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Fractionation of soybean phospholipids by preparative high-performance liquid chromatography with sorbents of various particle size

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Abstract

Normal-phase high-performance liquid chromatography was used on a preparative scale to seperate phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylcholine (PC) phospholipids from soybean. Separation was achieved using mixtures of three solvents, hexane, methanol and isopropanol. The optimized mobile phase compositions were experimentally determined while operating in a linear gradient mode using 15, 5–20, 25–40, and 40–63 μm preparative particles as well as 4 μm analytical particles. A gradient mobile phase was established on a commmercially available analytical Nova-Pak column such that hexane linearly decreased from 85 to 0 as isopropanol and methanol linearly increased in two gradient steps from 10 to 30 and 5 to 70 respectively. The total run time was 25 min at a flow-rate of 1.5 ml/min. A slight change in mobile phase composition was required to increase the resolution of phospholipids. The 15 μm particle size gave the best separation of the preparative particle sizes examined based on their resolutions between PE and PI and PI and PC. Finally, the retention factors of PE and PC were correlated in terms of mobile phase composition.

Keywords: Soya bean; Preparative chromatography; Mobile phase composition; Stationary phases, LC; Gradient elution; Particle size; Retention factor; Phospholipids; Lipids

1. Introduction

Phospholipids are found in all biological membranes and they contain extremely complex mixture of different classes, such as phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylcholine (PC) [1]. Phospholipids are functioning phosphatides in cell membranes and important substances in biomedical, pharmaceutical and nutrition research [2,3]. Phospholipids are commonly found in plant and animal tissue and serve as structural components in membranes in addition to playing a role in enzyme activation [4]. For this reason, they are widely based in the food and cosmetic industries, as well as industrial manufacturing [2,4]. For example, phospholipids are a key factor in determining phase-transition temperature, and membrane fluidity and therefore play important roles in events such as resistance to the freeze–thaw process, motility, acrosomal exocytosis or fusiogenic properties of the sperm [5].

Phospholipids separations have been studied by many research groups [4–6] and with numerous seperation methods employed. Commonly, quantita-

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tion and separation of phospholipids has been performed using thin-layer chromatography (TLC) [5,7]. This method has several disadvantages, including difficulties and possible inaccuracies in quantitative separation of individual species [8]. Recently, several reports have been published that describe successful separation of individual phospholipids by HPLC [5–7,9–11], especially normal-phase HPLC (NP-HPLC) [10] due to it's better reproducibility and reduced analysis times. It is known that simple isocratic elution of phospholipids results in excessive analysis times, as a result of difficulties in resolving peaks, because to the broad range of polarities of the phospholipids [10]. Consequently, in order to achieve proper separation of the phospholipids in an acceptable time using NP-HPLC, a gradient elution of the mobile phase is required. The packing sizes also have great effects on the resolution of the phospholipids. Resolution increases with a decrease in the particle size [12]. Limited research has been published on the separation of phospholipids using various sizes of packing materials.

The purpose of this work is to find the optimum mobile phase composition for the larger preparative packings which would be capable of separating phospholipids PE, PI and PC from soybean on a large-scale. We experimentally found the optimum linear gardient mobile phase compositions for the different particle size packings. Hexane, methanol and isopropanol were used as mobile phases. The optimum composition of mobile phase was based on the column efficiency and resolution of the phospholipids. The retention factors of phospholipids were also correlated in terms of the mobile phase composition.

2. Experimental

2.1. Reagents

HPLC-grade solvents, chloroform, hexane, isopropanol (IPA), and methanol were obtained from J.T. Baker (Philipsburg NJ, USA). Lecithin, labeled Percept 8140, a mixture of tthe major phospholipids, was supplied from Central Soya (FortWayne, Indiana, USA). Phospholipid standards, PC, PE and PI from soybean, were purchased from Sigma (St. Louis, MO, USA).

2.2. Sample preparation

Percept 8140 powder (50 mg) was dissolved in 10 ml of chloroform and then diluted to 5000 μg/ml. The concentrations of PC, PE and PI were 5000, 1000 and 1265 μg/ml, respectively. A constant injection volume of phospholipid solution, 10 μl was used for all the packings in this work.

2.3. Apparatus and method

HPLC was performed using Waters 600S solvent delivery system and 2487 UV dual channel detector (Waters, Milford, MA, USA). Data acquisition system was Millennium 3.2 installed in a HP Vectra 500 personal computer. The flow-rate of mobile phase was fixed at 1.5 ml/min. Dual UV wavelengths were set at 208 nm and 210 nm.

The mobile phases were degassed with helium. The mobile phases were hexane, IPA and methanol. The stationary phases, consisted of four different sizes of preparative packings: 5–20 μm, 15 μm, 25–40 μm, and 40–63 μm purchased from Merck (Darmstadt, Germany). They were packed in the laboratory with an aspirator (Tokyo Rikakikai, Tokyo, Japan). The performance of the preparative packings was compared with the commercially available Nova-Pak column (4 μm, Waters). The column size was fixed as 15×0.39 cm for the all of the preparative packings including the Nova-Pak column. Mobile phase composition was adjusted in order to set total run times between 25 and 50 min. All experiments were carried out at ambient temperature.

3. Results and discussion

3.1. Effect of packing sizes

The optimum mobile phase conditions required to separate the phospholipids from soybean on the four preparative packings and one commercial Nova-Pak column were investigated. The polarities of phospholipids studied, PE, PI, and PC are very different;

PE is almost nonpolar, while PC is very polar, and PI is in the middle range. In order to achieve the separation of these materials on a single column, gradient elution mode is required. One linear gradient of hexane–IPA–methanol (80:10:10 to 0:10:90, v/v, 25 min) step was tried to separate and identify phospholipids from soybean with the Nova-Pak column (data not shown). In the gradient, the composition of methanol increased, but that of hexane decreased at constant composition of IPA during 25 min. Although PC was eluted as a single peak, PE and PI were not resolved by the linear gradient step. To increase the efficiency of PC and PE and to isolate PI from PC, one or more gradient step needs to be added [13]. The resolution and separation times were considered to determine optimum conditions for seperation. The optimum mobile phase composition of hexane–IPA–methanol and the gradient conditions with the Nova-Pak were listed in Table 1. The experimental conditions were changed as the preparative packing materials were varied. The void volume in a chromatographic column is changed with packing sizes, the resulting change in conditions can be observed in others works [7,9]. The experimental variables were the gradient time and number of gradient steps (step-wise or linear) as well as the composition of mobile phases. At the constant column size and mobile phase flow-rate, the identification of PE, PI and PC contained in a real sample, was carried out by comparison to their respective external standards.

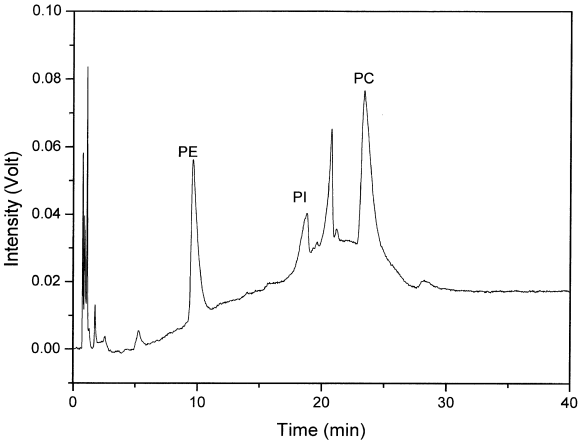

Fig. 1. Separation of phospholipids from soybean with 4 μm packing (refer to Table 1 for the mobile phase composition).

The chromatogram of phospholipids on the commercial 4 μm Nova-Pak column was shown in Fig. 1. The components of PE, PI, and PC were well

Table 1
Linear gradient conditions with the various analytical and preparative packings

Packing size (particle size)	Gradient conditions				
	Flow rate (ml/min)	Gradient time (min)	Hexane (%, v/v)	IPA (%, v/v)	Methanol (%, v/v)
Nova-Pak, 4 μm	1.5	0	85	10	5
		15	50	20	30
		25	0	30	70
15 μm	1.5	0	85	10	5
		10	50	20	30
		20	0	30	70
		25	0	0	100
5/20 μm	1.5	0	75	15	10
		10	20	30	50
		30	0	10	90
25/40 μm	1.5	0	85	10	5
		10	50	20	30
		20	0	30	70
		25	0	0	100
40/63 μm	1.5	0	90	3	7
		8	45	20	35
		15	10	5	85
		23	2	2	96

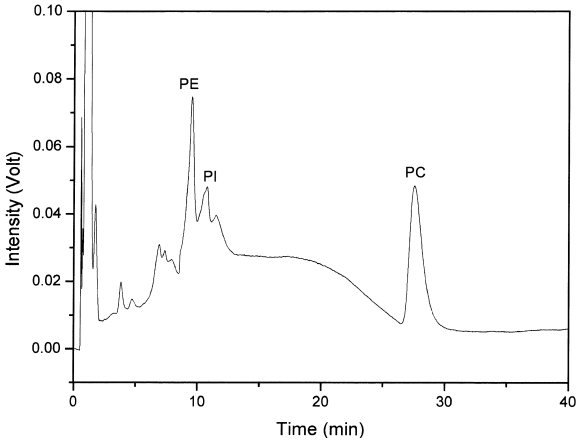

Fig. 2. Separation of phospholipids from soybean with 15 μm packing (refer to Table 1 for the mobile phase composition).

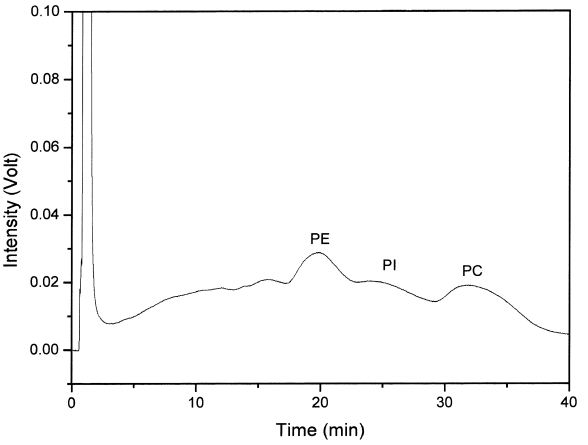

Fig. 4. Separation of phospholipids from soybean with 25–40 μm packing (refer to Table 1 for the mobile phase composition).

resolved, although the baseline was not flat due to the gradient mode applied in this work. As shown in Table 1, two or three linear gradients were used with the different packing sizes. The separation of phospholipids obtained on 15 μm, 5–20 μm and 25–40 μm preparative packings are shown in Figs. 2–4, respectively. The poorest resolutions of phospholipids were observed with the packing of 40–63 μm in Fig. 5. Also in the later part of experiment run, the baseline was quite unstable. The larger particle size of packing material on a fixed column, the more void volume. So the separation was not feasible under these conditions. The resolutions between PE and PI and PI and PC on 5–20 μm and 15 μm preparative packings were relatively better than on the larger 25–40 μm and 40–63 μm packing. PC was always eluted as a single peak for on all the packing materials except 40–63 μm packing. The band broadening of the peak was serious with larger packing size. The peak heights of PC were gradually lower with the larger packings. The peak appearance and the polarity-dependent retention time of PC was also highly reproducible. This implies that PC could be easily separated even on a commercial scale by controlling various packing sizes and solvent com-

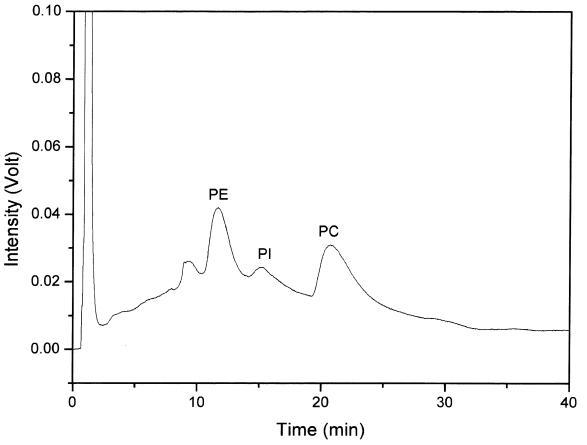

Fig. 3. Separation of phospholipids from soybean with 5–20 μm packing (refer to Table 1 for the mobile phase composition).

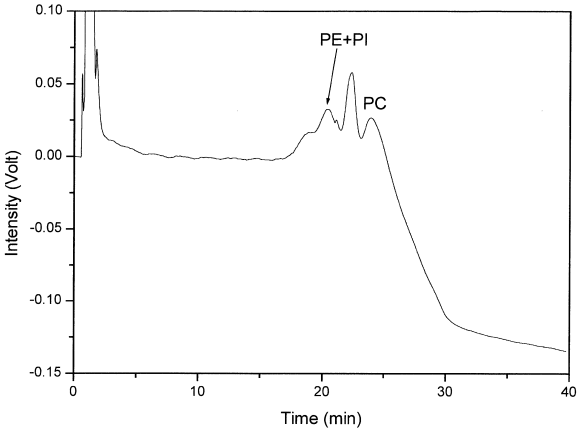

Fig. 5. Separation of phospholipids from soybean with 40–63 μm packing (refer to Table 1 for the mobile phase composition).

positions. PE and PI were relatively well separated on the 15 μm preparative packing in Fig. 2, while they were almost coeluted on the larger packings, 5–20, 25–40, and 40–63 μm in Figs. 3–5. This tendency can be explained by polarity-dependent retention time. That is, the elution of phospholipids having different polarity can be affected by changing polarity of mobile phase [9,12]. The particle size of packing material has a great influence on elution of phospholipids.

3.2. Column efficiency and resolution

The column efficiency of a peak is expressed as the number of theoretical plates, N, and it is calculated from the relationship:

$$N = 5.54 \cdot \left(\frac{t_R}{w_{1/2}} \right)^2 \quad (1)$$

where t_R denotes the retention time, and $w_{1/2}$ the peak width at half height. The larger the number of theoretical plates, the sharper the shape of the peak. Many factors affect the column efficiency, and in this work, it is used as a reference to confirm the effects of packing size on the number of theoretical plates. The relations between N and packing sizes were listed in Table 2. Generally, N is closely related to the physical properties such as viscosity and density of carriers. It amounts to 100 000 in gas chromatography, but to 1000 in liquid chromatography. The column efficiency of the analytical packings of 4 μm has the largest numbers of theoretical plates. The efficiency was deteriorated with lager packings. It was interesting that even in larger packing of 15 μm, the number of theoretical plates was increased due to the mobile phase composition and gradient conditions.

In order to investigate the resolutions of PE, PI and PC with the packing materials, the following equation was used:

$$R = \frac{t_{R2} - t_{R1}}{{}^1/_2 \cdot (W_1 + W_2)} \quad (2)$$

where t_{R1} and t_{R2} are retention times of the eluted peaks, and W_1 and W_2 are the widths of the peaks. Resolution depends on the two factors, the narrowness of the peaks, and the distance between the highest points of two peaks. Therefore, it is a function of column selectivity, that is, the nature of the stationary and mobile phases [14]. The separation at the baseline is usually designated as the value above 1. The relations between the calculated resolutions and the packing sizes are shown in Table 3. The resolutions with 15 μm packing were calculated as about 1.2 for PE and PI, and 7.3 for PI and PC. Those values were higher, compared to the other preparative packings. As the packing is smaller and more uniform, the axial dispersion is reduced and the paths of samples are shortened. Consequently, from the experimental results, it suggested the proper size of packings used for the preparative-scale operation should be 15 μm. The experimental result of the operating conditions might be extended to commercial scale. As the higher pressure drop was accompanied by larger packings, a pump with high-pressure rating would be necessary.

3.3. Estimation of retention factor of PE and PC

The proper packing material for preparative work was known to be 15 μm [15]. The relationship between the retention factor and the composition of ternary mobile phases was investigated to predict retention time of each phospholipid. The retention

Table 2
Calculated number of theoretical plate of phospholipids on the various packing materials

Packing size (μm)	N_{PC}	N_{PE}	N_{PI}
4	2304	395	2153
15	1058	1211	2401
5–20	371	294	337
25–40	177	256	231
40–63	169	224	58

Table 3
Comparison of resolutions between neighboring peaks obtained from the optimum operating conditions

Packing size (μm)	Resolution between PE and PI	Resolution between PI and PC
4	5.127	2.333
15	1.222	7.300
5–20	1.161	1.436
25–40	0.864	0.903
40–63	0.333	0.909

Table 4
Estimation of empirical coefficients in Eq. (3)

Phospholipids	*a*	*b*	*c*	*d*	*e*	r^2
PE	0.039	−0.811	−5.141	27.832	−59.051	0.9715
PC	0.008	−0.913	−0.539	3.562	66.202	0.999

factors were correlated into an empirical equation with mobile phase compositions. The retention factor, *k* was suggested in terms of mobile phase as follows:

$$k = aM^2 + bI^2 + cM + dI + e \quad (3)$$

M is the methanol, and *I* is the IPA content, both in % (v/v). The coefficients, a, b, c, d and e are empirical coefficients of each solute. PE and PC were analysed in an isocratic mode with various mobile phase compositions. PI, however, was not identified in isocratic mode. Moreover, PI detection using UV absorption was reported to be insensitive because of its weak chromatographic functionality in the 200–210 nm region [16]. PE and PC were eluted with 8 different isocratic modes. The calculated empirical coefficients were listed in Table 4. As the amount of organic modifier increased, the retention time of solute was shorter. PE was eluted in the hexane-rich mobile phase but PC was eluted in the methanol-rich mobile phase. The calculated and experimental retention factors were plotted in Fig. 6. Good agreement between calculated and experimented data was observed. It was conformed that PE was relatively close to non-polar species, but PC was polar species.

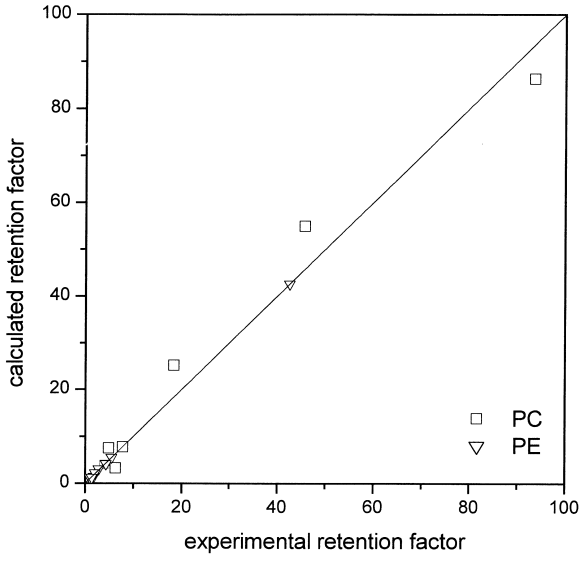

Fig. 6. Comparison between estimated retention factors and experimented retention factors of PE and PC on 15 μm packing material.

4. Conclusion

The optimum gradient mobile phase compositions were experimentally determined, on the basis of resolutions and separation times, for the analysis of the phospholipids (PE, PI and PC) from soybean using different particle sizes. The best separations was achieved when the particle size of the packing material was smaller and more uniform. Among the four larger preparative packings of 15, 5–20, 25–40, and 40–63 μm, 15 μm packing is recommended for preparative work. The relationship between the retention factors and ternary mobile phase compositions were correlated in a quadratic empirical equation. The correlation coefficients were over 0.97 for both PE and PC.

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