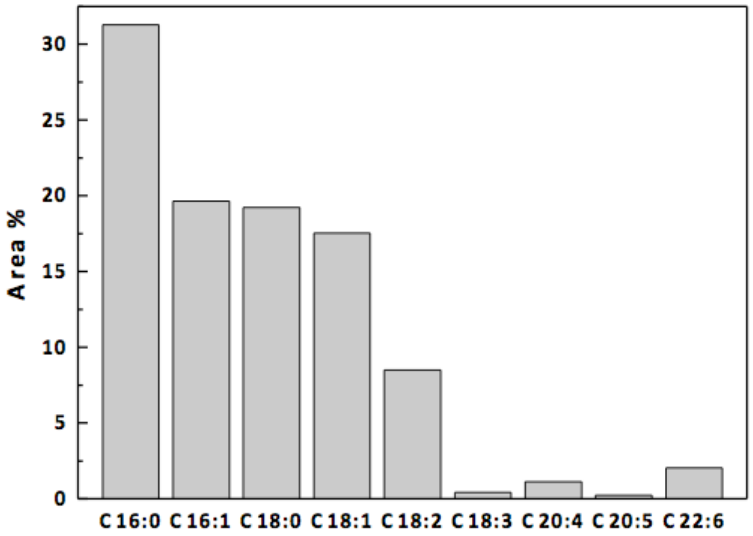


Figure 17 Relative percentage of each fatty acid on PC

In Figure 18, the relative percentage of each fatty acid on sn-1 position of PC was: palmitic acid (16:0, 41.43%), palmitoleic acid (16:1, 18.43%), stearic acid (18:0, 22.13%), oleic acid (18:1, 12.01%), linoleic acid (18:2, 5.11%), linolenic acid (18:3, 0.41%) and ALA (20:4, 0.47%).

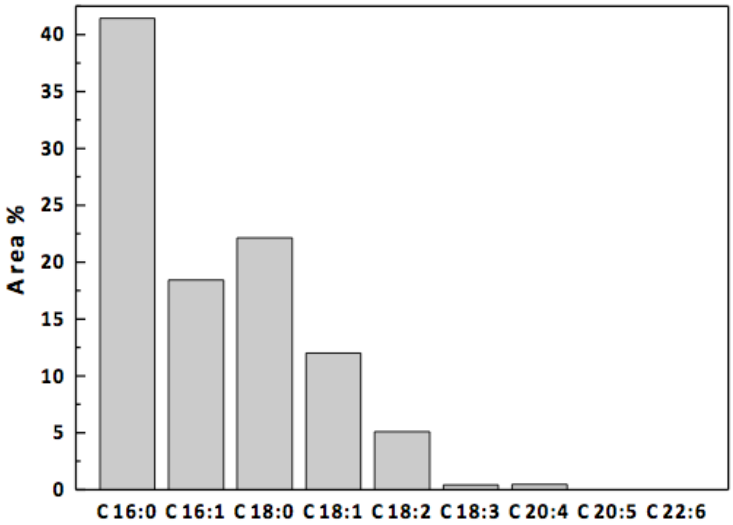


Figure 18 Relative percentage of each fatty acid on sn-1 position of PC

In Figure 19, the relative percentage of each fatty acid on sn-2 position of PC was: palmitic acid (16:0, 15.87%), palmitoleic acid (16:1, 14.77%), stearic acid (18:0, 11.64%), oleic acid (18:1, 32.73%), linoleic acid (18:2, 17.35%), linolenic acid (18:3, 0.63%), ALA (20:4, 2.63%), EPA (20:5, 0.25%) and DHA (22:6, 4.13%).

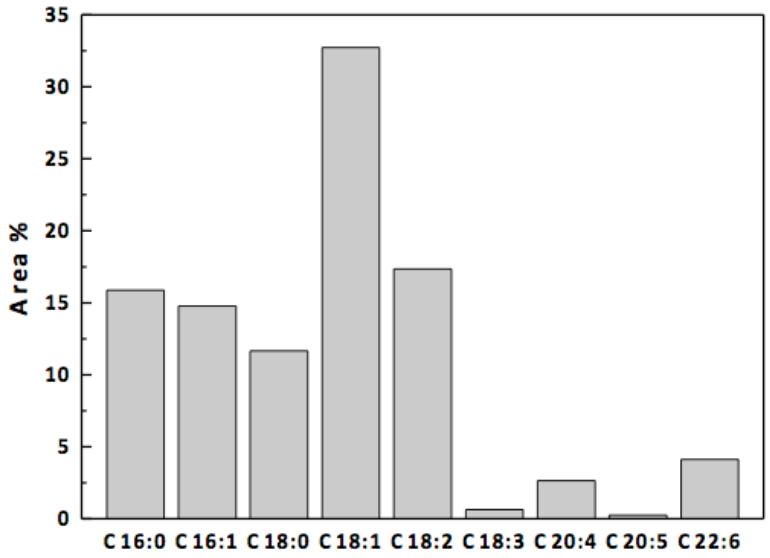


Figure 19 Relative percentage of each fatty acid on sn-2 position of PC

In the new method developed in the present study, enzymatic reaction and separation of total lipids and product mixtures were performed on one TLC plate, and thus reducing workloads, time and cost. Here is a potential mechanism of the *in-situ* enzymatic reaction. PC and enzymes are dispersed in a silica gel matrix of the TLC plate, and dropping wetting agent facilitates interactions between PC and enzymes to promote hydrolysis rate. Because *in situ* hydrolysis takes place in a small area on a TLC plate, while normal enzymatic incubations occur in vitro, the former one shows at a higher rate.

To be honest, complete PC hydrolysis, in our study, was hard to achieve. There were two main reasons to explain this result. On the one hand, as we know, enzymatic

hydrolysis of PC is a reversible reaction, and the PC/ hydrolysis product equilibrium would be reached concomitant with the *in-situ* enzymatic hydrolysis of PC. On the other hand, as we mentioned, this *in-situ* enzymatic hydrolysis occurred on a silica gel matrix, and hence the mass transfer between PC and phospholipases and the solubility of substrates and products are limited. Therefore it might be very difficult to achieve 100% hydrolysis rate.

However, from total lipids to separated FFAs, all components were reacted on one TLC plate, which means the possibility of components loss due to transfers is diminished, and thus our results would show higher accuracy then that of the conventional methods. Furthermore, from HPLC, each *in-situ* enzymatic reaction has only one kind of LPC, which means none acyl migration happened during both *in-situ* enzymatic hydrolysis. Thus, for *in-situ* positional analysis of PC, one enzyme would be good enough.

PC and unsaturated FFAs during *in-situ* enzymatic reaction are vulnerable to oxidation, since these compounds absorbed on TLC plate are exposed to atmospheric oxygen. But there is a potential solution for this oxidation problem. Recently, all TLC steps including sample application, solvents mixture, development and drying could be automated by "automated multiple development" technique[4]. In the near future, with the advent of new equipment, the oxidization problem might be solved by automatically performing the *in-situ* enzymatic hydrolysis in an oxygen-free environment.

As mentioned in the introduction, omega-3 fatty acids, among all fatty acids, are of great physiological interest. For those labs in which GC is inaccessible, there is an

alternative method to obtain information of positional distribution of omega-3 fatty acids on PC.

TLC plates impregnated with silver ions can separate fatty acids according to their unsaturation levels, for silver ions can reversibly form a complex with unsaturated lipids, which makes these unsaturated fatty acids show different mobility than other lipids[41]. The silver ion TLC can be easily made via immersing the plates in a solution of silver nitrate[41].

In addition, a band of interest on a TLC plate can be *in situ* quantitatively analyzed by a scanning densitometer. For a densitometer based on reflection mode, its light source and detector are located on a same side of the TLC plate[42]. The amount of light coming from the light source is altered at the sample zone, and the change is collected in the detector to develop calibration curves[42]. Considerable research has been devoted to the densitometry, which increases precision, sensitivity and repeatability of this quantitative measurement[46,47].

Hence, omega-3 fatty acids released from a specific position of PC, without further elution and derivatization, can be differentiated from other fatty acids on silver ion TLC and subsequently *in situ* quantified by photodensitometry. This opens a new opportunity to analysis PC structure without GC analysis, but further efforts are needed.

Conclusions

In this thesis, we developed a new PC structural analysis method, and found that most steps can perform on one TLC plate, which greatly saves time, labor and money. And there was no acyl migration occurred during *in-situ* enzymatic hydrolysis.

On HPLC we have already confirmed that PC can be quantitatively separated from total lipids using TLC and the separated PC with desirable purity. Then, the hardest part of *in-situ* enzymatic reaction: limited interactions between PC and phospholipase due to their dispersion in a silica gel matrix, was overcame by gradually dropping a wetting agent containing chloroform/ methanol/ water (65:24:4, v/v/v) on TLC plate. Under optimal conditions, 91.59% and 84.80% hydrolysis rates were obtained from PLA_1 enzymes and PLA_2 enzymes, respectively. Lastly, products were further separated via second-dimensional chromatography on the same plate and analyzed using GC-FID.

With the development of commercial pre-coated TLC plate, the convenience and repeatability of TLC have been greatly improved. And the potential oxidation of unsaturated fatty acids and PC on TLC plate might be overcame through combination with other techniques. It is obviously beneficial for the practical application of *in-situ* enzymatic reactions, and thus a broader spectrum of applications of TLC is achievable.

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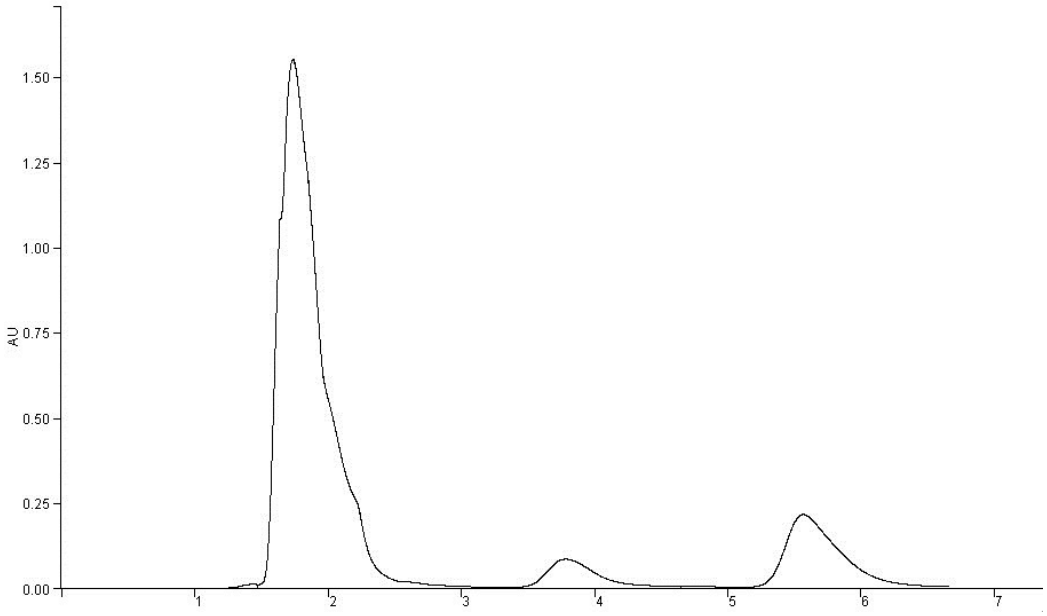
Appendices

HPLC images

PC hydrolyzed by 0.06ml PLA_1 enzymes



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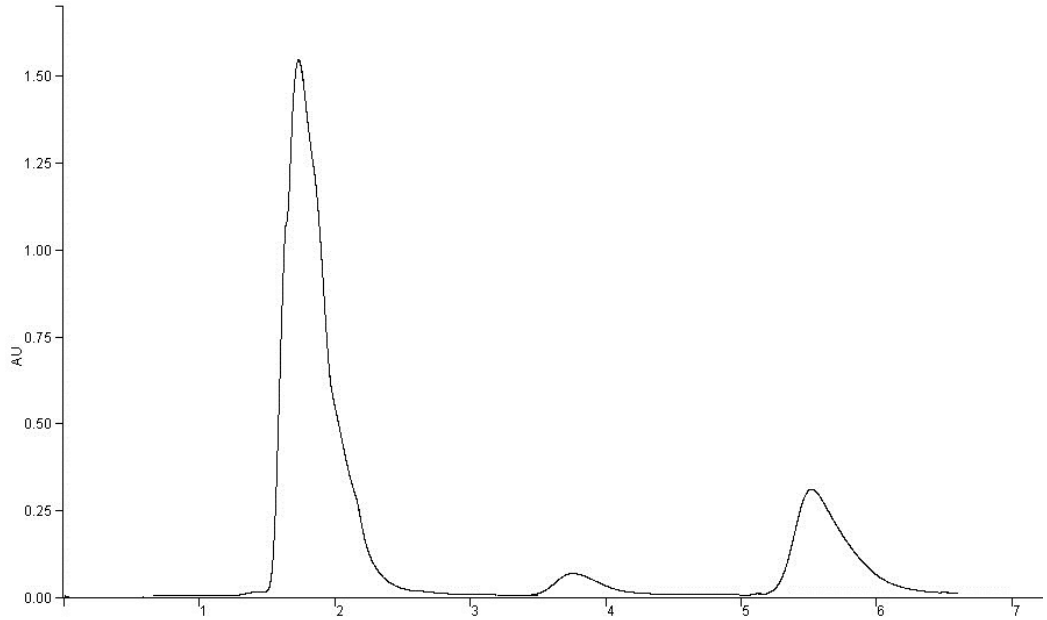

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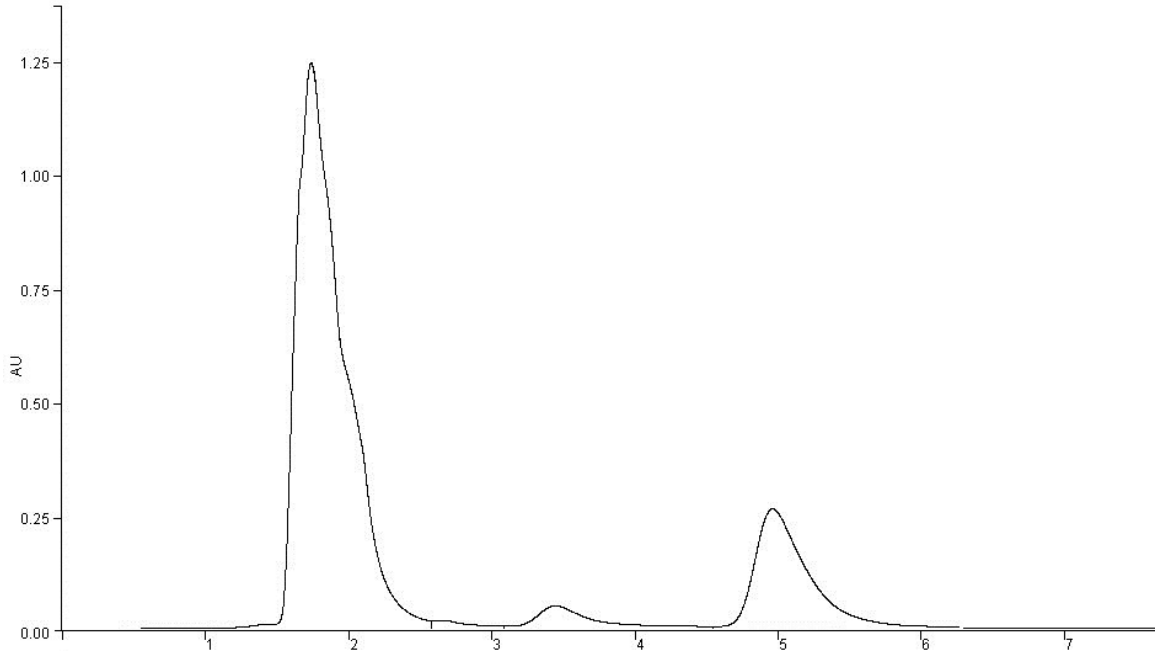
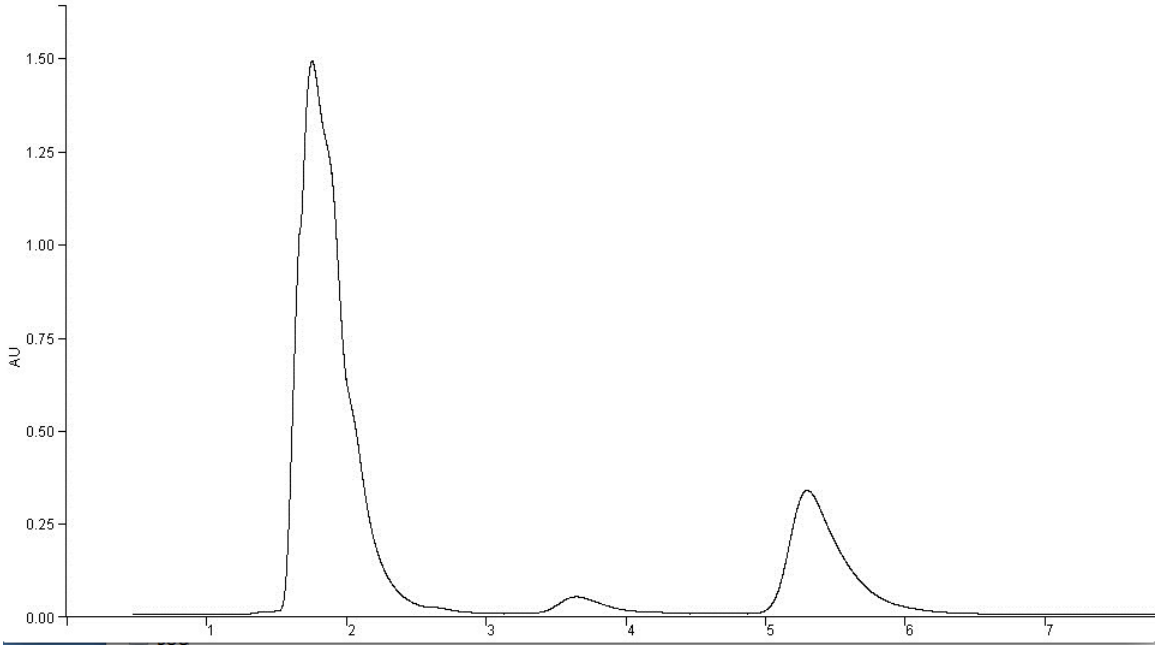
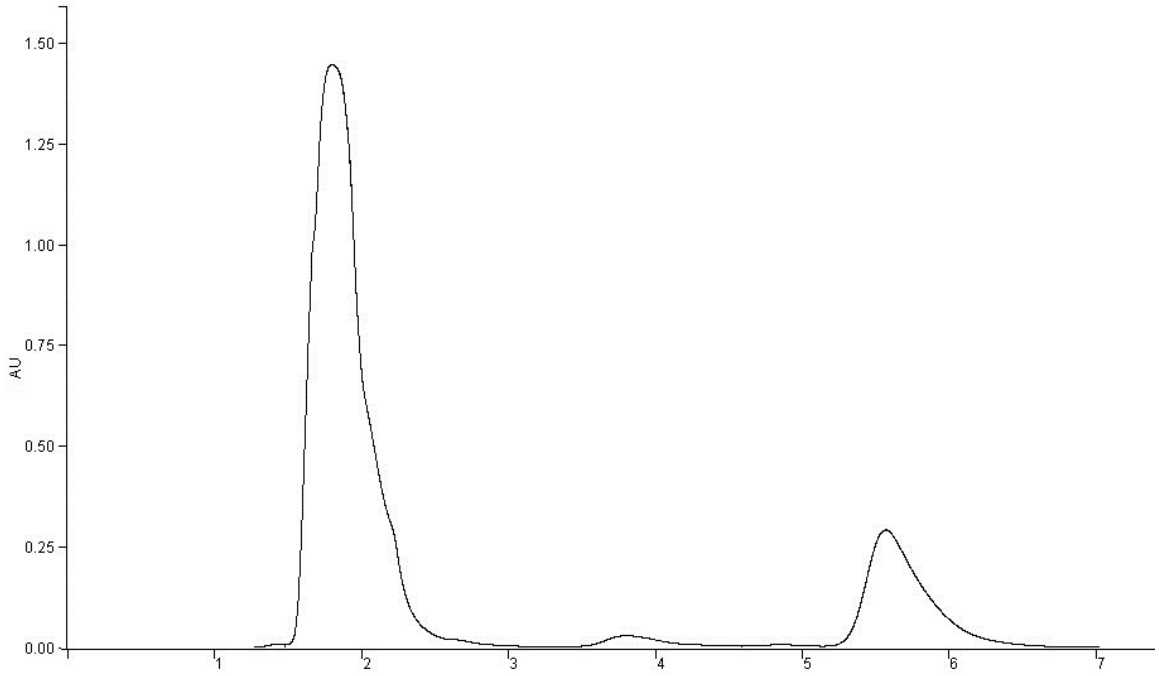


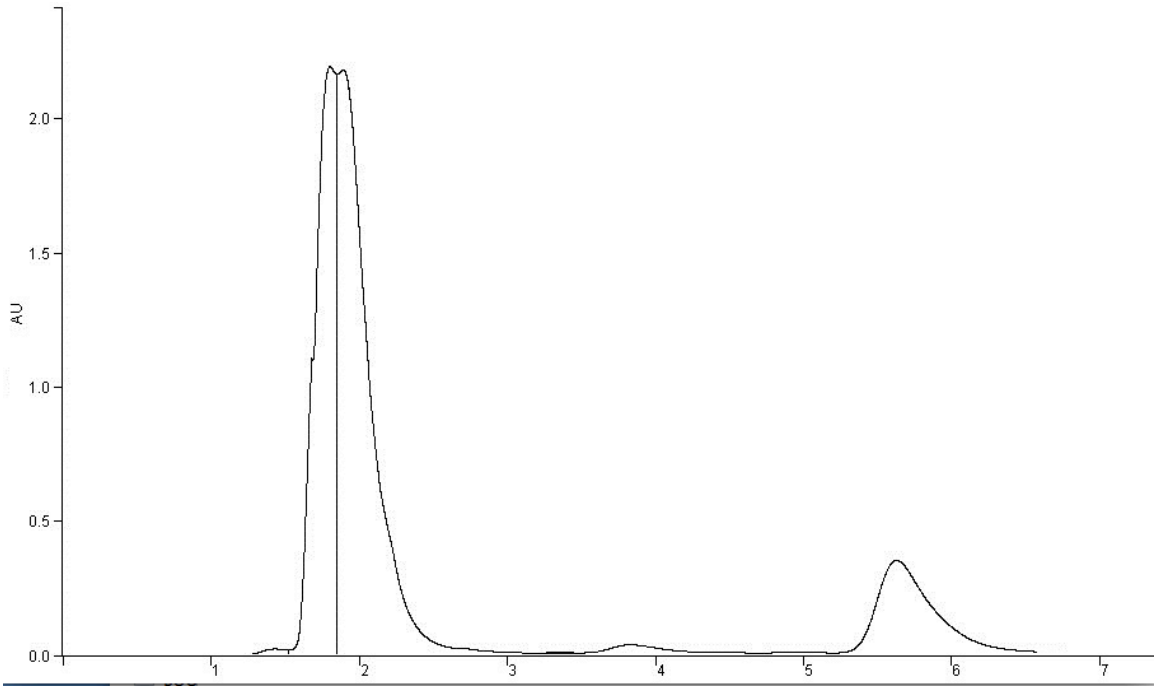
PC hydrolyzed by 0.24ml PLA_1 enzymes



PC hydrolyzed by 0.30ml PLA_1 enzymes



PC hydrolyzed by 0.36ml PLA_1 enzymes



PC hydrolyzed by 0.42ml PLA_1 enzymes