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Separation of phosphatidylcholine and phosphatidylethanolamine by using high-performance displacement chromatography

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Abstract

A binary mixture of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was successfully separated by high-performance displacement chromatography (HPDC) on an 150 mm × 4.6 mm analytical silica column (3–5 μm packing), using dichloromethane–methanol (9:1, v/v) as carrier and ethanolamine as displacer. The effects of displacer concentration, flow-rate, loading amount and the composition of the sample on separation efficiency were studied. Eighty-four milligrams sample (PE:PC 1:1.16) was separated perfectly by using 83 mM ethanolamine (in carrier) as displacer at the flow-rate of 0.1 ml/min. The yields of the pure PE and PC (100% purity) were 94.8% and 87.9%, respectively and the cycle time for a single separation was about 195 min. It was valuable that the optimum loading amount (the allowed maximum of sample loading) was investigated only by using the sample to be simulated the composition of the separated actual one, because the separation efficiency was significantly affected by the composition of the sample. For the same loading amount of 175 mg, the yields of the pure PE and PC were improved greatly from 31.4 and 16.9 to 56.0 and 77.6%, respectively, when the proportion of PE to PC was adjusted from 1:1.16 to 1:4. Furthermore, the separation of PE and PC in an actual sample (soybean phospholipids) was achieved using the proposed HPDC method.

Keywords: Displacement chromatography; Phosphatidylcholine; Phosphatidylethanolamine; Phospholipids; Lipids

1. Introduction

The displacement mode of chromatography was first recognized in 1906 by Tswett who found that sample displacement occurs under conditions of overloaded elution chromatography [1]. In 1943, the concepts of frontal, elution and displacement chromatographies were introduced by Tiselius [2]. The mode of displacement chromatography was non-linear, usually including four operation steps: equilibrium, loading, displacement and regeneration. The feed components were driven out of the column by the displacer that had stronger affinity to the stationary phase than any component in the feed. The feed components arranged themselves into a "displacement train" of adjoining square wave concentration pulses of the pure substance according to the order of the affinity strength to the stationary phase [3].

Applications of displacement chromatography, before the 1980s, were mainly concentrated on the fields of separation of rare earth element and isotope separation owing to the limit of inefficient chromatographic systems and packing [4,5]. However, since it was successfully utilized in separation and purification of biomolecules, displacement chromatography has attracted much attention. Horváth and co-workers successfully separated amino acid, peptide, pharmaceutical diastereoisomer, corticosterone, polymyxins and nucleic acids, etc. by displacement chromatography [6–13]; separation of protein and nucleic acids was achieved by Cramer and co-workers [14–18]; Qi and Huang tried to purify epimbicin from a raw product solution [19]; Freitag and co-workers compared separation efficiency of dairy whey protein on a packed column with a continuous-bed column, and they also investigated the effect of the mass of displacer on separation of protein by cation-exchange displacement chromatography [20,21]; Chevolot et al. improved the purification of sulfated oligofucan with ion-exchange displacement centrifugal partition chromatography [22].

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Compared with elution chromatography, displacement chromatography had some advantages such as large loading amount, high concentration of the product, little tailing, low solvent consumption and high efficiency of use of the stationary phase. Hence, it was appropriate to be used as preparative chromatography [2,3]. The loading amount of displacement chromatography is usually one or two order greater than that of elution mode when using the same column. Some of researchers have successfully performed the preparative displacement chromatography on analytical columns [11–22].

Natural phospholipids, main constituents of cell membranes, have a lot of important biological functions to all living organisms [23–25]. High-purity phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the fraction products of phospholipids, were extensively applied in the fields of nourishment, pharmaceuticals and cosmetics. At present, high-purity PC and PE were usually prepared by HPLC (elution mode) [26–29], in which there were some inherent shortcomings, i.e., low loading amount and high solvent consumption, which lead to high production cost and price. The high-purity PC and PE do not meet the needs of various fields because of the limit to their high prices. To reduce the cost, many researchers had done a great deal of work, but the results were not satisfactory. Yoon and Kim tried to simple the mobile phase for reducing the cost, and they successfully purified the egg PC by using pure methanol as mobile phase [30]. However, the innate shortcomings of elution mode couldn't be overcome. The purpose of our work in this paper was to solve this problem for the preparation of high-purity PC and PE by using displacement chromatography that is appropriate for preparative separation.

PC and PE were main components in natural phospholipids. The sum of PC and PE was about 70–90% of the total phospholipids in egg yolk [31]. Soybean and other phospholipids also could be purified to this level by using simple solvent extraction [32]. Consequently, binary mixture of PC and PE was used as object to be separated by using displacement chromatography. The effects of flow-rate of displacer, loading amount and displacer concentration on separation efficiency were also studied in this paper. We hope to establish a method with high efficiency and low cost based on the characteristic of displacement chromatography to be appropriate for preparative separation.

2. Experimental

2.1. Chemicals

Phosphatidylcholine and phosphatidylethanolamine were purchased from Sigma (Superior-chemicals & Instrument Co., Beijing, China). Soybean degummed oil residues were provided by Yonggu oil factory (Guangzhou, China). Silica gel G (100–200 mesh) used for TLC was obtained from Haiyang Chemical Group (Qingdao, China).

Methanol, ethanol, dichloromethane, chloroform, ethanolamine, diethanolamine, triethanolamine, acetonitrile, phosphoric acid and acetic acid were of analytical grade, obtained from Shanghai Medicine Co. (Shanghai, China). Water used in this work was re-distilled.

2.2. Apparatus

The high-performance displacement chromatography (HPDC) apparatus was consisted of a LC-6A pump (Shimadzu, Tokyo, Japan), an SPD-10A ultraviolet (UV) detector (Shimadzu) and a Rhecdyne 7725i injector with 0.7 ml sample loop (Cotati, CA, USA). The column used for HPDC was a 150 mm × 4.6 mm column packed with laboratory-made silica (3–5 μm).

The analytical HPLC apparatus was kindly provided by Dalian Elite Analytical Apparatus Co., which was consisted of a P200 II pump (Elite, Dalian, China), an UV200 II ultraviolet (UV) detector (Elite) and a Rhecdyne 7725i injector with 20 μl sample loop. The chromatographic data was acquired by chromatography working station Echrom98 (Elite). The HPLC analysis was performed on a 150 mm × 4.6 mm column packed with 5 μm laboratory-made silica.

2.3. Chromatography condition of analytical HPLC

Acetonitrile–methanol–85% phosphoric acid (180:3:1, v/v) was used as the mobile phase, filtrated and degassed before use [32]. The flow-rate was set at 0.5 ml/min. The loading volume was 20 μl. The detection wavelength of UV was 203 nm. All experiments were carried out at ambient temperature between 15 and 20 °C. The quantitative analyses were performed by using external standard based on the height of peak, which was detailed in ref. [32].

2.4. Procedures

2.4.1. Preparation of TLC

At first, the slurry was prepared by putting some silica gel G (100–200 mesh) into 0.2% carboxymethylcellulose (CMC) solution. And then the slurry was covered on some 25 mm × 75 mm glass plates with the thickness of about 0.5 mm. After dried, these plates were activated 2 h at 105 °C, and then were cooled in a desiccator before use.

2.4.2. Selection of mobile phase (carrier) and displacer by TLC

The candidate solvents were used as developer when the mobile phase (also called "carrier") has been selected. Standard PC and PE used as samples were spotted 5 mm above the solvent level, height of which was 10 mm from the bottom. The development was terminated when the solvent front moved to the location of 10 mm from the upper edge of the TLC plate. And then the plate was dried and colored by spraying with Dittmer–Lester reagent [33]. The blue dots were phospholipids. The R_F value of the sample was cal-

culated by the distance from dot to sample spotted location dividing that from solvent front to sample location.

After selection of the carrier, the candidate displacers were dissolved in these carrier solvents to a certain concentration, which were used as developers for displacement TLC. Standard PC, PE and mixture of both were spotted and developed, respectively. The other processes were the same as the above. The results of TLC were investigated to judge whether displacement took place or not.

The detailed procedures of selection of carrier and displacer by TLC referred to ref. [10].

2.4.3. Evaluation of regeneration efficiency of column and selection of regenerant

After the displacement was finished, the candidate solvent system used as regenerant was pumped into the column at flow-rate of 1 ml/min for a certain time. And then the HPDC system was equilibrated by carrier at flow-rate of 0.5 ml/min and 0.7 ml sample loop was replaced by 20 μl loop. The retention time of benzene and *p*-hydroxybenzoic acid was determined at wavelength of 254 nm. The regeneration efficiency was evaluated by the variances of the retention time.

2.4.4. Separation of standard phospholipids by HPDC

The system was first equilibrated with carrier at the flow-rate of 0.5 ml/min, then the drain was opened and the pump purged with the displacer solution. Turned off the pump and closed the drain, the injector valve was turned to "Load" position. The 0.7 ml feed loop was filled with the mixture of PC and PE. The pump was turned on when the valve was turned to "Injection" position. The feed was pushed into the column by displacer at a certain flow-rate. The effluent was collected in certain time intervals and analyzed by HPLC. After emergence of the displacer front, the column was regenerated by pumping regenerant into the column, and then it was equilibrated with carrier at 0.5 ml/min. The system could be used for next separation after equilibrium. The separation efficiency was optimized by changing the factors of flow-rate, loading amount and displacer concentration, and it was evaluated by two indices of the purity and yield of the product. The purity of a given product was defined for this study as the PE or PC percentage of the product. The yield of a given product was evaluated by dividing the quantity of the product by that of PE or PC in the loading sample. The fractions containing displacer were excluded when calculating the yield of the PC product.

2.4.5. Separation of soybean PC and PE by HPDC

The soybean degummed oil residues that contained rich phospholipids was usually used to prepare PC and PE. The crude PC was obtained from the degummed oil residues by the followed procedures in our work: acetone washing, 95% ethanol extraction, acetone precipitation and solvent recovery [32]. Besides PC and PE, the crude PC also contained a great deal of impurities. Thus, it was further purified by using a pre-column packed with silica according to Mounts's method [34]. Two hundred milligrams crude PC dissolved in a little chloroform was loaded on a 20 g silica gel column (100–200 mesh, irregular). It was eluted sequentially with chloroform (100 ml), acetone (50 ml) and methanol (150 ml). The methanol fractions were collected and removed from the solvents under vacuum at 50 °C. The residues were used as the raw product for separation by HPDC. This raw product was weighted and the PC and PE contents of it were determined by HPLC. And then it was dissolved in 1 ml carrier solvent for displacement separation.

3. Results and discussions

3.1. Selection of carrier and displacer by TLC

Selection of stationary phase, mobile phase (carrier) and displacer was interrelated in displacement chromatography. The order of selection in our work was stationary phase, carrier and displacer. It was usually a tedious and difficult work to choose the carrier and displacer. The solvents to be used as carrier must have characteristics of high solubility to dissolve the sample, weak elution strength, good chemical stability, low viscosity and to be removed from the sample easily, etc. The requests for the substance to be used as displacers were the strongest affinity to the stationary phase, good chemical stability and to be eluted out of the column (regeneration) easily and fast [2].

Normal-phase HPLC was usually used to analyze the phospholipids, in which silica and other bonded packings, e.g., amine bonded silica were used as stationary phases [30,35–37]. For reducing the production cost and facilitating to select the carrier and displacer by TLC, the silica was chosen as stationary phase for displacement separation of PC and PE in our work.

When the silica was used as stationary phase, it was proved that TLC was a very effective tool for selection of the carrier and displacer. Kalász and Horváth successfully selected the carrier, displacer and concentration of it for separation of corticosterone [10].

Dichloromethane and chloroform were solvents with high solubility to phospholipids. Hereby, both solvents were used as candidate carriers. From the results of TLC, we found that the spot of PC or PE did not move ($R_F = 0$) when using single pure solvent as developer, which indicated that the elution strength of pure dichloromethane or chloroform was too weak to be a desirable carrier for displacement separation of PE and PC. To improve the elution strength, methanol was added into dichloromethane and chloroform. The R_F values of PC or PE were different with different proportions of methanol to dichloromethane or chloroform. The results of TLC are listed in Table 1.

The solvents could be used as carriers if the R_F values of PC and PE were unequal and smaller than 0.1 when they were used as developers in TLC with the same stationary

Table 1
The R_F values of TLC of standard PE and PC with different solvents as developers

Solvents	R_F values	
	PE	PC
Chloroform	0	0
Chloroform–methanol (90:10, v/v)	0.07	0.01
Chloroform–methanol (80:20, v/v)	0.24	0.04
Chloroform–methanol (70:30, v/v)	0.56	0.1
Dichloromethane	0	0
Dichloromethane–methanol (90:10, v/v)	0.04	0.01
Dichloromethane–methanol (80:20, v/v)	0.14	0.03
Dichloromethane–methanol (70:30, v/v)	0.32	0.06

phase as that present in the column. From the data of the Table 1, we could conclude that dichloromethane–methanol (9:1, v/v) and chloroform–methanol (9:1, v/v) might be the acceptable carriers according to the above criterion. The R_F values of PC and PE were 0.01, 0.04 and 0.01, 0.07, respectively, when using above mixed solvents as developers.

About 5 ml candidate displacer dissolved in 95 ml dichloromethane–methanol (9:1, v/v) or chloroform–methanol (9:1, v/v) was used as developer for displacement TLC. If the spots colored by Dittmer–Lester reagent were all only one and their shapes were linear or narrow oblong being closely spaced at the front of the displacer for standard PC, PE or binary mixture of both as the sample, we could conclude that displacement was performed. Triethanolamine, diethanolamine and ethanolamine, etc. were investigated as candidate displacers. We found that displacement took place when dichloromethane–methanol (9:1, v/v) and ethanolamine were used as carrier and displacer, respectively. There was no displacement or only partial displacement in the other conditions. Consequently, dichloromethane–methanol (9:1, v/v) and ethanolamine were selected as carrier and displacer for HPDC.

3.2. Regeneration of column

The criteria used to select the composition of the regeneration solution is that the regenerant can remove displacer from the stationary phase quickly as well as it can be easily replaced by carrier for the next separation. It is helpful to achieve above goal that selecting the solvents of carrier as the main compositions of the regenerant. In this work, dichloromethane–methanol (9:1, v/v) and ethanolamine were used as carrier and displacer, respectively. Therefore, dichloromethane and methanol were selected as the basic compositions of the regenerant. For removing ethanolamine from stationary phase, acetic acid was added into above solvent system. The regeneration efficiencies of mixed solvents with different compositions were investigated, and we found that the appropriate proportion of dichloromethane–methanol–acetic acid was 60:30:10 (v/v). Hence, this mixed solvents was selected as regenerant.

After the displacement front emerged from the column, the regenerant containing 60% (v/v) dichloromethane, 30% (v/v) methanol and 10% (v/v) acetic acid was pumped through the column at a 1.0 ml/min flow-rate. After 90 min, the system was equilibrated by the carrier at flow-rate of 0.5 ml/min. Then, the retention time of benzene and *p*-hydroxybenzoic acid was determined at wavelength of 254 nm by using a 20 μl loop in place of the 0.7 ml loop. Compared with those determined before displacement, we found that the range of change was lower than ±10%. It could be considered that the regeneration efficiency was good and the column was regenerated completely.

3.3. Effect of displacer concentration on separation efficiency

Dichloromethane–methanol (9:1, v/v) and ethanolamine were used as carrier and displacer. Sample concentration was 120 mg/ml (PE:PC 1:1.16) and loading volume was 0.7 ml. In fact, the loading amount was 84 mg (120 mg/ml × 0.7 ml), which was separated at the flow-rate of 0.1 ml/min. Fractions of the column effluent were collected in 0.2 ml. Solvents of the fractions were removed by blowing N_2. The residues were weighted and then dissolved in chloroform–methanol (2:1, v/v) to a certain volume for determining the quantities of PC and PE by HPLC. The molar concentrations of fractions were calculated from above analytical results, which were used to construct the displacement chromatograms. The purity and yield of the corresponding products were also calculated from above results according to different ways to combine the fractions. In addition, the acidity or basicity of the fractions was determined by pH paper. It was indicated that displacer (ethanolamine) was effluent out of the column if the pH paper showed blue. The effect of displacer concentration on separation efficiency was evaluated by changing displacer concentration as the followed: 333, 167, 83 and 42 mM. Fig. 1A–D shows the displacement chromatograms of different displacer concentrations. The purity and yield of the products obtained with different ways to combine the fractions are listed in Table 2.

The yield of the pure PE (100% purity) increased with decreasing displacer concentration: 79.5% by 333 mM, 89.5% by 167 mM, 94.8% by 83 mM and 96.8% by 42 mM. Generally, the separation efficiency was better in lower concentration than higher concentration. There was only slightly different on separation efficiency between 42 and 83 mM. The yield of the PE product (95.8% purity) was 104.3% by 42 mM and that of the PE product (97% purity) was 102.7% by 83 mM, which were all better than those in higher concentrations (333 and 167 mM). The purity of the PE product was lower than 90% when the yield was almost 100% in higher concentrations (see Table 2). The concentrations of some PE fractions were higher than those of the PC fractions and displacer when the displacer concentration was 42 mM (see Fig. 1D), which might be resulted from this displacer concentration to be too low, so displace-

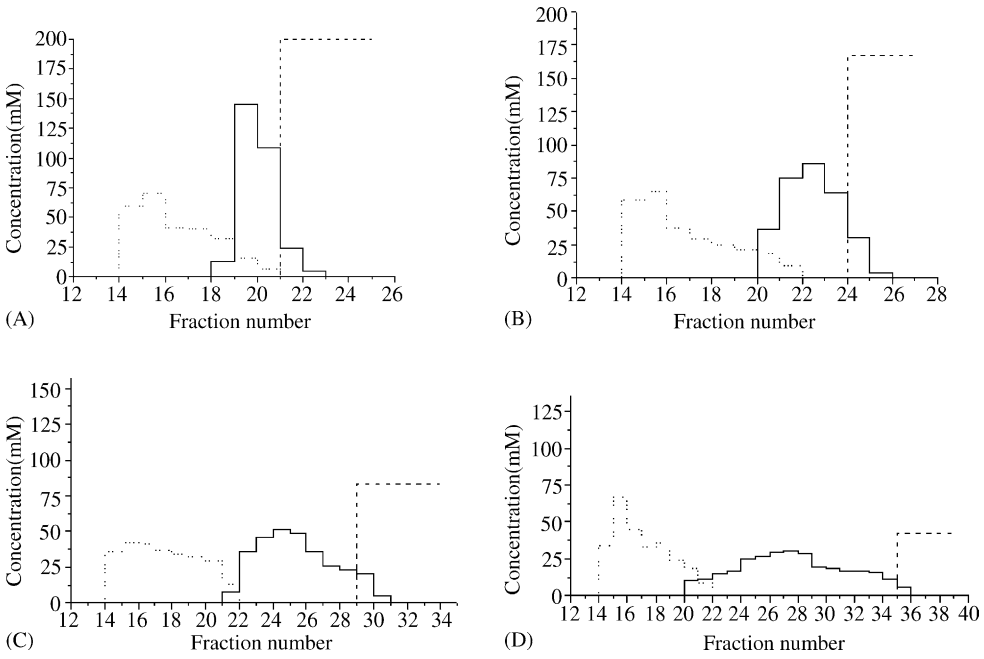

Fig. 1. Effect of displacer concentration on the resolution of standard PC and PE. Column, 150 mm × 4.6 mm silica column; carrier, dichloromethane–methanol (90:10, v/v); flow-rate, 0.1 ml/min; fraction volume, 0.2 ml; feed, 84 mg sample (PE:PC 1:1.16) in 0.7 ml; displacer, ethanolamine (A) 333 mM, (B) 167 mM, (C) 83 mM, (D) 42 mM. The concentrations of PE, PC and displacer are shown by dot, solid and dash lines.

ment not to be fully developed in present column length [6].

The effect of displacer concentration on the purity and yield of the PC product was great. The purity of the product decreased with increasing the yield at the same displacer concentration. No pure PC (100% purity) was obtained by 333 mM, however, with the purity to be decreased to 83.9%, the yield increased to 107.6% by 333 mM. The yield of the pure PC (100% purity) was 60.7% and that of the PC product (96.7% purity) was 89.2% by 167 mM. There was also not an obvious difference on the purity and yield of the PC product between 83 and 42 mM, in which the yields of the pure PC (100% purity) were 87.9 and 91.0%, respectively. The yield was 94.6% when the purity of the PC product was 95.7% by 83 mM. The PC product with high purity and yield was also obtained by 42 mM. The yield of the high purity PC product (97.5% purity) was 97.3% by 42 mM (see Table 2).

In summary, the separation efficiency was perfect when displacer concentration was 83 mM or 42 mM. In above conditions, the yields of the pure PE and PC (100% purity) were all high. The separation efficiency was also good when displacer concentration was 167 mM. The concentrations of the products and separation time were determined by displacer concentration when the other conditions were fixed. The higher displacer concentration was used, the higher concentration of the product could be obtained and the less

Table 2
Effect of displacer concentration on separation efficiency

Displacer concentration (mM)	PE		PC	
	Purity (%)	Yield (%)	Purity (%)	Yield (%)
333	100.0	79.5	100.0	0
	84.5	97.1	94.6	38.8
			90.9	53.4
			83.9	107.6
167	100.0	89.5	100.0	60.7
	86.7	111.3	96.7	89.2
			91.8	107.6
83	100.0	94.8	100.0	87.9
	97.0	102.7	95.7	94.6
42	100.0	96.8	100.0	91.0
	95.8	104.3	97.5	97.3
			92.0	106.5

separation time would be taken [6], which was the same as our result (see Fig. 1). The advantages of high concentration of the product were that the consumption of solvent would be decreased and the energy for recovery of solvent would be saved. The period of production would be shortened when separation time was reduced. All these would result in the decrease of the production cost, which was a primary factor to be considered for preparative separation. Therefore, the higher displacer concentration should be used on the condition of separation efficiency to be guaranteed. Amongst several concentrations chosen in our work, 333 mM was too high to obtain satisfactory separation and 42 mM was too low because separation time was taken too much despite the purity and yield of the product being high. 83 and 167 mM were desirable concentrations, and which one was used should depend on the requirement for quality of the product. If the purity of the product was required more than 95% and the yield was also needed high, 83 mM should be used. However, if the requirement was rather low, 167 mM could be used for saving the solvent and energy consumption and reducing the separation time.

3.4. Effect of the flow-rate of displacer on separation efficiency

The displacer concentration was 167 mM and the other conditions and procedures were the same as in Section 3.3 in addition to the flow-rate. The effect of the flow-rate of displacer on separation efficiency was investigated by changing the flow-rate from 0.1 to 0.5 ml/min. Fig. 2A and B shows the displacement chromatograms under conditions of the different flow-rate. The purity and yield of the products are given in Table 3.

The yields of the pure PC and PE (100% purity) decreased with the increase of the flow-rate. As the flow-rate increased from 0.1 ml/min to 0.2 ml/min and 0.5 ml/min, the yield of the pure PE decreased from 94.8 to 78.6 and 62.2%, and that of the pure PC decreased greatly from 87.9 to 60.2 and 0.0% (see Table 3). Consequently, the separation efficiency was the best when the flow-rate was 0.1 ml/min and both the purity and yield of the product were high. If the purity of the product was not strictly required, the flow-rate of 0.2 ml/min also could be used. In this condition, the yield of the PE product (85.7% purity) was 112.1% and that of the PC product (90.2% purity) was 96.2%. Although the purity and yield of the product were lower at the flow-rate of 0.2 ml/min than those at 0.1 ml/min, the separation time would be reduced half. The separation efficiency was bad at the flow-rate of 0.5 ml/min, it was indicated that the flow-rate was too fast to fully develop the displacement train in our HPDC system. Hence, the flow-rate of 0.1 or 0.2 ml/min should be chosen according to different requirements for the quality of the product.

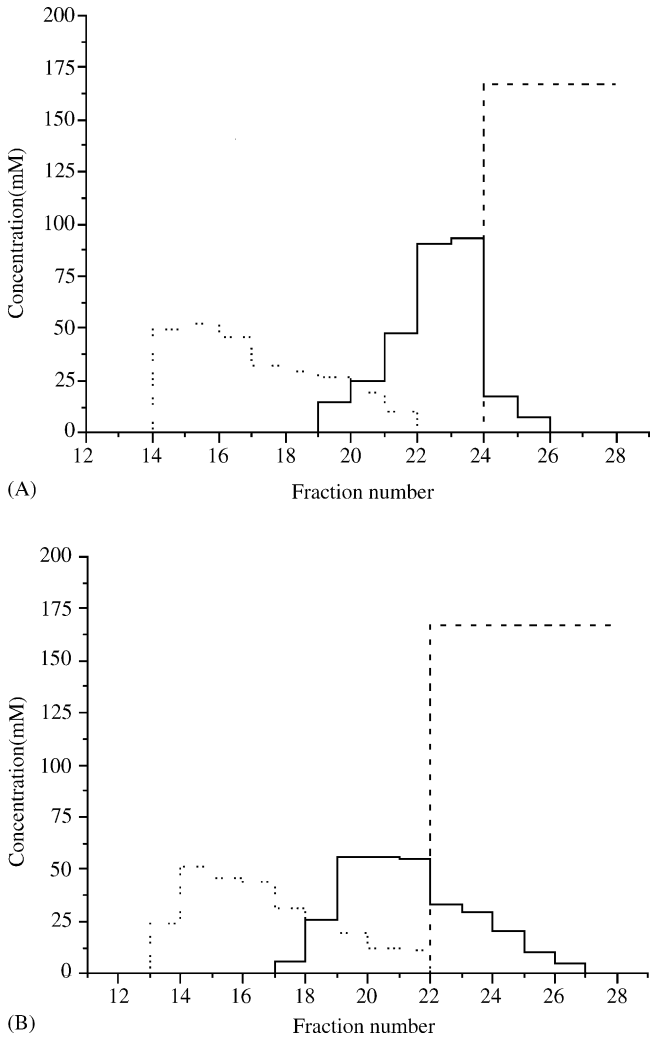

Fig. 2. Effect of flow-rate on the resolution of standard PC and PE. Column, 150 mm × 4.6 mm silica column; carrier, dichloromethane–methanol (90:10, v/v); flow-rate, (A) 0.2 ml/min, (B) 0.5 ml/min; fraction volume, 0.2 ml; feed, 84 mg sample (PE:PC 1:1.16) in 0.7 ml; displacer, 167 mM ethanolamine. The concentrations of PE, PC and displacer are shown by dot, solid and dash lines.

Table 3
Effect of flow-rate on separation efficiency

Flow-rate (ml/min)	PE		PC	
	Purity (%)	Yield (%)	Purity (%)	Yield (%)
0.1	100.0	94.8	100.0	87.9
	97.0	102.7	95.7	94.6
0.2	100.0	78.6	100.0	60.2
	85.7	112.1	90.2	96.2
			83.6	109.9
0.5	100.0	62.2	100.0	0
	86.9	96.9	80.4	70.1
	72.3	126.6	74.8	87.1
			67.4	99.5

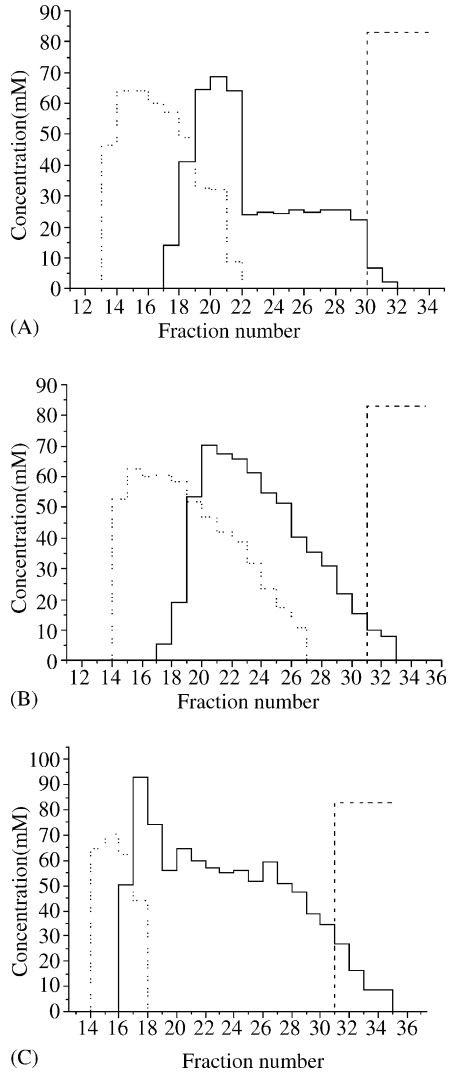

Fig. 3. Effect of loading amount and composition of the sample on the resolution of standard PC and PE. Column, 150 mm × 4.6 mm silica column; carrier, dichloromethane–methanol (90:10, v/v); flow-rate, 0.1 ml/min; fraction volume, 0.2 ml; feed, (A) 130 mg sample (PE:PC 1:1.16) in 0.7 ml, (B) 175 mg sample (PE:PC 1:1.16) in 0.7 ml, (C) 175 mg sample (PE:PC 1:4) in 0.7 ml; displacer, 83 mM ethanolamine. The concentrations of PE, PC and displacer are shown by dot, solid and dash lines.

3.5. Effect of loading amount and composition of the sample on separation efficiency

The displacer concentration was 83 mM and the other conditions were the same as in Section 3.3 expect loading amount. The effect of loading amount on separation efficiency was evaluated by changing the loading sample concentration as follows: 186 mg/ml (PE:PC 1:1.16), 250 mg/ml (PE:PC 1:1.16) and 250 mg/ml (PE:PC 1:4). Fig. 3A–C shows displacement chromatograms of different loading amount. The purity and yield of the product are presented in Table 4.

Because loading volume was 0.7 ml, the factual loading amounts were 130 mg (PE:PC 1:1.16), 175 mg (PE:PC 1:1.16) and 175 mg (PE:PC 1:4).

Table 4
Effect of loading amount and composition of the sample on separation efficiency

Loading amount	PE		PC	
	Purity (%)	Yield (%)	Purity (%)	Yield (%)
130 mg (PE:PC 1:1.16)	100.0	56.7	100.0	43.2
	95.1	74.1	96.9	59.1
	85.3	96.5	89.6	80.8
			85.1	101.8
175 mg (PE:PC 1:1.16)	100.0	31.4	100.0	16.9
	94.5	43.6	93.3	25.2
	91.9	57.3	88.0	36.3
			83.8	48.9
175 mg (PE:PC 1:4)	100.0	56.0	100.0	77.6
	78.7	103.9	95.1	92.4
			89.4	104.3

Loading amount in every production period was a vital index for preparative separation. The time would be consumed more for the equal quantity sample if the loading amount was too low, which would lead to increase the production cost. However, the loading amount could not be too large, otherwise, the separation efficiency would be bad when it surpassed a certain point (the allowed maximum of loading). When the column was overloaded, the larger or longer column or slower flow-rate was required to achieve separation [2]. Hence, selection of an appropriate loading amount (the allowed maximum of loading) was an important factor for preparative displacement separation.

With the increase of the loading amount from 84 mg (PE:PC 1:1.16) to 130 mg (PE:PC 1:1.16) and 175 mg (PE:PC 1:1.16), the purity and yield of the products decreased greatly. The yield of the pure PE (100% purity) decreased from 94.8 to 56.7 and 31.4%, and that of the pure PC (100% purity) decreased from 87.9 to 43.2 and 16.9%. The yield of the PE product (97% purity) was 102.7% and that of the PC product (95.7% purity) was 94.6% in 84 mg (see Table 2). When the loading amount increased to 130 mg (PE:PC 1:1.16), the yield of the PE product (95.1%) decreased to 74.1% and that of the PC product (89.6% purity) decreased to 80.8%. When the loading amount further increased to 175 mg (PE:PC 1:1.16), the yields of the PE product (94.5% purity) and the PC product (88.0% purity) were only 43.6 and 36.3%, respectively (see Table 4). The above results illustrated that the yield and purity of the product decreased with the increase of the loading amount in our work, which was the same as the result of ref. [2]. When the loading amount was 130 mg (PE:PC 1:1.16), the separation efficiency was inferior to that of 84 mg (PE:PC 1:1.16). However, it could be accepted if the requirement for the quality of the product was not high. Therefore, for obtaining the good separation, the loading amount must be lower than 130 mg (PE:PC 1:1.16).

The effect of composition of the sample on separation efficiency was also investigated in this work. The separation

efficiency was distinctly different even if the loading amount was equal when the proportion of PE to PC was different in the sample. Although the loading amount were the same as 175 mg, the yields of the pure PE and PC (100% purity) were improved from 31.4 and 16.9 to 56.0 and 77.6% when the proportion of PE to PC was adjusted from 1:1.16 to 1:4 (see Table 4). The result was also demonstrated that the effect on the purity and yield of the PC product was greater with the increase of the PC proportion in the sample. The yield of the PC product (93.3% purity) was only 25.2% when the proportion of PE to PC was 1:1.16, however, that of the PC product (95.1% purity) was as high as 92.4% when that was 1:4.

As a whole, the separation efficiency was not only correlated with the loading amount, but also with the composition of the sample under a certain condition. Because the compositions of each natural phospholipids were different, the optimum loading amount (the allowed maximum of loading) should be selected by using the simulating sample, the composition of which was the same as the actual sample to be separated.

The purpose of using the binary mixture (PE:PC 1:4) as the sample was just to simulate the compositions of egg phospholipids and ethanol extracted soybean phospholipids [31], which was the basis for further research of these actual samples by using HPDC method.

3.6. Separation of soybean PC and PE by HPDC

About 132 mg raw product was obtained from 200 mg crude PC. The contents of PC and PE of the raw product are given in Table 5. Fig. 4 shows the HPLC chromatogram of the raw product.

The raw product was separated by using 83 mM ethanolamine and dichloromethane–methanol (9:1, v/v) as displacer and carrier. The flow-rate of displacer was set at 0.1 ml/min. The loading volume was 0.7 ml and the actual loading amount was 92.4 mg (132 mg/ml × 0.7 ml). The effluents were collected in 2 min intervals. The other procedures were the same as above. Fig. 5 shows the displacement chromatogram constructed by depending on the analytical results. From Fig. 5, we could found that 13–17 fractions contained PE, 19–26 fractions contained PC, fraction 18 contained little PE and a great deal of PC and the displacer

Table 5
The compositions of the raw product, soybean PE and PC product and yield of the soybean PE and PC product

	PE (%)	PC (%)	Others (%)	Yield (%)
Raw product	17.3	66.5	16.2	
PE product	72.2[a]	ND[b]	27.8	117.9
PC product	1.3	88.9[a]	9.8	95.1

[a] The content of PE or PC in the soybean PE or PC product is the purity of that.
[b] ND refers PC to be not detected.

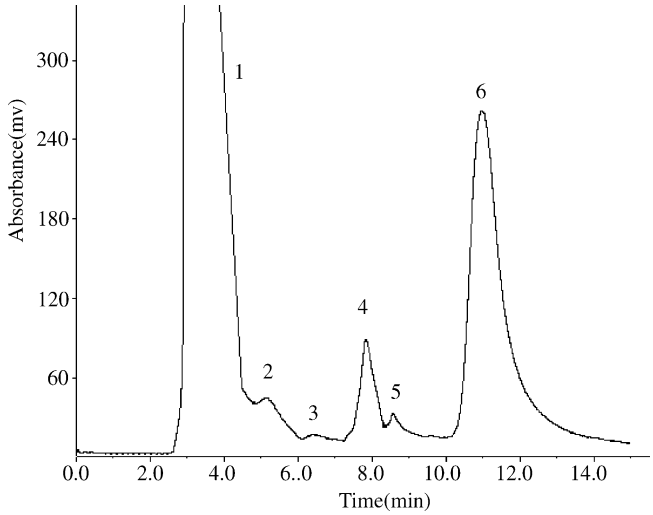

Fig. 4. Chromatogram of the raw product. Column, 150 mm × 4.6 mm silica column; mobile phase, acetonitrile–methanol–85% phosphoric acid (180:3:1, v/v); injection volume, 20 μl; flow-rate, 0.5 ml/min; sample concentration, 1 mg/ml (in chloroform–methanol, 2:1, v/v). Peaks: (1) solvents + impurities; (2, 3 and 5) impurities; (4) PE; (6) PC.

emerged after fraction 27. Thus, fraction 13–17 and 18–26 were combined to recover PE and PC by removing solvents under vacuum at 50 °C, respectively. The main components and yield of the soybean PE and PC product are presented in Table 5. Figs. 6 and 7 show the HPLC chromatograms of the soybean PE and PC product.

Compared with the result of displacement separation of standard PC and PE, the purity of the soybean PE and PC product was slightly low. The purity of the soybean PE product was only 72.2% and that of the soybean PC product was 88.9% when the yield was 117.9 and 95.1%, respectively. Although the purity of the product was not very high, it was

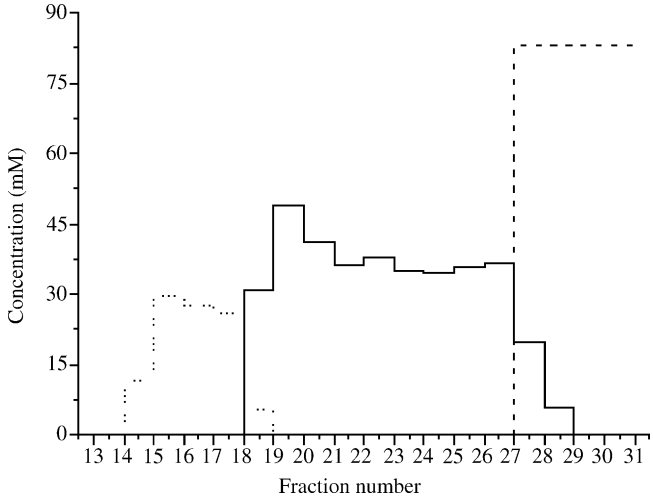

Fig. 5. Displacement chromatogram of soybean PE and PC. Column, 150 mm × 4.6 mm silica column; carrier, dichloromethane–methanol (90:10, v/v); flow-rate, 0.1 ml/min; fraction volume, 0.2 ml; feed, 92.4 mg in 0.7 ml; displacer, 83 mM ethanolamine. The concentrations of PE, PC and displacer are shown by dot, solid and dash lines.

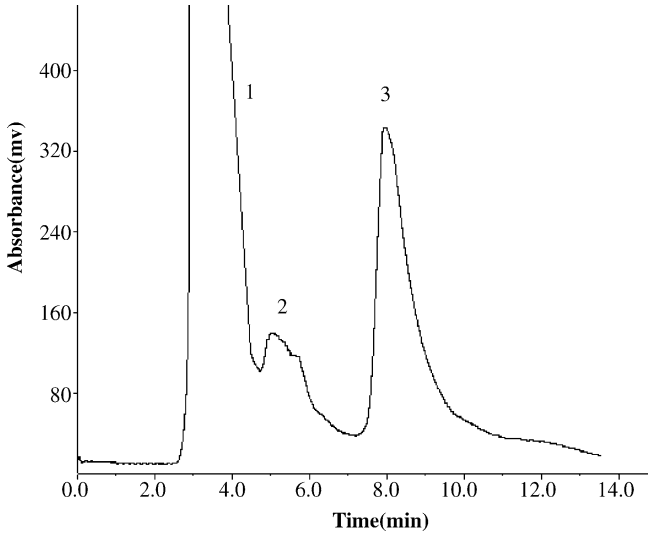

Fig. 6. Chromatogram of the soybean PE product. Column, 150 mm × 4.6 mm silica column; mobile phase, acetonitrile–methanol–85% phosphoric acid (180:3:1, v/v); injection volume, 20 μl; flow-rate, 0.5 ml/min; sample concentration, 1 mg/ml (in chloroform–methanol, 2:1, v/v). Peaks: (1) solvents + impurities; (2) impurities; (3) PE.

demonstrated that displacement separation of PE and PC in soybean phospholipids was performed. PC was not detected in the PE product, and there was only 1.3% of PE in the PC product (see Table 5). The product purities were not high due to a number of impurities existing in the raw product besides PE and PC. These impurities were eluted out of the column accompanying PE or PC because their adsorption characteristics did not meet the requirements for displacement under the conditions in this work. Consequently, the purity of PE or PC product was affected by these impurities, especially for the former (impurity of 27.8% in the soybean PE product and only 9.8% in the soybean PC product, see Table 5).

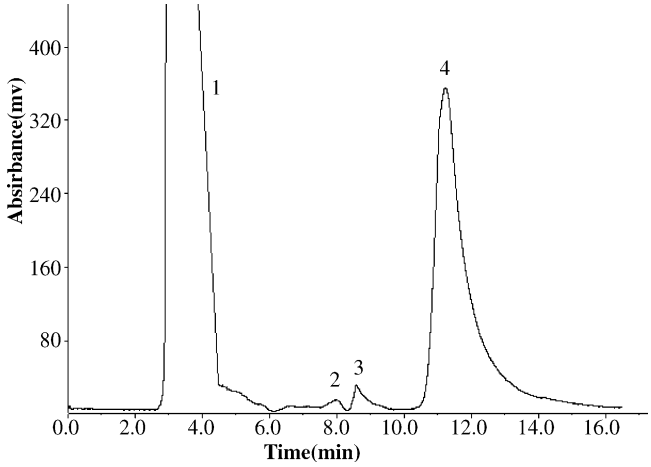

Fig. 7. Chromatogram of the soybean PC product. Column, 150 mm × 4.6 mm silica column; mobile phase, acetonitrile–methanol–85% phosphoric acid (180:3:1, v/v); injection volume, 20 μl; flow-rate, 0.5 ml/min; sample concentration, 1 mg/ml (in chloroform–methanol, 2:1, v/v). Peaks: (1) solvents + impurities; (2) PE; (3) impurities; (4) PC.

From the displacement chromatogram in Fig. 5, it was also illustrated that PE and PC were separated completely. Hence, the displacement chromatography used for separation of phospholipids was feasible.

4. Conclusion

TLC was a simple and effective method for selection of carrier and displacer when silica was used as stationary phase in normal-phase HPDC.

Eighty-four milligrams binary mixture of PC and PE (PE:PC 1:1.16) was successfully separated by HPDC on an 150 mm × 4.6 mm analytical silica column at the flow-rate of 0.1 ml/min when dichloromethane–methanol (9:1, v/v) and 83 mM ethanolamine were used as carrier and displacer, and the yield of the pure PE and PC were 94.8 and 87.9%. If the proportion of PE to PC was adjusted to 1:4, 175 mg mixture could be successfully separated. Under this condition, the yield of the pure PC (100% purity) was 77.6% and even when the purity of the PC product was 95.1%, the yield of that was as high as 92.4%. The cycle time for a single separation was about 195 min.

The separation efficiency of displacement chromatography was affected by various factors such as displacer concentration, flow-rate of displacer, loading amount, etc., and it was also affected by the composition of the sample. Even though for the equal loading amount, the separation efficiency was very different under the same chromatography condition due to the different contents of the components in the sample. Hence, it was not enough to investigate the allowed maximum of loading if the composition of the sample was not considered. Only the composition of sample was similar to the actual one to be separated, the results obtained from these experiments would be valuable.

As a chromatography mode appropriate for preparative separation, the displacement chromatography exhibits the potentiality of application in the field of preparation of phospholipids owing to inherent advantages such as high loading amount and concentration of the product, low solvent consumption, etc.

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