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Optimization of parameters of high-performance displacement chromatography for separation of soybean phosphatidylcholine and phosphatidylethanolamine

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Abstract

Hundred milligrams of soybean phospholipids were successfully separated by using high-performance displacement chromatography (HPDC) on a 150 mm × 4.6 mm analytical silica column (3–5 μm packings) with dichloromethane–methanol (9:1, v/v) as carrier and ethanolamine as displacer. From the viewpoint of preparative separation, the effects of loading amount, concentration and flow-rate of displacer on separation efficiency were investigated using throughput and recovery as indices. The parameters were optimized by orthogonal test design and statistical analysis method. Under the optimum conditions, namely displacer concentration being 167 mM, the flow-rate of displacer at 0.2 ml/min and concentration of sample being 211 mg/ml (factual loading amount 211 mg/ml × 0.7 ml = 148 mg), the purity, throughput and recovery of obtained soybean phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were 80.2%, 65.7 mg/h, 70.9% and 90.5%, 272.6 mg/h, 88.3%, respectively. In addition, selections of regenerant and appropriate regeneration condition were also studied.

Keywords: Displacement chromatography; Phosphatidylcholine; Phosphatidylethanolamine; Soybean phospholipids; Lipids

1. Introduction

Phospholipids, main component of cell membrane, have a lot of important biological functions [1–3]. High purity phospholipids fractions, i.e., phosphatidylcholine (PC) and phosphatidylethanolamine (PE), have been used widely in the fields of nourishment, pharmaceuticals and cosmetics due to their perfect emulsification and penetration abilities. Especially on pharmaceuticals, the demand for high purity phospholipids fractions are rapidly increasing with liposomes formed from pure phospholipids being applied as ideal carriers for drugs which have so called "target" and can improve cure efficiency and reduce pain of patients [4–10]. At present, soybean phospholipids, the major source of commercial phospholipids, are usually prepared as by-product of oil refinery. High purity phospholipids fractions were usually prepared by HPLC (elution mode) [11–14], in which there were some inherent drawbacks, i.e., low loading amount and high solvent consumption, which lead to high production cost and price. It limits the application of phospholipids. Thus, it becomes an urgent problem need to be solved to find a new preparation method with high efficiency and low cost.

Compared with elution chromatography, displacement chromatography has some advantages such as large loading amount, high product concentration, little tailing, low solvent consumption and high efficiency of use of the stationary phase. Hence, it is fit to be used as preparative chromatography. The theory and application of displacement chromatography were detail studied and reported by Horváth, Cramer and other scientists [15–31]. In order to overcome the innate shortcomings of elution mode, displacement chromatography was successfully tried to separate soybean PC and PE in our previous work [32].

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The purpose of this paper is to optimize parameters of displacement chromatography in order to base for large-scale use. Selections of regenerant and regeneration conditions were also studied. The effects of loading amount, concentration and the flow-rate of displacer on separation efficiency were investigated, and these parameters were optimized by orthogonal test and statistical analysis method.

2. Experimental

2.1. Chemicals

Standard phosphatidylcholine and phosphatidylethanolamine were purchased from Sigma (Superior-chemicals & Instrument Co., Beijing, China). Soybean phospholipids powder (acetone insolubility >90%) was provided by Jingban Food Co. (Shanghai, China). Silica gel (100–200 mesh, irregular) used for column chromatography (400 mm × 30 mm) was obtained from Haiyang Chemical Group (Qingdao, China).

Methanol, ethanol, chloroform, dichchloromethane, ethanolamine, 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 consisted of a LC-6A pump (Shimadzu, Tokyo, Japan), an SPD-10A ultraviolet (UV) detector (Shimadzu) and a Rheodyne 7725i injector with a 0.7 ml and a 20 μl sample loop (Cotati, California, USA). The column used for HPDC was a 150 mm × 4.6 mm column packed with home-made silica (3–5 μm).

The analytical HPLC apparatus was kindly provided by Dalian Elite Analytical Apparatus Limited Company, which consisted of a P200 II pump (Elite, Dalian, China), an UV200 II ultraviolet (UV) detector (Elite) and a Rheodyne 7725i injector with 20 μl sample loop (Cotati, California, USA). 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 home-made silica. The acetonitrile–methanol–85% phosphoric acid (180:3:1, v/v) was used as the mobile phase, filtrated and degassed before use [33]. 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. [33].

2.3. Preparation of raw material

About 50 g soybean phospholipids powder was pretreated by the followed procedures: acetone washing, 95% ethanol extraction, acetone precipitation and solvent recovery. The solid phospholipids was further purified by using a 400 mm × 30 mm column packed with silica according to Mounts's method and adjusted as the follows [34]: it was eluted sequentially with chloroform (500 ml), acetone (250 ml), chloroform–methanol (3:1, v/v) (250 ml) and methanol (750 ml). The methanol fractions were collected and removed solvents under vacuum at 50 °C. The residues were used as raw material for separation by displacement chromatography.

2.4. Selections of regenerant and regeneration conditions

The system was first equilibrated with carrier at the flow-rate of 1 ml/min and wavelength was 254 nm. The retention time ($t_{R,b}$) for *p*-hydroxybenzoic acid was determined by loading 20 μl sample. And then the column was eluted with ethanolamine (dissolving into carrier) of certain concentration at a certain flow-rate, and the wavelength was adjusted at 203 nm. When the breakthrough curve was observed, it illustrated that the column was saturated. The column was eluted by a candidate regenerant replacing ethanolamine. After a period, the system was re-equilibrated by carrier. And then the retention time ($t_{R,a}$) of 20 μl *p*-hydroxybenzoic acid was determined by adjusting detection wavelength at 254 nm. Regeneration efficiency (RE, %) was evaluated by the followed formula:

$$\mathrm{RE}\,(\%) = \left(1 - \frac{t_{R,a} - t_{R,b}}{t_{R,b}}\right) 100\%$$

This formula used for evaluation of RE depended on the variation extent of retention time for *p*-hydroxybenzoic acid between before and after regeneration. When RE > 90%, which meant variation of retention time for *p*-hydroxybenzoic acid being less than 10%, it could be considered thorough regeneration. If RE < 90%, the column was then continuously eluted by above regenerant and took down the elution time. Repeated above evaluation procedure till RE was more than 90%. After evaluating one kind of regenerant, the system was equilibrated by carrier again and then repeated above procedures to evaluate other candidate regenerants.

The effect of regeneration conditions on RE was investigated by changing the flow-rate of selected regenerant. The operation process and estimate method were the same as above.

2.5. Effect of chromatography conditions on separation efficiency

Throughput and recovery are two of the most important indices for preparative separation. Throughput of a given certain purity product was defined as product quantity of unit time (mg/h). In this paper, consumption time referred to the time during displacement separation period. Recovery was evaluated by dividing the quantity of the product by that of

PE or PC in the loading sample. Two indices could be comprehensively evaluated by the followed formula [35]:

$$Y_i = \alpha T_i + \beta R_i \quad (1)$$

where Y_i was a comprehensive evaluation index; T and R referred to throughput and recovery, respectively; i was footnote representing different conditions; α and β were coefficients that could be calculated by the following formula:

$$\alpha = \frac{a}{T_{max} - T_{min}} \quad (2)$$

$$\beta = \frac{b}{R_{max} - R_{min}} \quad (3)$$

where T_{max}, T_{min} and R_{max}, R_{min} referred to the maximum, minimum throughput and maximum, minimum recovery for a series of changing conditions; a and b were weight coefficients determined by depending on relatively important of two indices ($a + b = 100$), which were equal important in this paper, thus $a = b = 50$.

Owing to PE and PC product to be obtained simultaneously, the total comprehensive evaluation including two products was needed as the followed when evaluating separation efficiency under certain condition:

$$CV_i = \frac{Y_{PE,i} + Y_{PC,i}}{2} \quad (4)$$

Where CV_i was total evaluation index; $Y_{PE,i}$ and $Y_{PC,i}$ were indices comprehending throughput and recovery of PE and PC product, respectively, which could be calculated according to formula (1)–(3).

2.5.1. Effect of displacer concentration on separation efficiency

The system was first equilibrated with carrier, dichloromethane–methanol (90:10, v/v), at the flow-rate of 1 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 soybean phospholipids of which concentration was 150 mg/ml. The pump was turned on when the valve was turned to "Injection" position. The feed was pushed into the column by displacer at the flow-rate of 0.1 ml/min. The effluent was collected in 0.2 ml intervals and analyzed by HPLC. The collected effluent was combined to ensure the purity of PE and PC to be more than 80 and 90%, respectively, depending on HPLC analysis results. The combined fractions including PE or PC were removed solvents under vacuum at 50 °C. The PE and PC product were weighted and the purity of them were determined using HPLC by re-dissolving them into chloroform–methanol (2:1, v/v). Throughput, recovery and the total evaluation index could be calculated according to above formulas. After emergence of the displacer front, the column was regenerated by pumping regenerant into the column, and then it was equilibrated with carrier. The system could be used for next separation after equilibrium.

The effect of displacer concentration on separation efficiency was studied by changing displacer concentration as 42, 83, 167 and 333 mM.

2.5.2. Effect of flow-rate of displacer on separation efficiency

The displacer concentration was 83 mM and the other conditions and procedures were the same as in Section 2.5.1 except the flow-rate. The effect of flow-rate of displacer was investigated by changing the flow-rate as 0.1, 0.2 and 0.3 ml/min.

2.5.3. Effect of loading amount on separation efficiency

The displacer concentration was 83 mM and the other conditions were the same as in Section 2.5.1 in addition to loading amount. The effect of loading amount was evaluated by changing the loading sample as 150, 211 and 318 mg/ml.

2.6. Optimization of parameters of HPDC for separation of soybean PC and PE

The separation parameters of HPDC were optimized by orthogonal test design. Loading amount, concentration and flow-rate of displacer were taken as factors to be optimized. The investigated levels of each factor were selected depending on above experiment results. Throughput and recovery were used as indices to evaluate separation efficiency.

3. Results and discussions

3.1. Selection of regenerant and regeneration condition

The solution to be selected as regenerant must meet two requirements. It can remove displacer from the stationary phase quickly as well as can be easily replaced by carrier for the next separation. It is helpful to achieve above goal when selecting the solvents of carrier as the main compositions of the regenerant. In this work, dichloromethane–methanol (90:10, 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 different proportions of three solvents were investigated, and the analysis results are listed in Table 1.

Firstly, the proportion of acetic acid was fixed and just changed proportions of dichloromethane and methanol. When proportion of dichloromethane–methanol–acetic acid was 60:30:10 (v/v), RE was 84.9% after regenerating 60 min. It was completely regenerated till 75 min, RE being 91.3% (>90%) at this moment. When proportion of dichloromethane was increased to 70:20:10 (v/v), it was needed 105 min to achieve regeneration requirement (RE = 92.3%). With the proportion of dichloromethane further being increased to

Table 1
The regeneration efficiencies of different regenerants

Regenerants	Regeneration efficiency (RE) (%)[a]			
	60 min	75 min	90 min	105 min
Dichloromethane–methanol–acetic acid (60:30:10, v/v)	84.9	91.3	–	–
Dichloromethane–methanol–acetic acid (70:20:10, v/v)	77.0	85.5	88.5	92.3
Dichloromethane–methanol–acetic acid (80:10:10, v/v)	49.6	64.7	78.4	85.1
Dichloromethane–methanol–acetic acid (60:25:15, v/v)	92.5	–	–	–
Dichloromethane–methanol–acetic acid (60:35:5, v/v)	78.6	85.7	90.3	–

[a] Regeneration efficiency (RE) is evaluated by the following formula: RE (%) = $[1 - (t_{R,a} - t_{R,b})/t_{R,b}] \times 100\%$, where $t_{R,b}$ and $t_{R,a}$ refer to the retention time of *p*-hydroxybenzoic acid before the displacement and after regeneration, respectively.

80:10:10 (v/v), the system was not regenerated completely till 105 min (RE = 85.1% < 90%), see Table 1. Consequently, RE decreased with the increase of proportion of dichloromethane when acetic acid was fixed. Relative low proportion of dichloromethane was helpful to enhance RE. However, the proportion of dichloromethane could not too low due to the proportion of dichloromethane in carrier being 90%. A long time was needed to re-equilibrate the system if the difference between proportion of dichloromethane in regenerant and carrier was large, which was a disadvantage for improving throughput. From the above analyses, the proportion of dichloromethane was thus selected at 60%.

Secondly, the proportion of dichloromethane was fixed at 60% and the proportions of acetic acid and methanol were changed. When dichloromethane–methanol–acetic acid was 60:35:5 (v/v), the system was thoroughly regenerated till 90 min (RE = 90.3%). However, just 60 min was needed to finish regeneration (RE = 92.5%) when proportion of three solvents was 60:25:15 (v/v), see Table 1. Notwithstanding RE increased with the increase of acetic acid in regenerant, the proportion of acetic acid was unfit to be too high. High acetic acid proportion would result in damage of chromatography system and long time for re-equilibrium. Hence, it was appropriate to select dichloromethane–methanol–acetic acid (60:30:10, v/v) as regenerant.

Regeneration time would directly affect the throughput of product. It was determined by the flow-rate of regenerant when other conditions did not vary. RE of regenerant at different flow-rate is presented in Table 2. RE was 90.9%, which needed 75 min at the flow-rate of 1 ml/min. When the flow-rate was increased at 1.5 ml/min, 60 min was needed (RE = 94.4%). With further increasing at 2 ml/min, just only 45 min was needed (RE = 92.8%), see Table 2. Hereby, regeneration time decreased with the increase of the flow-rate. Because high flow-rate would result in high column pressure which was a disadvantage for system, relatively high flow-rate should be used to save time if permission of column pressure.

Table 2
Effect of the flow-rate of regenerant on regeneration efficiency[a]

Flow-rate (ml/min)	Regeneration efficiency (RE) (%)[b]			
	30 min	45 min	60 min	75 min
1	25.6	62.7	83.1	90.9
1.5	64.0	86.9	94.4	–
2	87.4	92.8	–	–

[a] Dichloromethane–methanol–acetic acid (60:30:10, v/v) is used as regenerant.
[b] The method of evaluation of regeneration efficiency is the same as that in Table 1.

3.2. Effect of HPDC conditions on separation efficiency

The raw material used for HPDC separation was pretreated by using solvent extraction and column chromatography, which contained 18.8% PE and 72.2% PC.

The purity, throughput and recovery of PE and PC product under different conditions of loading amount, concentration and flow-rate of displacer are listed in Tables 3–5, respectively. The calculated comprehensive evaluation indices (*Y*) according to formula (1)–(3) are also presented in Tables 3–5. The total evaluation index (CV) was calculated from Y_{PE} and Y_{PC} according to formula (4). The value of CV could be used to evaluate the separation efficiency. The effects

Table 3
Effect of displacer concentration on separation efficiency

Displacer concentration (mM)	PE				PC			
	Purity (%)	Throughput (mg/h)	Recovery (%)	Y_{PE}[a]	Purity (%)	Throughput (mg/h)	Recovery (%)	Y_{PC}[a]
42	79.7	18.2	84.8	262.0	92.4	66.6	94.5	503.9
83	81.5	23.2	94.3	308.4	90.4	78.8	93.7	520.8
167	80.0	26.7	88.9	319.5	93.2	92.0	94.0	543.8
333	81.3	23.5	66.9	260.6	92.4	96.9	82.5	503.9

[a] Y_{PE} and Y_{PC} are comprehensive evaluation of two indices of throughput and recovery for PE and PC products, respectively. They are calculated depending on the formula (1)–(3) in the text.

Table 4
Effect of the flow-rate of displacer on separation efficiency

Flow-rate (ml/min)	PE				PC			
	Purity (%)	Throughput (mg/h)	Recovery (%)	Y_{PE}[a]	Purity (%)	Throughput (mg/h)	Recovery (%)	Y_{PC}[a]
0.1	81.5	23.2	94.3	155.9	90.4	78.8	93.7	180.2
0.2	81.4	38.5	62.7	176.5	91.0	190.3	91.4	216.6
0.3	79.1	39.0	37.8	156.1	89.3	218.3	62.9	180.5

[a] Y_{PE} and Y_{PC} are comprehensive evaluation of two indices of throughput and recovery for PE and PC products, respectively. They are calculated depending on the formula (1)–(3) in the text.

Table 5
Effect of loading amount on separation efficiency

Concentration of sample (mg/ml)	PE				PC			
	Purity (%)	Throughput (mg/h)	Recovery (%)	Y_{PE}[a]	Purity (%)	Throughput (mg/h)	Recovery (%)	Y_{PC}[a]
150	81.5	23.2	94.3	668.6	90.4	78.8	93.7	287.2
211	80.8	25.0	71.4	688.8	92.4	97.5	84.3	286.0
318	81.9	25.2	49.1	668.9	90.0	126.8	70.9	287.1

[a] Y_{PE} and Y_{PC} are comprehensive evaluation of two indices of throughput and recovery for PE and PC products, respectively. They are calculated depending on the formula (1)–(3) in the text.

of HPDC conditions on separation efficiency are shown in Figs. 1–3.

With increasing displacer concentration from 42 to 333 mM, the separation efficiency increased from low to high till at 167 mM to maximum, and then dropped at 333 mM (see Fig. 1).

With increasing the flow-rate of displacer from 0.1 ml/min to 0.3 ml/min, the separation efficiency first increased and then dropped. The maximum was at 0.2 ml/min (see Fig. 2).

There was similar trend as above for loading amount changing. With increasing the loading amount, the separation efficiency also first increased and then dropped. The maximum separation efficiency was observed when loading sample concentration was 211 mg/ml (see Fig. 3).

From above analyses, we found that there was an optimum condition leading to the highest separation efficiency. Horváth obtained similar result when investigating the effect of chromatography conditions on throughput [36]. In this paper, separation efficiency comprehended two indices of throughput and recovery. When the loading amounts, concentrations and flow-rate of displacers surpassed the certain values (optimum conditions), throughput and recovery for PE and PC would decrease due to overlap of PE, PC and displacer peaks increasing.

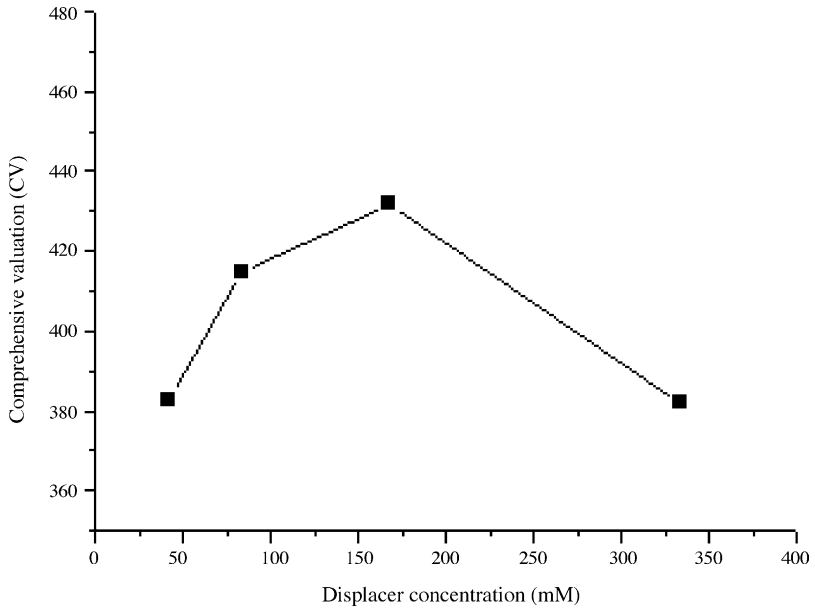

Fig. 1. Effect of displacer concentration on separation efficiency. Column, 150 mm × 4.6 mm silica column; carrier, dichloromethane–methanol (90:10, v/v); flow-rate, 0.1 ml/min; fraction volume, 0.2 ml; sample concentration, 150 mg/ml (in carrier solvent); injection volume, 0.7 ml.

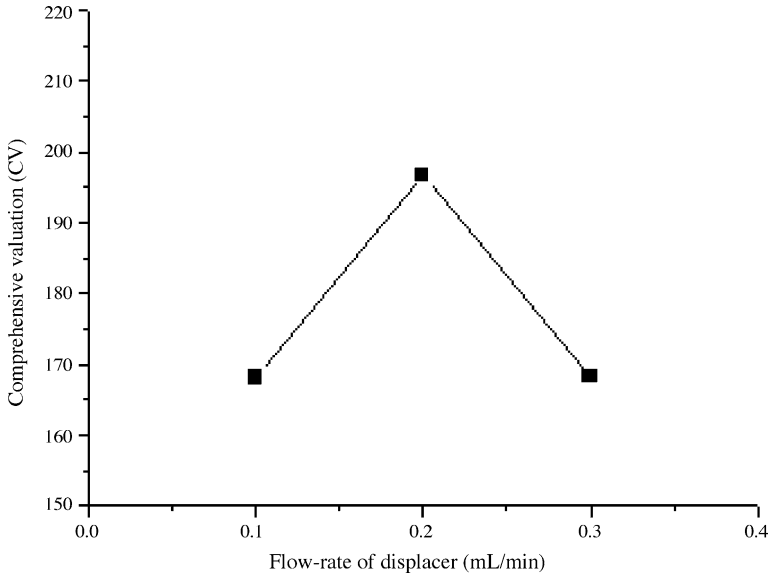

Fig. 2. Effect of flow-rate of displacer on separation efficiency. Column, 150 mm × 4.6 mm silica column; carrier, dichloromethane–methanol (90:10, v/v); displacer, 83 mM ethanolamine; fraction volume, 0.2 ml; sample concentration, 150 mg/ml (in carrier solvent); injection volume, 0.7 ml.

3.3. Result and analysis of orthogonal test

Owing to the separation efficiency being the highest under optimum conditions, it was very important for preparative separation to look for these optimum parameters. The effect of changing single factor on separation efficiency was studied as above (see Figs. 1–3). However, it was not enough to judge what parameter was optimum because other factors were fixed under this condition. The optimum parameters should be obtained by using reasonable test design and mathematical analysis.

The orthogonal test was designed to optimize parameters on the base of above experiment results. Three factors, concentration (A) and the flow-rate (B) of displacer, loading amount (C), were selected for optimization. Three levels of each factor were investigated. In order to look for optimum parameters, the levels were adjusted on the base of single factor experiment result. The selected factors and levels are listed in Table 6.

Throughput and recovery were direct investigated indices. The total evaluation index was used to analysis by statistical method.

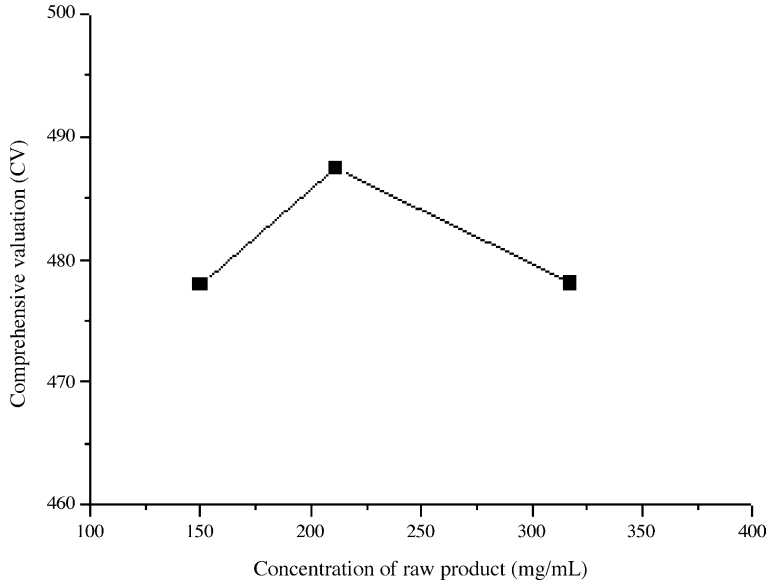

Fig. 3. Effect of loading amount on separation efficiency. Column, 150 mm × 4.6 mm silica column; carrier, dichloromethane–methanol (90:10, v/v); flow-rate, 0.1 ml/min; fraction volume, 0.2 ml; displacer, 83 mM ethanolamine; injection volume, 0.7 ml.

Table 6
Factors and levels for orthogonal test

Level	A (displacer concentration) (mM)	B (flow-rate of displacer) (ml/min)	C (loading amount) (mg/ml)
1	83	0.1	114
2	167	0.2	150
3	250	0.3	211

The results of orthogonal test and extreme difference analysis are presented in Table 7. The analysis of variance was performed by statistical software SPSS 11.0 and the result is listed in Table 8.

From analysis of extreme difference, the influential order of three factors on separation efficiency was A > B > C, and the optimum condition was $A_2B_2C_3$ (see Table 7).

The value of P (significance) for model was 0.004 in analysis of variance, which illustrated that this model was available. Owing to the values of P for three factors being relatively high, it was shown that the contributions of three factors for separation efficiency were slightly different and no significant factor. From the values of P, A ($P = 0.116$) < B ($P = 0.218$) < C ($P = 0.368$), the affections were different and the order was A > B > C (see Table 8), which was agreed with result of extreme difference analysis.

Integrating the results of extreme difference and variance analysis, the optimum parameters were obtained as the followed: displacer concentration being 167 mM, the flow-rate of displacer at 0.2 ml/min and sample concentration being 211 mg/ml.

3.4. Separation of soybean phospholipids by using HPDC

Soybean phospolipids were separated by HPDC under the optimum parameters. dichloromethane–methanol (90:10, v/v) and 167 mM ethanolamine were used as carrier and displacer, respectively. Sample concentration was 211 mg/ml and loading volume was 0.7 ml. In fact, the loading amount was 148 mg (211 mg/ml × 0.7 ml), which was separated at the flow-rate of 0.2 ml/min. Fractions of the column effluent were collected in 0.2 ml intervals. 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

Table 7
Result of orthogonal test and extreme difference analysis

	A[a]	B[a]	C[a]	PE				PC				
				Purity (%)	Throughput (mg/h)	Recovery (%)	Y_{PE}[b]	Purity (%)	Throughput (mg/h)	Recovery (%)	Y_{PC}[b]	CV[c]
1	1	1	1	83.1	13.6	80.6	88.8	93.8	57.0	95.4	139.4	114.1
2	1	2	2	81.4	38.5	62.7	95.9	91.0	190.3	91.4	163.4	129.7
3	1	3	3	80.8	51.0	36.0	82.8	90.2	281.3	57.9	138.9	110.9
4	2	1	2	80.0	26.7	88.9	109.2	93.2	92.0	94.0	145.3	127.3
5	2	2	3	80.2	65.7	70.9	129.7	90.5	272.6	88.3	177.4	153.6
6	2	3	1	78.4	47.7	62.3	104.4	94.1	186.2	73.0	138.1	121.3
7	3	1	3	82.4	29.5	63.6	88.1	92.5	132.1	83.7	140.4	114.3
8	3	2	1	80.4	26.3	52.9	75.0	93.2	137.7	80.2	137.0	106.0
9	3	3	2	82.8	40.7	35.6	72.5	91.6	241.5	61.5	134.9	103.7
I	354.7	355.7	341.4									
II	402.2	389.3	360.7									
III	324.0	335.9	378.8									
R[d]	78.2	53.4	37.4									

[a] A, B and C refer to factors of displacer concentration, flow-rate of displacer and loading amount, respectively.
[b] Y_{PE} and Y_{PC} are comprehensive evaluation of two indices of throughput and recovery for PE and PC products, respectively. They are calculated depending on the formula (1)–(3) in the text.
[c] CV is the total comprehensive valuation for PE and PC product, which is calculated depending on the formula (4) in the text.
[d] R refers to the result of extreme analysis.

Table 8
Result of analysis of variance

Source	Sum of squares	d.f.	Mean square	F	Significance
Model	131570.023[a]	7	18795.718	276.801	0.004
A[b]	1034.887	2	517.443	7.620	0.116
B[b]	485.840	2	242.920	3.577	0.218
C[b]	233.207	2	116.603	1.717	0.368
Error	135.807	2	67.903		
Total	131705.830	9			

[a] $R^2 = 0.999$ (adjusted $R^2 = 0.995$).
[b] A, B and C refer to factors of displacer concentration, flow-rate of displacer and loading amount, respectively.

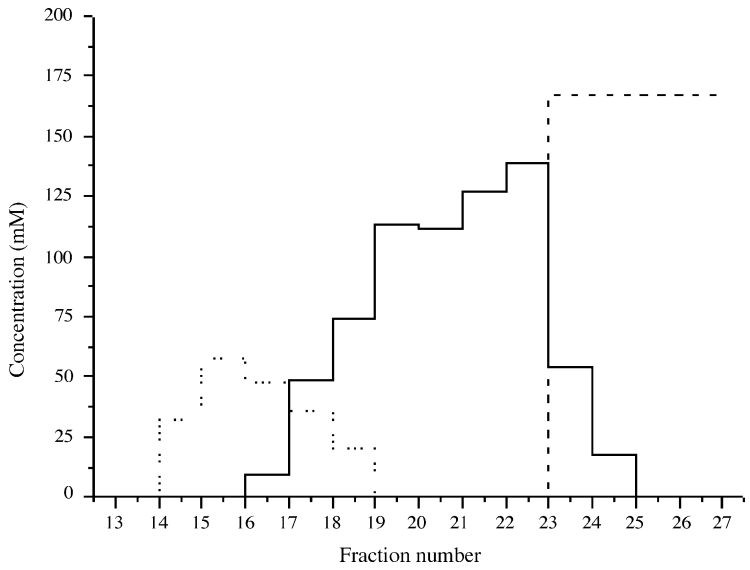

Fig. 4. Displacement chromatogram of soybean phospholipid. Column, 150 mm × 4.6 mm silica column; carrier, dichloromethane–methanol (90:10, v/v); flow-rate, 0.2 ml/min; fraction volume, 0.2 ml; sample concentration, 211 mg/ml (in carrier solvent); injection volume, 0.7 ml; displacer, 167 mM ethanolamine. The concentrations of PE, PC and displacer are shown by dot, solid and dash lines.

certain volume for determining the quantities of PC and PE by HPLC. Depending on above analytical results, the displacement chromatogram was constructed in Fig. 4. Fraction 14–16 and 17–22 were combined to recover PE and PC by removing solvents under vacuum at 50 °C, respectively. The PE and PC product were weighted and analyzed by HPLC. HPLC chromatograms of the raw material, PE and PC product are shown in Figs. 5–7, respectively. The purity, throughput and recovery of the soybean PE product were 80.2%, 65.7 mg/h and 70.9%, and those of the soybean PC product were 90.5%, 272.6 mg/h and 88.3%, respectively. From Fig. 4, we found that displacer eluted out of column after fraction 23. At this moment, displacement was stopped and then the system was regenerated by dichloromethane–methanol–acetic acid (60:30:10, v/v) eluting at the flow-rate of 1 ml/min. After eluting 75 min, the system was re-equilibrated by carrier for next separation.

It was shown that there was little PC in the PE product and little PE in the PC product in Figs. 6 and 7, which illustrated that the resolution of PE and PC was high in our HPDC system. The purity of the product being not high resulted from effect of some impurities and that was analyzed in a previous

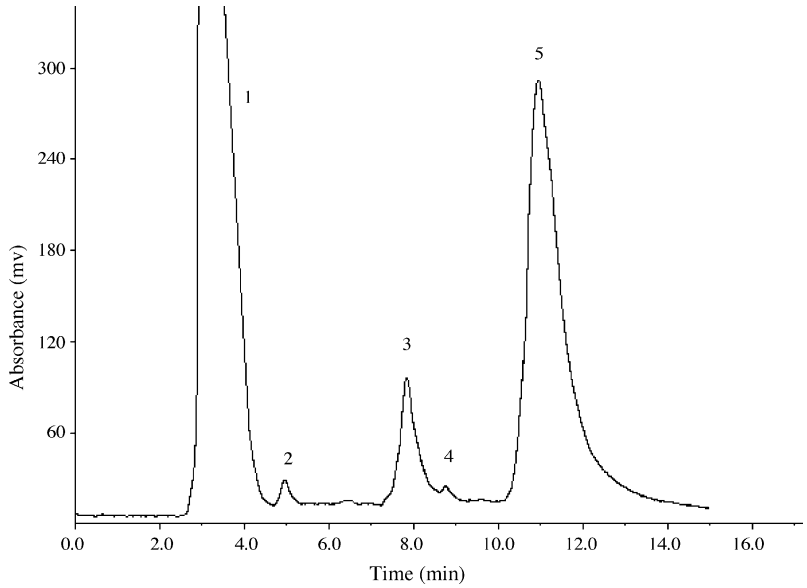

Fig. 5. HPLC chromatogram of the raw product. Column, 150 mm × 4.6 mm silica column; mobile phase, acetonitrile–methanol–85% phosphoric acid (180:3:1, v/v); injection volume, 20 μl; flow-rate, 0.5 ml/min; sample concentration, 1 mg/ml (in chloroform–methanol, 2:1, v/v). Peaks: (1) solvents; (2) and (4)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

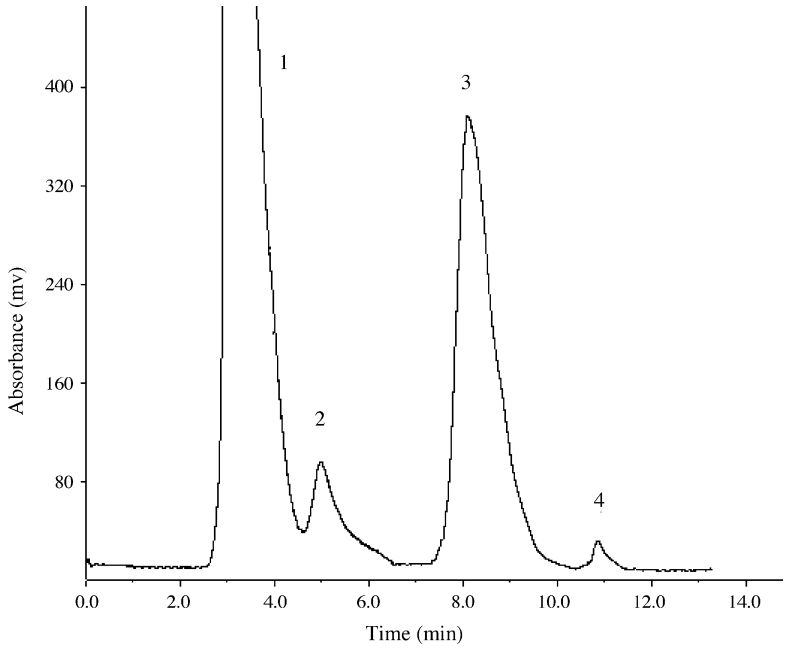

Fig. 6. HPLC chromatogram of the soybean PE product. Column, 150 mm × 4.6 mm silica column; mobile phase, acetonitrile–methanol–85% phosphoric acid (180:3:1, v/v); injection volume, 20 μl; flow-rate, 0.5 ml/min; sample concentration, 1 mg/ml (in chloroform–methanol, 2:1, v/v). Peaks: (1) solvents; (2)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

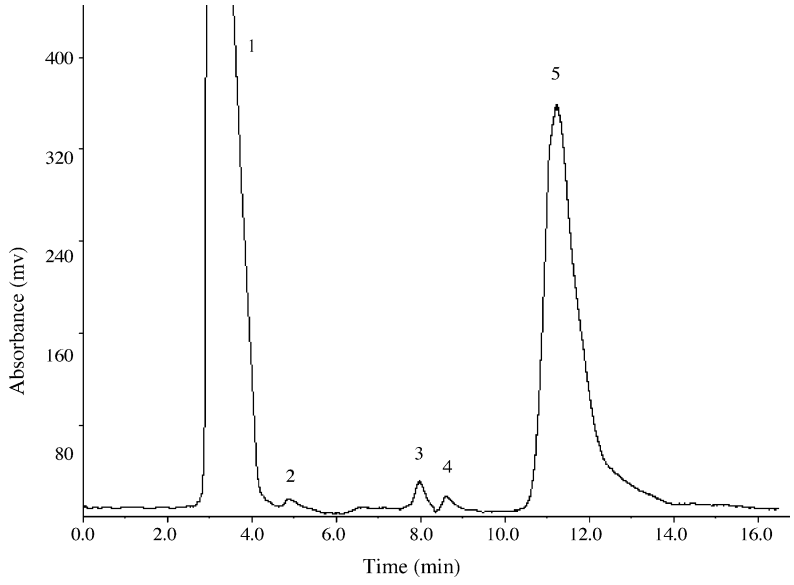

Fig. 7. HPLC chromatogram of the soybean PC product. Column, 150 mm × 4.6 mm silica column; mobile phase, acetonitrile–methanol–85% phosphoric acid (180:3:1, v/v); injection volume, 20 μl; flow-rate, 0.5 ml/min; sample concentration, 1 mg/ml (in chloroform–methanol, 2:1, v/v). Peaks: (1) solvents; (2), (4) impurities; (3) PE; (5) PC.

paper [32]. For improving the product purity, it was necessary to further study a new pre-treat method to remove impurities.

4. Conclusions

About 148 mg soybean phospholipids were successfully separated by HPDC on a 150 mm × 4.6 mm analytical silica column under optimized parameters of loading amount, concentration and the flow-rate of displacer. It was illustrated that HPDC used for preparative separation of natural phospholipids was feasible. Meanwhile, the displacement chromatography exhibits great application potentiality for separation of natural phospholipids because of its high loading amount, little tailing, low solvent consumption and high product concentration, etc.

In our paper, just two components, PE and PC, were separated. It was needed to study new HPDC system for separation of phospholipids containing more components before this technology to be commercial used.

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