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SHORT COMMUNICATION

PREPARATIVE SEPARATION OF PHOSPHOLIPIDS FROM SOYBEAN BY NP-HPLC

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Abstract – Soybean phospholipids-phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC)-were separated by normal-phase high performance liquid chromatography. The system was operated in a gradient mode. The experimental variables were gradient time and mobile phase composition. The experimental results showed that PE, PI and PC were resolved by three step-change gradient modes which employed ternary systems of hexane/1-propanol/water (58/40/2 and 56/40/4 by vol.%) and methanol/1-propanol/water (80/18/2, by vol.%) for the gradient times of 10, 30 and 76 min, respectively.

Key words: *Phospholipids, Soybean, Preparative Separation, HPLC*

INTRODUCTION

Soybean lecithin is a commercial by-product in vegetable oil production. Due to its high phospholipid content, this material is widely used as a natural emulsifier, stabilizer, baking improver, and wetting agent. Moreover, in recent years, numerous applications in dietetics, cosmetics and pharmaceuticals have been reported [Gunstone, 1994]. Commercially available products are usually complex mixtures of neutral lipids, phospholipids, and glycocipids. Many analytical high-performance liquid chromatographic procedures for separating phospholipid classes have been reported [Balazs et al., 1996; Moreau, 1994; Meeren et al., 1992]. HPLC columns generally contain solid supports of silica or bonded phase. Silica columns have been used because they are relatively cheaper than the bonded phase. For separating the soy phospholipids, NP-HPLC with a UV detector is the most common method.

A few papers on the preparative-scale separation of the phospholipids are recently published. Hurst et al. [1996] experimented a column load of 180 mg in a 300×57 mm preparative column. Amari et al. [1990] obtained phosphatidylcholine (PC) from 236 mg of crude egg york phospholipids in a 200×50 mm preparative column. A higher column load of 2.1 g glycol-insoluble lecithin was separated on a 300×47 mm column [Hanras and Perrin, 1991]. A gram-scale lecithin fractionation was also performed by Meulenaer [1995a] and Meeren [1990a]. Furthermore, to reduce solvent consumption, the procedure was optimized using a method-development column [Meulenaer et al., 1995b].

The mobile phase in HPLC can be isocratic or gradient. An isocratic system is simple and more stable but it limits the number of compounds to be separated. However, this difficulty can be overcome by using the gradient mobile phase. In this work, systematic approaches of changing mobile phase compositions were studied to separate the phospholipids from soybean into PE, PI, and PC by NP-HPLC.

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EXPERIMENTAL

1. Chemicals

Powdered soybean-lecithin was provided by Doosan Technical Center (Yongin, Korea), and the concentration of the lecithin dissolved in chloroform was 100 mg/ml. The identification of peaks was confirmed with standard solutions of PE, PI and PC provided by Doosan Technical Center (Yongin) and Thin Layer Chromatography (TLC). For preparative purpose, rather than HPLC-grade solvents, extra-pure grade solvents of hexane, 1-propanol, IPA, and methanol were purchased from Yakuri Pure Co. (Japan). The water used was distilled and deionized.

2. HPLC Equipment

For experiments, we used the following equipments: Waters Model 600 liquid chromatograph (Waters Associates, Milford, MA, U.S.A.) equipped with the Waters 600E Multisolvent Delivery System, a UV-visible tunable wavelength absorbance detector (Waters 486), a U6K injector (2 ml sample loop). The data aquisition system was CHROMATE (V. 3.0, Interface Eng., Korea) installed in a personal computer. A preparative column (500×9.8 mm) packed by irregular-shaped silica packings of 10-40 μm particle size (YMC Co.) was used. Initially, the packings were charged and tapped, and then mobile phase was pumped through the column. Small amounts of packings were refilled later. After this procedure was repeated for several times, the final weight of the column came out to be 24.56 g.

3. Methods

Absorbance was monitored at 216 nm with a sensitivity of 2 and 0.001 a.u.f.c. The dead volume measured by introducing hexane of 100 μl was 20.40 ml. The lecithin solutions of 0.3-2.0 ml were directly injected into HPLC column. Elution experiments were performed by using gradient protocols at a flow rate of 5 ml/min. All separations were done at am-

Table 1. Gradient conditions used in this experiments

	1st step		2nd step		3rd step		4th step	
	Comp.	Time	Comp.	Time	Comp.	Time	Comp.	Time
Fig. 1	H/I/W	(min)	H/I/W	(min)	H/I/W	(min)		
	60/40/0	10	57/40/3	77	61/35/4			
Fig. 2	H/P/W	(min)	H/P/W	(min)	H/P/W	(min)		
	60/40/0	10	59/40/1	60	61/35/4			
Fig. 3	H/P/W	(min)	H/P/W	(min)	H/P/W	(min)	M/P/W	(min)
	60/40/0	10	58/40/2	30	56/40/4	76	80/18/2	
Figs. 4, 5	H/P/W	(min)	H/P/W	(min)	M/P/W	(min)		
	58/40/2	10	56/40/4	30	80/18/2	76		

(H: hexane, I: IPA, W: water, P: 1-propanol, M: methanol)

bient laboratory temperature. Prior to use the HPLC column, solvents were filtered through a 0.5 μm filter (Micropore Co.) to avoid particulate contamination of the column. The solution used in TLC was a mixture of chloroform, acetone, acetic acid, methanol and water (50 : 20 : 10 : 10 : 5 by vol.%). Iodine (Mallinckrodt Chemical Co.), a TLC plate coated with Silica gel 60H (5-40 μm, 60 Å, Merk), and Silica gel 60H (5-40 μm, 60 Å, Merk) were placed in a TLC chamber with a developing solution.

RESULTS AND DISCUSSION

Since preparative-scale separation and isolation of individual phospholipid classes by using mobile phases of hexane/IPA/water [Blank and Snyder, 1983; Patton et al., 1983] were reported, we first used a mobile phase of hexane/IPA/water to separate PE, PI and PC in an isocratic mode. However, it was very hard to resolve three components from soy phospholipids in the mobile phase. The gradient method allows to increase the number of phospholipids to be separated. Throughout this work, the compositions of mobile phase were changed in step-change and their gradient conditions were listed in Table 1. Fig. 1 shows the chromatogram obtained by gradient mode. A ternary mobile phase was hexane/IPA/water. The water content was slightly increased at different steps to elute out PC. Each peak was identified by comparing the collected phospholipids with standard samples and by examining TLC chromatograms. The three phospholipids were not well soluble in the mobile phase, so poor resolution was ob-

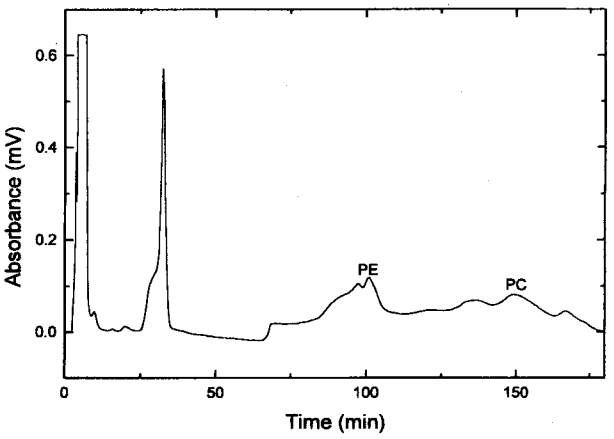

Fig. 2. Separation of phospholipids from soybean on a preparative column.
(Mobile phase condition in Table 1, same concentration and injection volume as in Fig. 1).

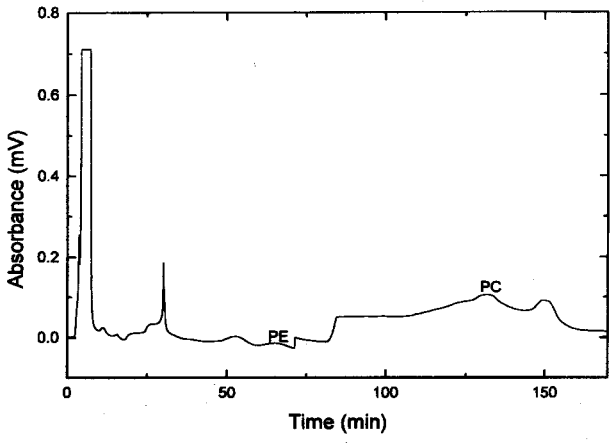

Fig. 1. Separation of phospholipids from soybean on a preparative column.
(Mobile phase condition in Table 1, concentration 100 mg/ml, injection volume=0.3 ml).

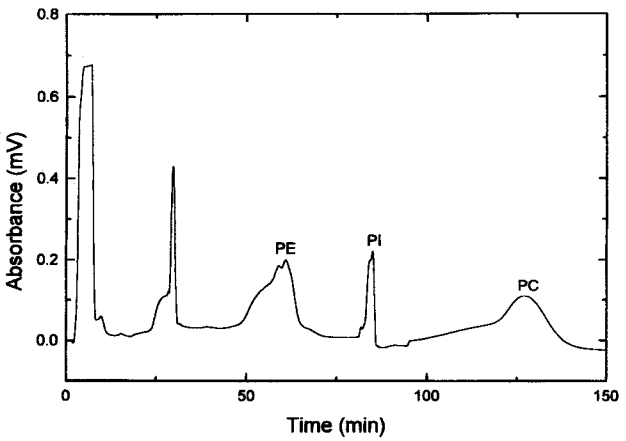

Fig. 3. Separation of phospholipids from soybean on a preparative column.
(Mobile phase condition in Table 1, concentration 100 mg/ml, injection volume=0.5 ml).

served. When replacing IPA with 1-propanol, the peaks of soy phospholipids were similar at almost same compositions of mobile phase, as shown in Fig. 2. The solubilities of PE and PC decreased with length of the alkyl group of alcohols, whereas PI was dissolved only in methanol and seemed to be insoluble in higher alcohols [Meeren et al., 1990b]. To get the sharp peak of PE and PC, and to resolve PI from PC, one more gradient step was added. Methanol was a major constituent in the last gradient step (see Fig. 3 in Table 1). In this figure, it was shown that PI was entirely resolved from PC due to the high content of methanol in the last step. Finally we could separate the three separated phospholipids-PE, PI, and PC. To reduce the total run time (145 min) which means less consumption of solvent, water was added in the first step as shown in Fig. 4 (see condition in Table 1). The effects of water content in the last step were very critical [Hanras and Perrin, 1991]. The resolution of PI and PC was significantly changed by slight variation in the water content. Column conditioning appeared to be related to precision of data. The major problem of NP-HPLC used with bare silica occurred in the existence of water so it delayed the equilibration of the column. Frequent conditionings were done to ensure good retention time and reproducibility. The composition of the mobile phase in the last step was 80/18/2 by vol.% (methanol/1-propanol/water). The influence of sample load was studied by increasing injection volume. In Fig. 4, the amount of sample injected was 50 mg. Fig. 5 shows that the resolution is similar even with the sample of 200 mg. It implies that a higher loading may be possible in our system. In Figs. 4 and 5, sharp and relatively symmetric peaks were obtained for the three phospholipids without sacrifice of resolution and recovery. When considering total run time of 120 min, solvent consumption did not exceed 0.6 *l*. In this operating condition, pressure drop was approximately 306 psi.

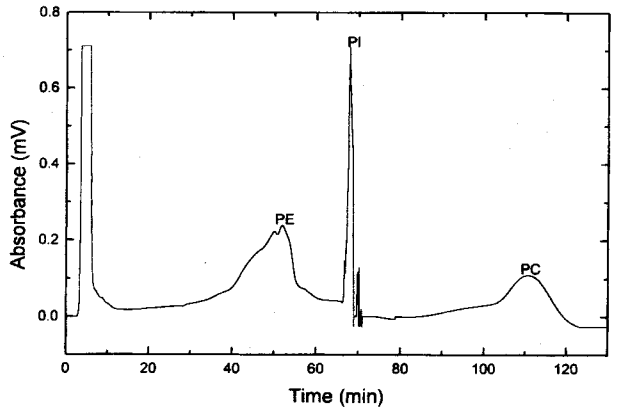

Fig. 4. Separation of phospholipids from soybean on a preparative column.
(Mobile phase condition in Table 1, same concentration and injection volume as in Fig. 3).

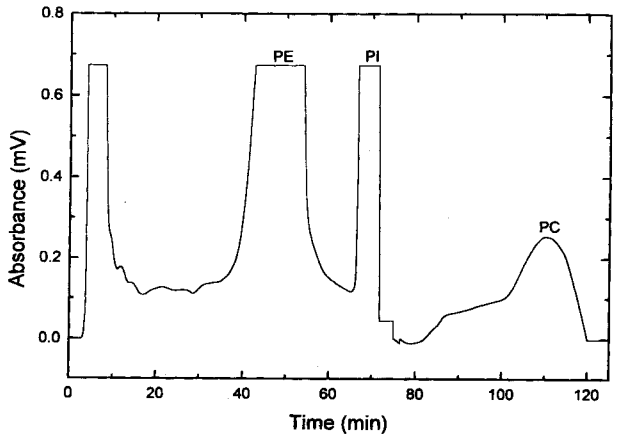

Fig. 5. Separation of phospholipids from soybean on a preparative column.
(Mobile phase condition in Table 1, concentration 100 mg/ml, injection volume=2.0 ml).

CONCLUSIONS

Soybean phospholipids (PE, PI, and PC) were separated by NP-HPLC in a gradient mode. The phospholipids were separated by three step-change gradient modes, which employ ternary systems of hexane/1-propanol/water (58/40/2 and 56/40/4, by vol.%) and methanol/1-propanol/water (80/18/2, by vol.%). Three gradient times were 10, 30 and 76 min, respectively.

The experimental condition could be extended to a gram-level fractionation method. An advantage of this work is that a low-cost and irregular-shaped silica gel was used as packing materials. Furthermore, fractionation was accomplished by a gradient mode of step-change, so that a simple isocratic pump, together with a normal valve for changing mobile phases, could be used. To ensure the possibility of scale-up system, the experimental data on large sample loads will be published in near future.

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