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Predicted Separation of Phospholipids from Soybean by Chromatography on Silica with Changes in Solvent Composition

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ABSTRACT

Normal-phase HPLC was used to separate the useful phospholipids phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC) from soybean lecithin. The mobile phase used in this experiment consisted of hexane, isopropanol, and methanol. The step-gradient mode was applied because the three components could not be separated by isocratic mode. To find the optimum separation conditions, the concentration profiles of effluents from a column were simulated by the retention factor and the plate theory in the step-gradient mode. The retention factor was correlated by the equation $\ln k' = A + BF + CF^2 + DG + EG^2$, where the constants A, B, C, D, and E were experimentally determined. F and G are the volume fractions of isopropanol and methanol, respectively. From the calculated results, PE was separated with hexane/isopropanol/methanol (90/5/5 vol%) in the isocratic mode, while PI and PC were resolved in the operating conditions of 15 minutes of gradient time and a second mobile phase of hexane/isopropanol/methanol (50/20/30 vol%) in the step-gradient mode. The agreement between the calculated concentration profile and the experimental data was fairly good, so the methodology developed in this work can be used to obtain useful separation conditions for stepwise elution.

Key Words. Phospholipids; Step-gradient; HPLC; Separation; Plate theory

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INTRODUCTION

Phospholipids are important physiological substances naturally occurring in animal and plant cell membranes. Soybean phospholipid (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, and wetting agent (1). Moreover, in recent years, numerous applications in dietetics, cosmetics, and pharmaceuticals have been reported (2). Commercially available products are usually complex mixtures of neutral lipids, phospholipids, and glycolipids.

HPLC columns generally contain solid supports of silica or a bonded phase. Silica columns have been used because they are cheaper than a bonded phase. For separating the soy phospholipids, NP-HPLC (normal-phase high-performance liquid chromatography) with a UV detector is the most common method. Some preparative as well as analytical high-performance liquid chromatographic methods for separating phospholipid classes have been reported (3–6). The plate theory was used in this work to obtain the experimental conditions. The chromatographic theory relates to the development of the theoretical plates conception, where the solutions for the concentrations of mobile phase and stationary phase are determined with respect to individual points along the column length (7, 8).

The complexity of the separation of lecithin is caused by the very large differences in polarity of the compounds. The retention factors of neutral lipids are much different from those of polar lipids. Therefore, to elute nonpolar and polar lipids simultaneously in a single run, it is necessary to use the gradient method. A change in the composition of the mobile phase needs to be selected to resolve the phospholipids by a chromatographic column. To apply this method in real separation work, the retention factor of each component should be optimized by adjusting the solvent compositions and by determining the gradient mode of change, e.g., stepwise or linear. Compared to an isocratic system, a gradient mode increases the number of compounds to be separated in a limited time. In this experiment the ternary mobile phases were hexane, isopropanol, and methanol, and the retention factor of phosphatidyle-thanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC) was correlated into a quadratic equation with the composition of the mobile phase. To choose the gradient condition of the gradient time and the solvent compositions effectively, a mathematical model based on plate theory and the retention factor correlated with mobile phase composition was developed and adopted to separate the phospholipids from soybean into PE, PI, and PC by NP-HPLC.

THEORY

The retention volume of a compound in a chromatographic column changes with the polarity of the mobile phase. The retention factor (k') is defined by

$$k' = \frac{V_R - V_m}{V_m} \tag{1}$$

where V_m denotes the dead volume.

The polarity of mobile phase can be adjusted by the quantity of isopropanol and methanol. The composition of the ternary mobile phase in this experiment was expressed as the volume percent of isopropanol and methanol, so the following relationship between the retention factor and the composition was suggested:

$$\ln k' = A + BF + CF^2 + DG + EG^2 \tag{2}$$

A, B, C, D, and E are experimentally determined constants, and F and G are the volume percentages of isopropanol and methanol, respectively.

We assumed that a single stepwise-gradient mode was applied in one experimental run to separate PE, PI, and PC from soybean lecithin. In such case the retention volumes of the phospholipids PI and PC were predicted from the following equation (9):

$$V_R = V_m(1 + k_2') + V_{g,1}\frac{k_1' - k_2'}{k_1'} \tag{3}$$

where k_1' and k_2' are the retention factors of a compound in the first and second mobile phases, respectively. $V_{g,1}$ is the gradient volume of the first mobile phase just before the step-gradient from start-up.

At a given composition of the ternary mobile phase, the retention factor was calculated by Eq. (1). For the three phospholipids, each retention factor as a function of the composition of isopropanol and methanol was experimentally obtained and fitted into Eq. (2). Therefore, individual empirical constants A–E, were obtained for PE, PI, and PC. By combining Eqs. (2) and (3), the retention volume of the step-gradient mode can be calculated in the arbitrary conditions of mobile phase composition and gradient volume. The assumptions used to calculate the elution profile of the outlet concentration from plate theory were that the number of theoretical plates remained unchanged with the composition of mobile phase in the isocratic mode, and that the peak width was determined only by the mobile phase applied during the gradient mode. The calculated profiles were obtained as follows (7):

$$C_N = C_0 \sum_{i=N-r}^{N-1} \frac{(aV)^r}{i!} e^{-aV} \tag{4}$$

where C_N and C_0 are the concentrations of the Nth plate and the feed injection, respectively, V is the volume of the mobile phase, and a is a constant which contains the equilibrium constant K:

$$a = \frac{1}{v_m + Kv_s} \tag{5}$$

β is the phase ratio v_s (volume of stationary phase in a plate)/v_m (volume of mobile phase in a plate), and also the proportional constant between the retention factor and the partition coefficient:

$$k' = \beta K \tag{6}$$

We assumed that β was 0.75 (10).

The retention factor was obtained by Eq. (1), where the retention volume was calculated by Eqs. (2) and (3), with the assumption that a theoretical plate was uniform for the phospholipids and the average values of the number of theoretical plates (N) were obtained by

$$N = 16\left(\frac{V_R}{w}\right)^2 \tag{7}$$

where V_R is the retention volume and w is the peak width in units of volume. Finally, the resolution, R, was calculated by

$$R = \frac{\sqrt{N}}{2}\,\frac{a-1}{a+1}\,\frac{k'}{1+k'} \tag{8}$$

The elution profiles were generated by Eq. (4). The resulting calculations were done by the commercial software Mathematica (Ver. 2.2).

EXPERIMENTS

The soybean-lecithin was provided by Doosan Technical Center (Yongin, Korea), and the concentration of the lecithin dissolved in chloroform was 10 mg/mL. The peaks were identified by use of thin-layer chromatography with PE, PI, and PC standards provided by Doosan Technical Center (Yongin, Korea). HPLC-grade solvents of hexane, isopropanol, and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). The water used was distilled and deionized.

The following HPLC system was used. A Waters Model 600 liquid chromatograph (Waters Associates, Milford, MA, USA) equipped with the Waters 600E Multisolvent Delivery System, a UV-visible tunable wavelength absorbance detector (Waters 486), and a U6K injector (2 mL sample loop). The data aquisition system was CHROMATE (V.3.0, Interface Eng., Korea).

A Waters column (150 × 3.9 mm) packed with Nova-Pak silica of 4 μm particle size was used. The modifier concentrations of isopropanol and methanol were ranged from 0 to 55% (v/v). Aliquots of 20 μl were injected directly for HPLC analysis. Absorbance was monitored at 208 nm with a sensitivity of 2 and 0.001 a.u.f.s. The dead volume measured by applying hexane of 100 μl was 1.344 ml. Elution experiments were performed by using gradient protocols at a flow rate of 1.5 ml/min. All separations were done at ambi-

ent laboratory temperature (20 ± 1°C). Prior to use the HPLC column, solvents were filtered through a 0.5 μm filter (Micropore Co.) to confirm the cleanliness of HPLC-grade solvents. 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, 60Å, Merk), and Silica gel 60G (5–40 μm, 60Å, Merk) were placed in a TLC chamber with a developing solution.

RESULTS AND DISCUSSION

As the retention factors of the useful phospholipids PE, PI, and PC differ substantially, the gradient mode should be applied to separate them. The retention factors of PE and PC were obtained in the isocratic mode with wide variations in the mobile phase composition, while that of PI was calculated by experimental data of the gradient mode. Table 1 lists the empirical constants, *A–E* for PE, PI, and PC. The coefficients of PI are close to those of PC. In the binary mobile phase of water/acetonitrile and water/methanol in the reversed mode, good agreement among the values calculated by the quadratic equation and the experimental retention factor were reported (11).

The retention factor in normal-phase HPLC is greatly affected by the polar solvent in the mobile phase because such solvents are adsorbed on the polar surface of bare silica, whereas the interaction between the nonpolar hexane and the stationary phase is negligible. When the two volume percentages of isopropanol and methanol are fixed, that of hexane is automatically known, so the volume percentage of hexane was not included in Eq. (2). The elution profiles of PE, PI, and PC can be predicted with the retention factors of Eq. (2) even in gradient mode.

Complete separation of PE, PI, and PC in the isocratic mode was not feasible. Figure 1 shows that at higher polarity of mobile phase, PC was eluted in a reasonable time, but the resolution of PE and PI was incomplete, and neutral lipids are coeluted with PE and PI (not shown in Fig. 1, but we confirmed it by experiment). The chloroform peak overwhelmed the peaks of PE and PI as well as of neutral lipids. In Fig. 2, at a lower polarity of the mobile phase, the neutral lipids and PE were separated, but PI was not resolved with PC. At the lower polarity of the mobile phase, neutral lipids and PE were separated from PI and PC, while at the higher polarity, PI was separated from PC. To separate the phospholipids in a single run, the gradient mode needs to be applied. We limited the gradient mode to a step function. PE was eluted in the isocratic mode before the second mobile phase, and the composition of the first mobile phase was hexane/isopropanol/methanol (90/5/5, vol%).

The mixer volume, $V_{m,1}$, was 5.33 mL. The number of theoretical plates of PE and PC could be experimentally measured because they were resolved in

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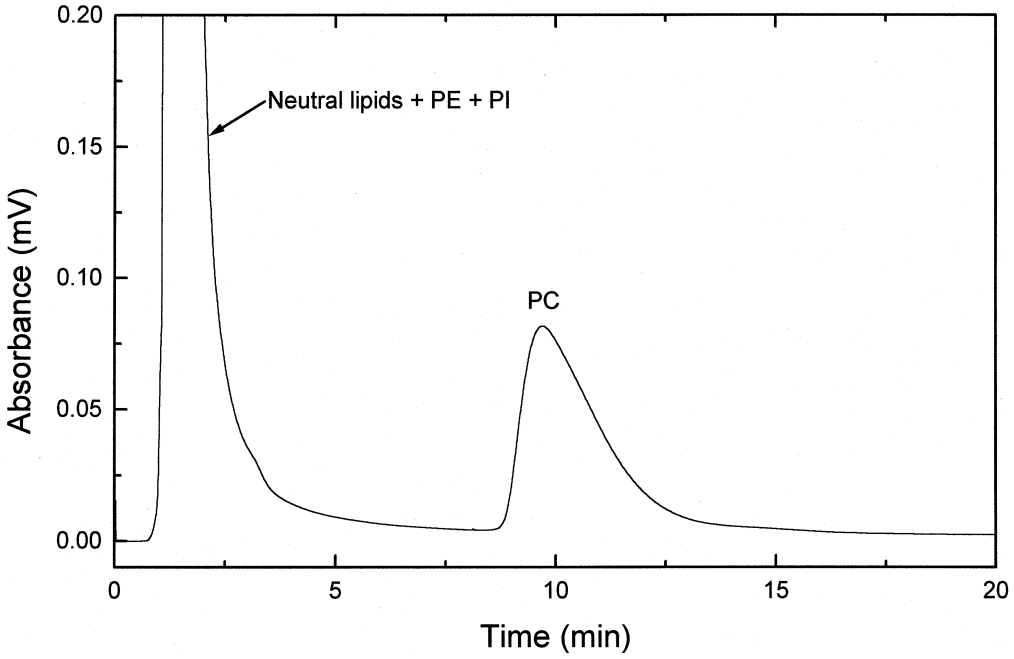

FIG. 1 Calculated elution profile of phospholipids in isocratic mode (hexane/isopropanol/methanol = 50/30/20 vol%).

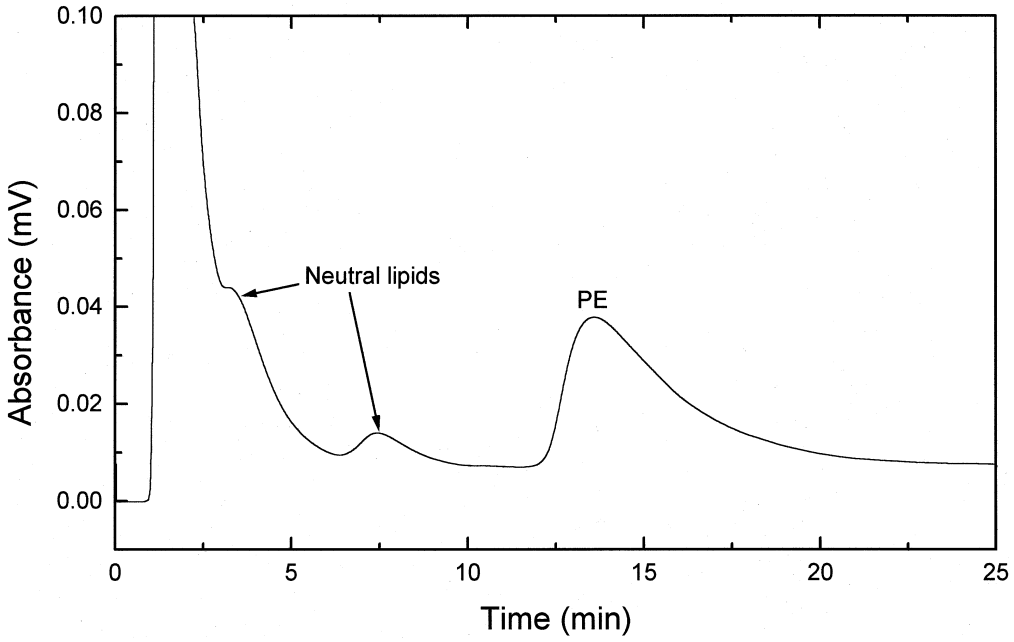

FIG. 2 Calculated elution profile of phospholipids in isocratic mode (hexane/isopropanol/methanol = 92/5/3 vol%).

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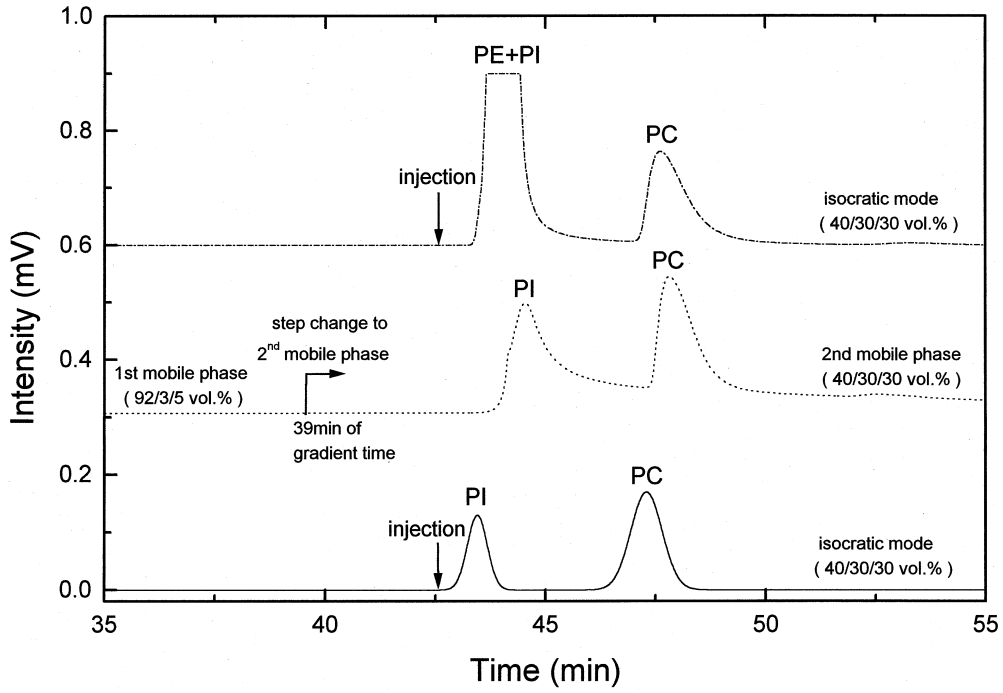

FIG. 3 Comparison of the experimental data (dotted line) and the calculated elution profile (solid line) by isocratic and gradient modes.

the isocratic mode. Comparison of the two experimental peaks of PC in the isocratic and gradient modes (Fig. 3) showed that the two numbers of theoretical plates were almost coincident at the same mobile phase of hexane/isopropanol/methanol (40/30/30 vol%). The elution time was corrected in the isocratic mode by adding the gradient time of 39 minutes and the mixer time (dividing the mixer volume by the flow rate of the mobile phase). This was not the case for PI. To calculate the number of theoretical plates of PI, the retention factors of PI in terms of the mobile phase composition need to be known. By using Eq. (3) combined with Eq. (2), experiments were preformed to determine the coefficients of PI in Eq. (2), and they are shown in Table 1. The

TABLE 1
Empirical Coefficients of PE, PI, and PC Used in Eq. (2)

Material	*A*	*B*	*C*	*D*	*E*
PE	0.41	1.36	-9.39×10^{-2}	-7.07×10^{-1}	2.90×10^{-2}
PI	5.50	-1.00×10^{-2}	-1.00×10^{-4}	-2.00×10^{-2}	-1.30×10^{-2}
PC	5.46	2.55×10^{-2}	-3.67×10^{-4}	-2.28×10^{-1}	2.67×10^{-3}

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elution profiles of PI and PC in the isocratic condition of hexane/isopropanol/methanol (40/30/30 vol%) were calculated by Eq. (4) and are shown in the bottom chromatogram of Fig. 3. The calculated profile of PI was similar to the experimental data.

The retention factors of PE, PI, and PC expressed as Eq. (2) were used to investigate the effects of the organic modifiers isopropanol and methanol in the mobile phase (Figs. 4–6, respectively). As the quantity of methanol increased, the retention factors of PE, PI, and PC decreased. The effect of the amount of isopropanol in the mobile phase is shown in the figures. At higher amounts of methanol, the retention factor are changed negligibly. However, at a very low methanol content the retention factors of PE and PC increase with the content of isopropanol, but above a certain point they decrease with iso-

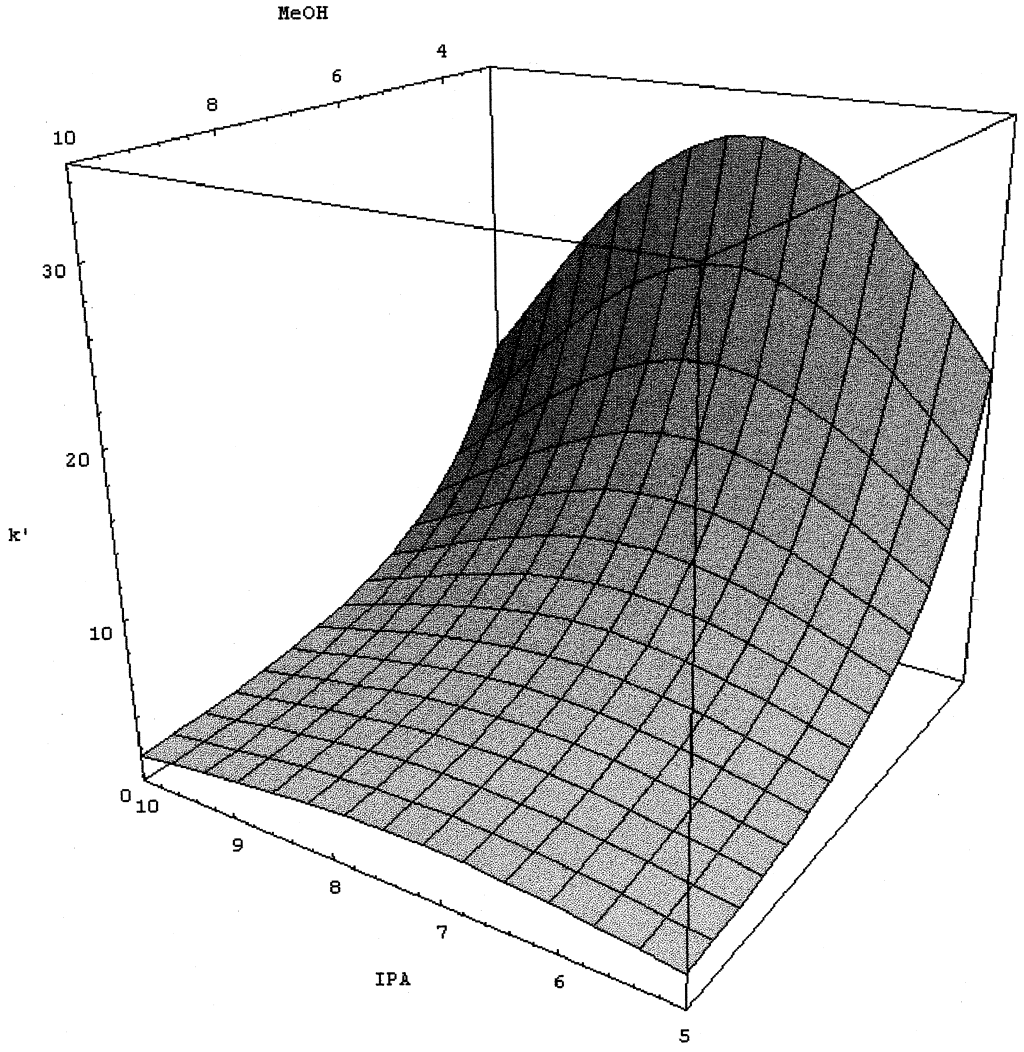

FIG. 4 3-D plot of retention factors of PE with the mobile phase compositions.

propanol. It seems that PE and PC are more adsorbed on silica packings at a lower isopropanol content while isopropanol itself is adsorbed at a higher content and the retention factors are decreased. The proper amount of organic modifier was needed to elute the two components. The solubilities of PE and PC decreased as the length of the alkyl group of alcohols increased, whereas PI dissolved only in methanol and seemed to be insoluble in higher alcohols (12).

The next task was to determine the gradient time and the composition of the second mobile phase composed of hexane, isopropanol, and methanol in order to separate PI and PC in the gradient mode. To investigate the effects of the mobile phase composition and the gradient time of the two phospholipids, the elution profile was simulated by Eq. (4), and the resolution was calculated by

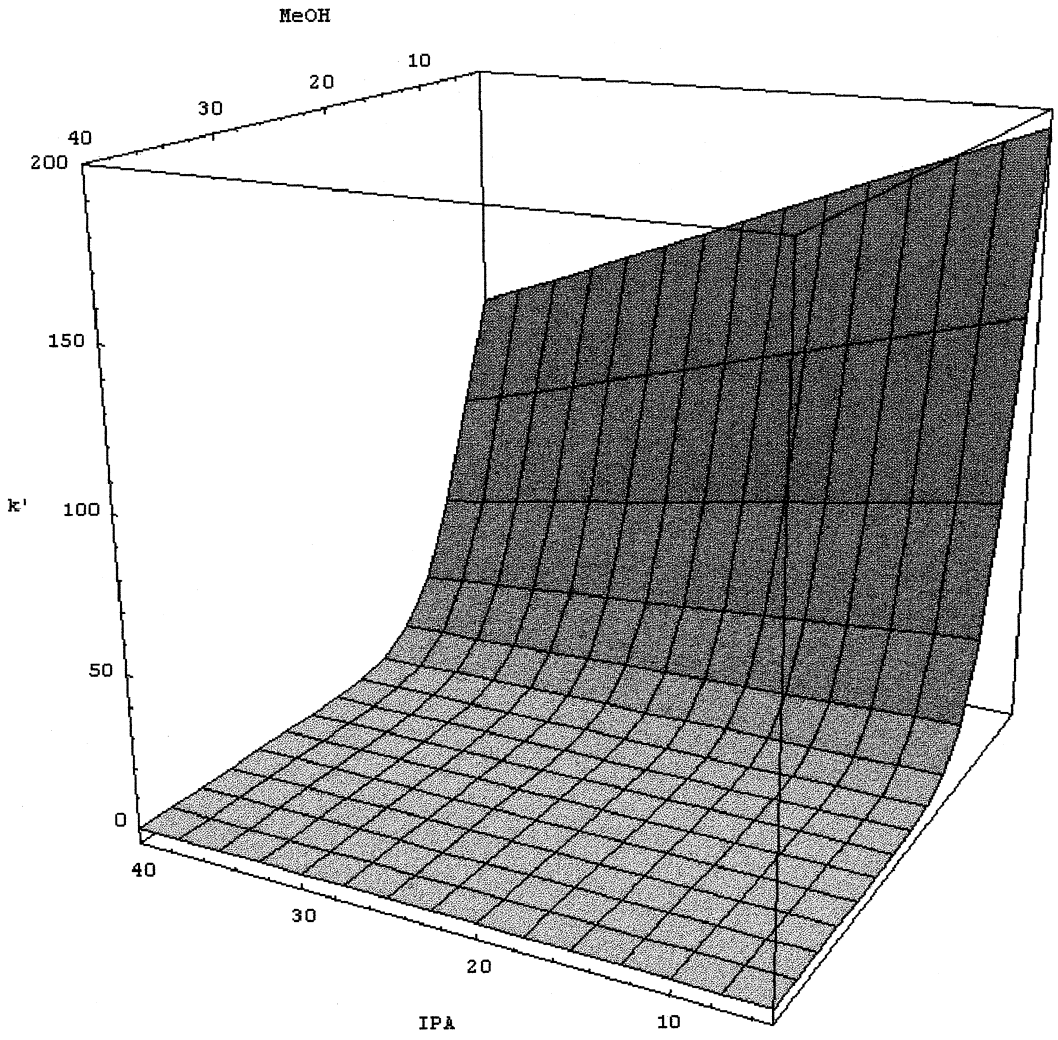

FIG. 5 3-D plot of retention factors of PI with the mobile phase compositions.

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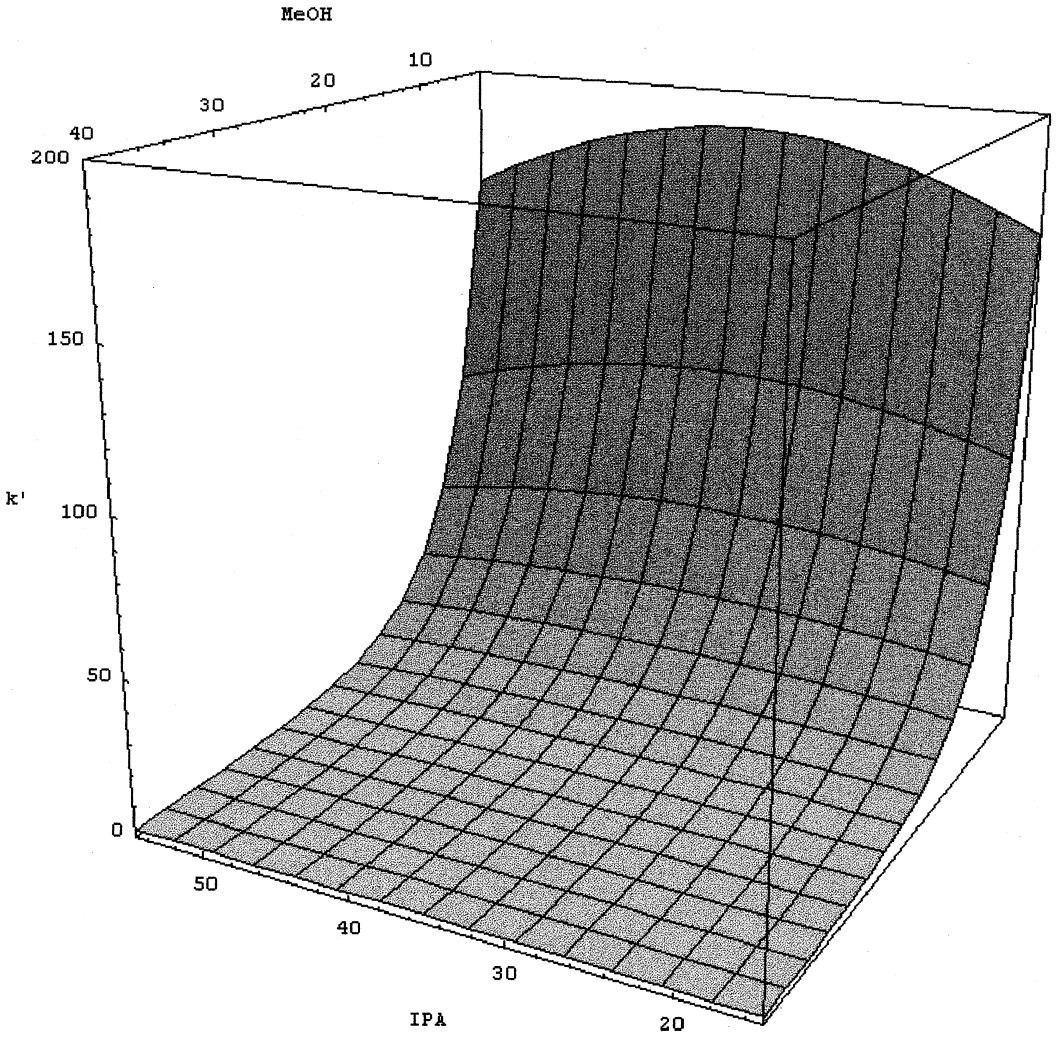

FIG. 6 3-D plot of retention factors of PC with the mobile phase compositions (gradient time: 15 minutes).

Eq. (8). The calculation results are shown in Figs. 7–9. The effect of the quantity of methanol in the second mobile phase on the resolution is shown in Fig. 7. Within the 10–40 vol% methanol range the resolution of PI and PC is higher than 2 but it was deteriorated with an increase in methanol because the difference between the retention times of the two phospholipids became less. At lower contents of methanol and isopropanol the composition was relatively similar to that of the first mobile phase, so the column efficiency was not greatly affected by the step-gradient mode but did result in lower resolution. The resolution was largest at 40 vol% isopropanol. Because PI dissolves better in methanol than the retention factor of PI was less in hexane and isopropanol, at a lower isopropanol content so the resolution was worse. Figure 8 shows the effects of isopropanol on the resolution of PI and PC. The resolu-

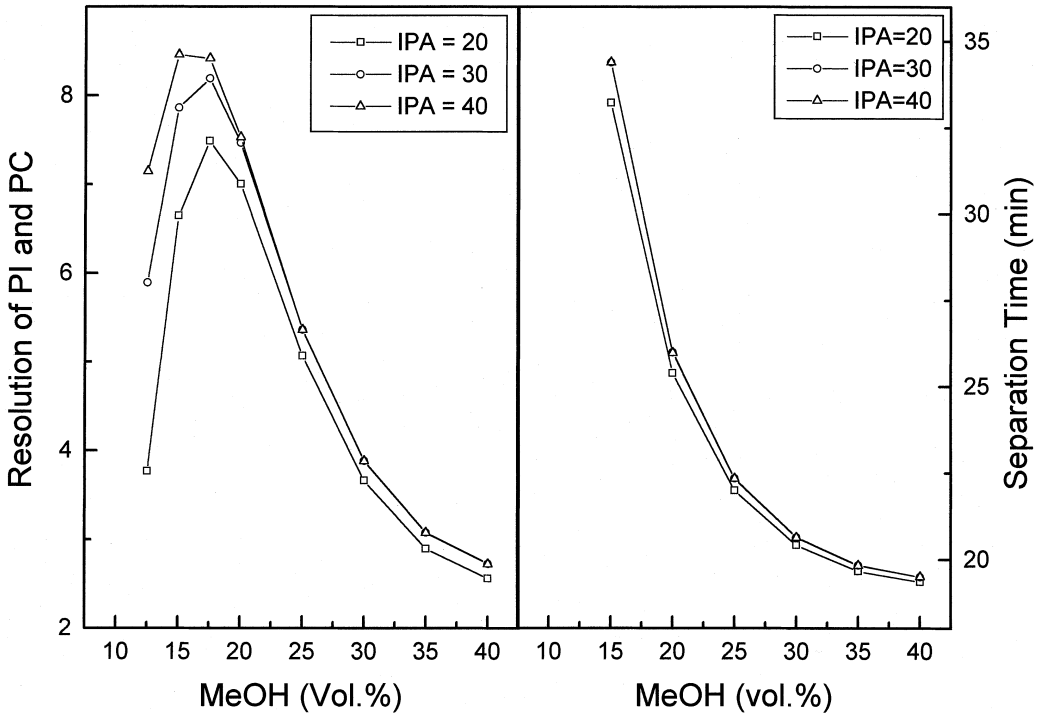

FIG. 7 Changes of resolution and separation time in methanol content of the second mobile phase (gradient time: 15 minutes).

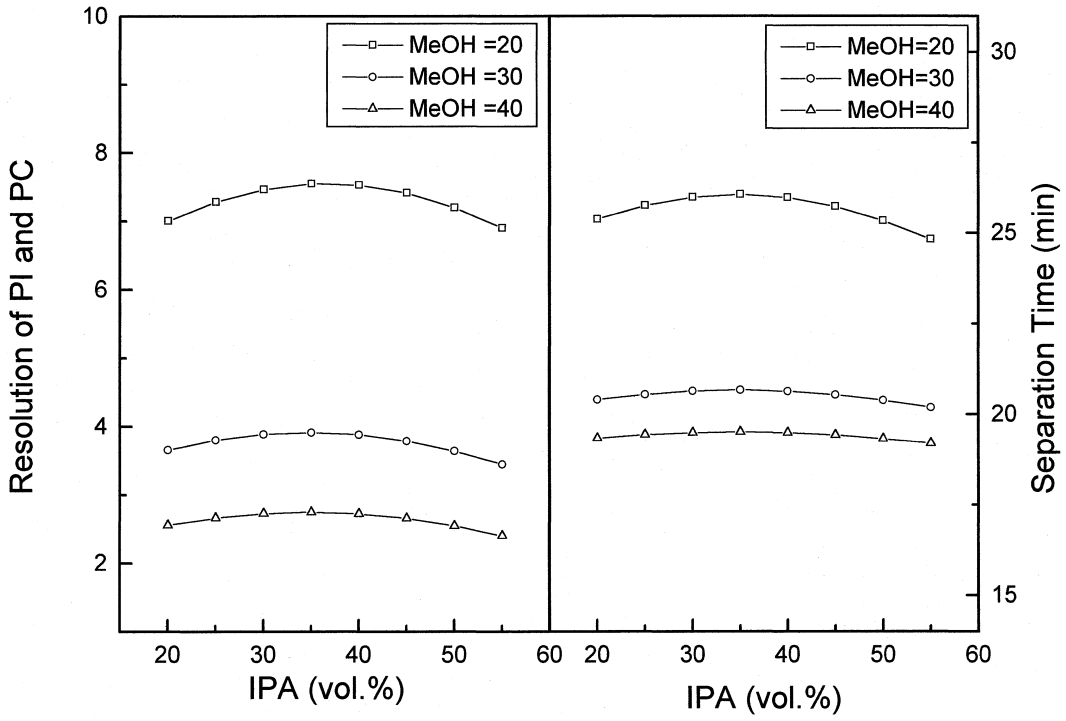

FIG. 8 Changes of resolution and separation time in isopropanol content of the second mobile phase (gradient time: 15 minutes).

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tion was best at 35 vol% isopropanol, and it decreased with a large amount of methanol. The effect of the gradient time on resolution is shown in Fig. 9, where the contents of isopropanol and methanol were in 20–30 and 20–40 (vol%) ranges, respectively, based on the results of Figs. 7 and 8. As the gradient time was increased, a larger amount of the first mobile phase of lower polarity passed the column, and therefore the resolution of PI and PC decreased linearly.

In optimizing the experimental conditions, the separation time as well as the mobile phase composition needs to be considered. The separation times of the *y*-coordinates in Figs. 7–9 were fixed as the termination time of PC elution, i.e., they were determined by the retention time of the last eluting component, PC. The gradient time was fixed at 15 minutes. The separation time was mainly affected by the methanol content in the second mobile phase (Fig. 7). The separation time for the isopropanol content was longer, around 35 vol% (Fig. 8). Contrary to methanol, isopropanol did not have a great effect on the retention time of PC, so the variation of the separation time was almost negligible. As shown in Fig. 9, the separation time increased linearly with the gradient time. The polarity of the first mobile phase, hexane/isopropanol/methanol (90/5/5 vol%), is so low that the retention time of PC was not

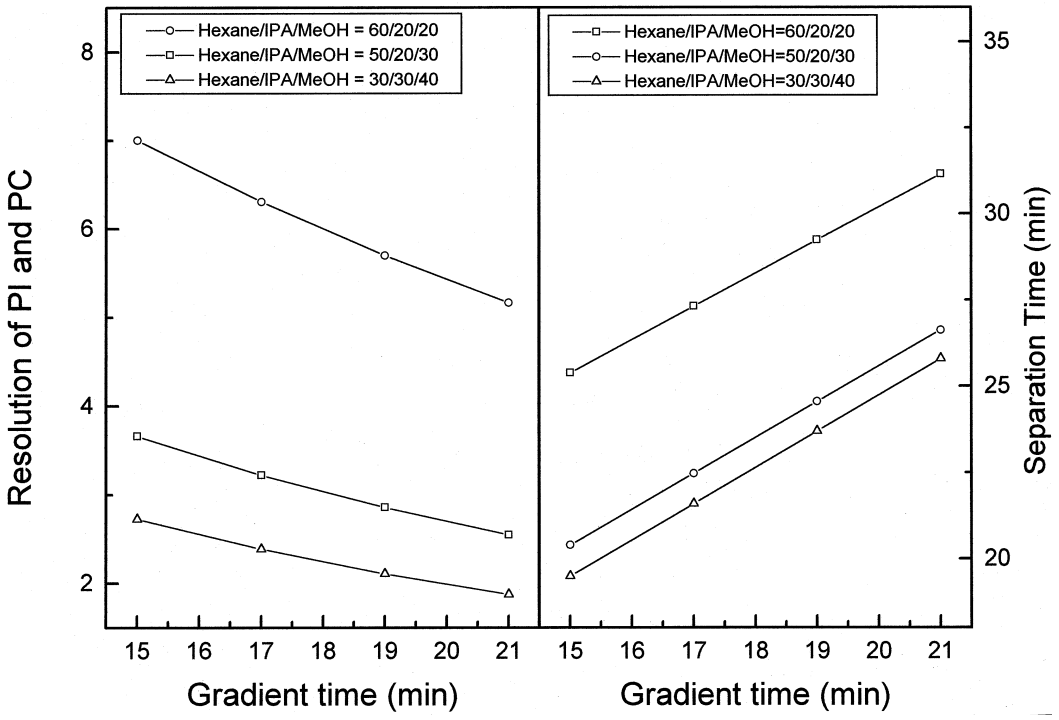

FIG. 9 Changes of resolution and separation time in gradient time and the second mobile phase.

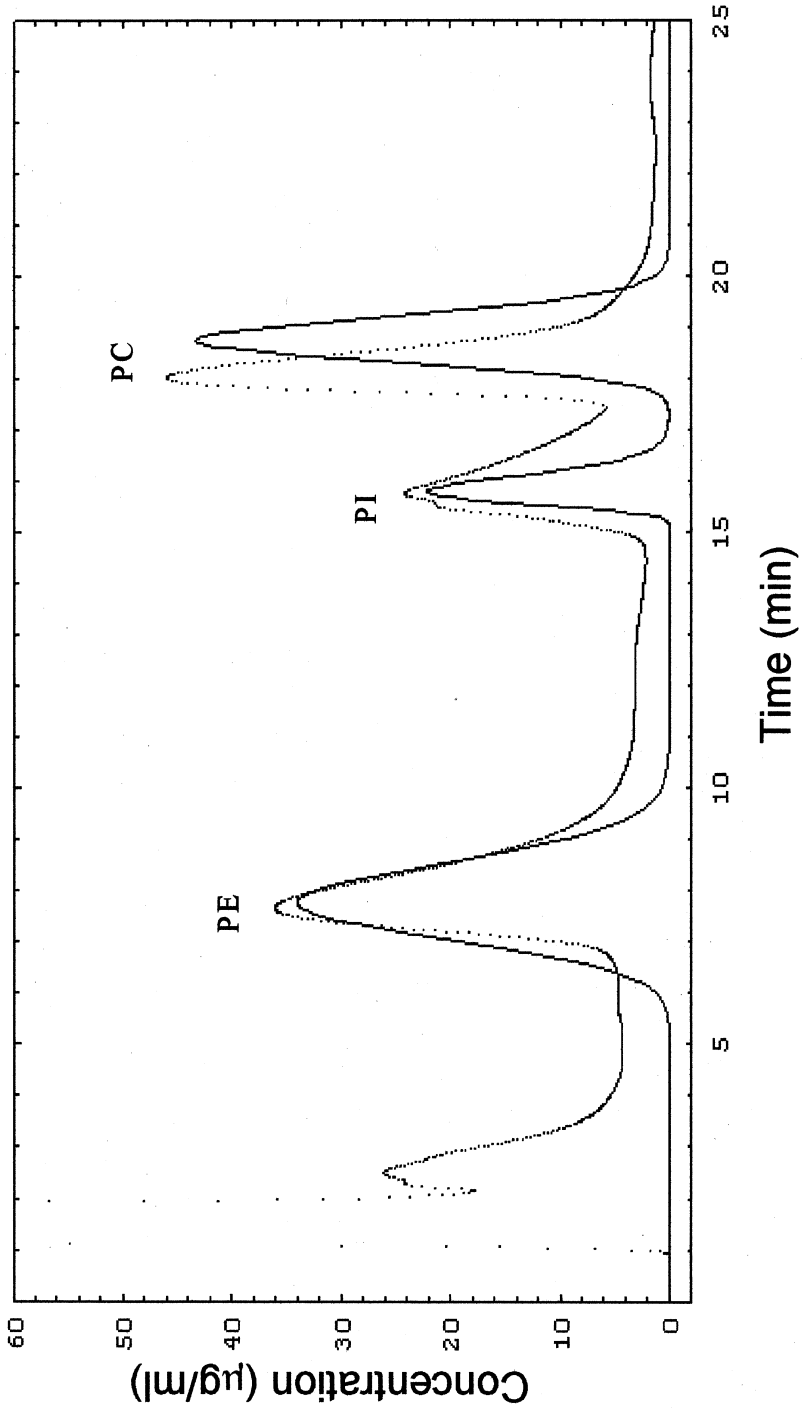

FIG. 10 Comparison of experimental and calculated elution profiles (the first mobile phase, hexane/isopropanol/methanol, 90/5/5 vol%, gradient time 15 minutes the second mobile phase, 50/20/30 vol%).

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changed with the composition of the second mobile phase (refer to the insets in Fig. 9).

The polarity of the first mobile phase had to be quite low to separate neutral lipid and PE, and the methanol content had to be increased to resolve PI and PC. Based on the assumption that the peak width, *w*, of PI and PC was determined by the second mobile phase, it was determined that

$$w = 4V_R N^{1/2} \tag{9}$$

Combining *w* with the retention time by Eq. (3), the number of theoretical plates needed in the gradient mode was calculated, and then the elution profile of PI and PC was calculated by Eq. (4). It took approximately 10 minutes to obtain the optimal condition. From the calculation results based on plate theory, the optimum conditions were for a second mobile phase of hexane/isopropanol/methanol (50/20/30, vol%) and 10 minutes of gradient time. A comparison was made between the calculated elution profile and the experimental data for these experimental conditions. As shown in Fig. 10, the agreement was relatively good.

CONCLUSIONS

The elution profile calculated by plate theory is a symmetric Gaussian curve. In an analytical column characterized by small sample size, elution profiles in the step-gradient mode were simulated by the number of theoretical plates and the retention factor with the mobile phase compositions needed to separate PE, PI, and PC from soybean lecithin.

In our separation scheme based on calculations, the neutral lipids and PE were separated by the isocratic mode while PI and PC were resolved by the step-gradient mode. The optimum composition of the second mobile phase for the step-gradient mode and gradient time were determined by comparing the resolution of PI and PC and the separation times. The resolution was best at 35 vol% but the separation time was longest. After collecting PI and PC from the chromatographic column in a final evaporation stage and considering the relatively higher boiling point of isopropanol (83°C) and the separation time, the second mobile phase was slightly modified to 20 vol% isopropanol and 30 vol% methanol. It is important that the separation time and the resolution be investigated simultaneously in order to determine desirable experimental conditions.

NOMENCLATURE

A, B, C, D, E	empirical constants
a	empirical constant

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c_0	concentration of injected solute (mg/mL)
c_N	concentration of solute in Nth plate (mg/mL)
F, G	volume percent of isopropanol and methanol in the mobile phase, respectively
k'	retention factor
N	number of theoretical plate, column efficiency
r	number of sample-loading plates
R	resolution
$V_{g,1}$	gradient volume (mL)
V_m	dead volume (mL)
V_R	retention volume (mL)
V_{Rg}	retention volume in gradient mode (mL)
v_m, v_s	volume of mobile phase and stationary phase in one plate, respectively (mL)
ω	peak width (mL)

Subscripts

1	first mobile phase zone
2	second mobile phase zone

Greek Letter

β	ratio of mobile phase to stationary phase

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