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GRADIENT SEPARATION OF SOYBEAN PHOSPHOLIPIDS WITH RETENTION FACTORS OF A NEW POLYNOMIAL CORRELATION

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Abstract–The separation mechanism of solutes under gradient conditions has been studied. The separation of a mixture of soybean phospholipids (phosphatidylethanolamine, phosphatidyl-inositol, and phosphatidylcholine) in the ternary mobile phase of methanol, hexane, and isopropanol was investigated. An analytical expression was obtained to calculate the retention volume under a two step-gradient mode. The following new polynomial equation was also presented, $\log k'=A+B\times F+C\times F^2+D\times G+E\times G^2$ where A, B, C, D, and E are empirical coefficients and F and G are volume percentage of isopropanol and methanol in the mobile phase based on hexane, respectively. The resulting equations of the three phospholipids were used to describe the effect of the composition of the ternary mobile phase on the retention factor under step-gradient conditions.

Key words : Phospholipids, Retention Factor, Polynomial Correlation, Step-Gradient Mode

INTRODUCTION

Optimization of gradient elution is very important for analytical HPLC and scale-up column chromatography. The theory of gradient elution processes contains the general problems. One of the problems is connected with a total theory of solute migration under stepwise gradient conditions. Under the assumption that the relationships between the retention factor and composition of the mobile phase are known, this problem was handled in most cases [Markowski and Golkiewicz, 1988].

Predicting the value of the retention factor (k') for any composition of the multicomponent mobile phase is another general problem. More often the correlations are used on a linear dependence log k' via content of one or more components in the mobile phase for binary [Snyder and Quarry, 1987], and ternary [Xie et al., 1991] mixtures. One study [D'Agostino et al., 1988] demonstrated that linear models are apparently not applicable to ternary and quarterly mixtures. The proposed procedure [D'Agostino et al., 1988] is based on the calculation of multiple polynomial regressions between k' and the mobile phase composition, and it includes the five empirical coefficients, by regression analysis.

The problem of phospholipids separation is fully solved [Hurst et al., 1986; Van der Meeren et al., 1990; Hathout et al., 1994; Abidi et al., 1994; Klein and Dudenhausen, 1994; Abidi and Mounts, 1994; Row and Lee, 1997]. The authors used the gradient mode of normal-phase liquid chromatography where water was one of the components of the mobile phase in all cases. We use a ternary mixture (methanol, isopropanol, and hexane), taking into account a continuation of this work for scale-up optimizations. Gradient mode is usually used to separate the phospholipids from soybeans, because the separation was not done by isocratic mode. In this work, to predict the retention times of the three phospholipids in step-gradient mode, a polynomial equation in terms of the ternary mobile phase composition was suggested and utilized.

THEORETICAL

1. Prediction of Retention Factors

Normally, the prediction of retention volume is based on the retention factor dependence on mobile phase composition. The relationship of retention volume and retention factor is expressed by the following :

$$V_R=V_m\,(1+k') \qquad (1)$$

where V_R is retention volume, V_m is dead volume, and k' is retention factor. The relationship of log k' and ternary mobile phase (hexane, isopropanol, methanol) composition is expressed by a new simple polynomial of quadratic form

$$\log k'=A+B\times F+C\times F^2+D\times G+E\times G^2 \qquad (2)$$

where A, B, C, D, and E are empirical coefficients and F and G are volume percentage of isopropanol and methanol in the mobile phase based on hexane. Note that Eq. (2) is a quadratic polynomial without the interaction term of F and G.

2. Gradient Elution

Consider the solute migration in a chromatographic column under stepwise gradient conditions (see Fig. 1). The V axis represents the volume of the mobile phase used during the chromatographic process and the Y axis represents the migration of the solute in the chromatographic column. The mobile phase and the solute start at point 0. Assuming the chromatographic column is in an equilibrium condition, the demixing effects can be neglected. The function, Y(V), is mathematically equal to the migration of solute. The slope of Y(V) is

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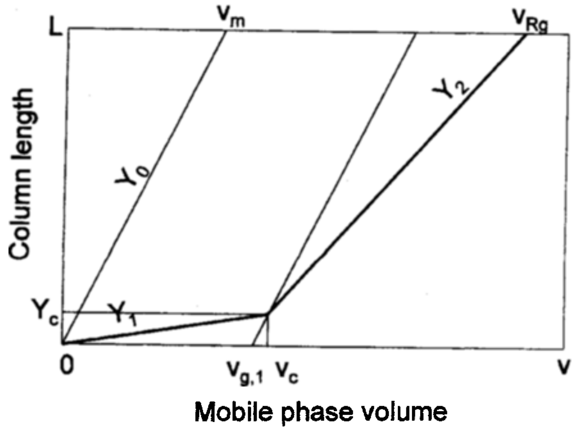

Fig. 1. Solute migration in stepwise gradient mode.

equal to the speed of migration which can be determined by the retention factor.

The solute migrates in the first mobile phase zone, and in this case, the retention factor is expressed as k'_1. The slope of the migrating function (a_1) is expressed as

$$a_1 = L/[V_m \times (1+k'_1)] \tag{3}$$

where L is column length.

The migrating function (Y_1) in the first mobile phase zone is through a point 0. Thus this formula can be expressed as follows :

$$Y_1=(L/[V_m \times (1+k'_1)]) \times V \tag{4}$$

where V is mobile phase volume.

The mobile phase changes to the next mobile phase during dead volume. Therefore this formula can be expressed as follows :

$$Y_0=(L/V_m)(V-V_{g,1}) \tag{5}$$

where $V_{g,1}$ is first mobile phase volume.

Using Eqs. (4) and (5), the initial point of migrating function in the second mobile phase can be found as follows :

$$V_c=[V_{g,1}(1+k'_1)]/k'_1 \tag{6a}$$

$$Y_c=(V_{g,1} \times L)/(V_m \times k'_1) \tag{6b}$$

The migrating function in the second mobile phase can be expressed as follows :

$$a_2=L/(1+k'_2) \tag{7}$$

$$Y_2-Y_c=[L/V_m(1+k'_2)](V-V_c) \tag{8}$$

After substitution of Eq. (5) into Eq. (6) the following equation is obtained :

$$Y_2=(L/[V_m(1+k'_2)]) \times V+[L \times V_{g,1}(k'_2-k'_1)]/[V_m \times k'_1(1+k'_2)] \tag{9}$$

When the solute leaves the column the Y_2 value is L and the V value is the retention volume in stepwise gradient elution (V_{Rg}), and it is expressed as follows :

$$V_{Rg}=V_m(1+k'_2)+V_{g,1}(k'_1-k'_2)/k'_1 \tag{10}$$

where k'_1 and k'_2 are retention factors in the first and second mobile phases.

EXPERIMENTAL

Soybean lecithin of food grade was supplied from Doosan Technical Center. The mixture of soybean phospholipid includes phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC). The mixture was dissolved in HPLC-grade chloroform and the total concentration was 10 mg/ml. Hexane, isopropanol, and methanol were purchased from J. T. Baker (Philipsburg NJ, USA). The HPLC system was equipped with the Waters 600E Multisolvent Delivery System, the UV-visible tunable wavelength absorbance detector, and the U6K injector (2 ml sample loop). For more details of the HPLC system see [Kim et al., 1995]. The data acquisition system was CHROMATE (Ver. 2.1, Interface Eng.) installed in a PC. A Nova-Pak silica column (3.9×150 mm) was used.

RESULTS AND DISCUSSION

The retention factor is one of the most important factors for predicting the retention volume in the chromatographic process of separation. If the retention factor is expressed by a function of the mobile phase composition, the retention volume of the solute can be calculated for the any changes of the mobile phase composition. To find the parameters experimentally determined in Eq. (2), we performed experimental runs at different compositions of the mobile phase. It is easier to separate the phospholipids of PE and PC under isocratic conditions. Experimental conditions and retention volumes of PE and PC are presented in Table 1. These data were used to calculate the empirical coefficients of Eq. (2), which are listed in the Table 2.

Table 1. Experimental conditions and retention of PE and PC under isocratic conditions

Material	Mobile phase (vol%)			Retention time (min)
	Hexane	Isopropanol	Methanol	
PE	85	10	5	6.17
	83	10	3	13.57
	80	10	10	2.45
	87	10	7	3.75
	90	5	5	7.67
	80	5	10	2.50
	92	5	3	21.73
PC	40	20	40	3.20
	50	20	30	4.77
	60	20	20	9.70
	65	20	15	16.93
	30	30	40	3.25
	20	40	40	3.32
	10	50	40	3.15
	5	55	40	3.02
	40	30	30	5.07
	30	40	30	5.07
	55	15	30	4.12
	50	30	20	10.01
	83	10	7	75.67
	80	10	10	30.17

Table 2. Empirical coefficients of 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}

The results show the next characteristic properties of phospholipids resolution under the isocratic mode of elution. The solute PE is easily separated in the mobile phase of lower polarity, and PC in the mobile phase of higher polarity. This is illustrated by Figs. 2 and 3. PI did not exist alone in either case. In the mobile phase of low polarity the retention volume of PI is similar to PC and the peak band is very broad. In the mobile phase of high polarity, the retention volume of PI cannot be determined under isocratic conditions. PE and PC were separated in isocratic mode, but PI was not separated in isocratic mode. In the high polarity mobile phase, the retention volume of PI is similar to PE and PI peak is hidden by chloroform peak. Thus the detection of PI in isocratic elution is impossible in this system.

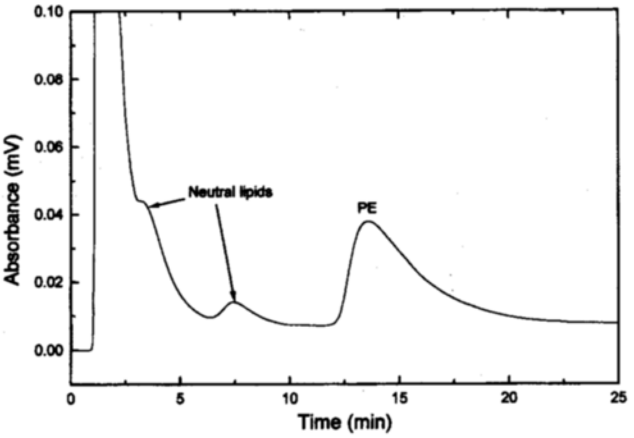

Fig. 2. Isocratic chromatogram of phospholipids.
(hexane/isopropanol/methanol=87/10/3 vol%, 1.5 ml/min)

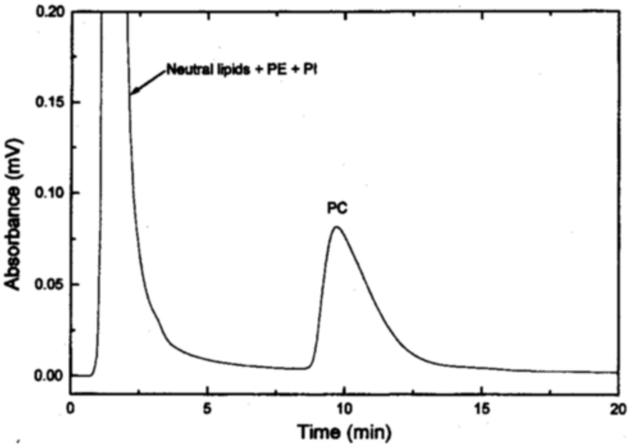

Fig. 3. Isocratic chromatogram of phospholipids.
(hexane/isopropanol/methanol=60/20/20 vol%, 1.5 ml/min)

To calculate the retention factor of PI, the gradient mode was used. All three phospholipids were separated under some stepwise gradient conditions (see for example Fig. 4). The retention time of PI was determined under gradient conditions. The coefficients of Eq. (2) for PI can be calculated as follows. Substituting Eq. (2) into Eq. (10), the resulting nonlinear equation with the five empirical coefficients can be solved with the five experimental runs which are presented in the Table 3. The iteration method was applied to obtain the coefficients. The initial value was tried as the corresponding value of PI. Each coefficient was taken within the error of 5 %

Fig. 5 shows the order of the A's coefficient determination. The number in the box indicates the experimental run of Table 3. The error was minimum at 5.5, and this trends was observed in the other four coefficients, B, C, D, and E. The good agreement between the calculated retention factor of PI and the experimental data of Table 3 is shown in Fig. 6. Finally, Table 2 lists the coefficients in Eq. (2) for PE, PI, and PC. The retention factors of PE, PI, and PC expressed by Eq. (2) were used to investigate the effects of organic modifiers (methanol and isopropanol) and are presented in Figs. 7 to

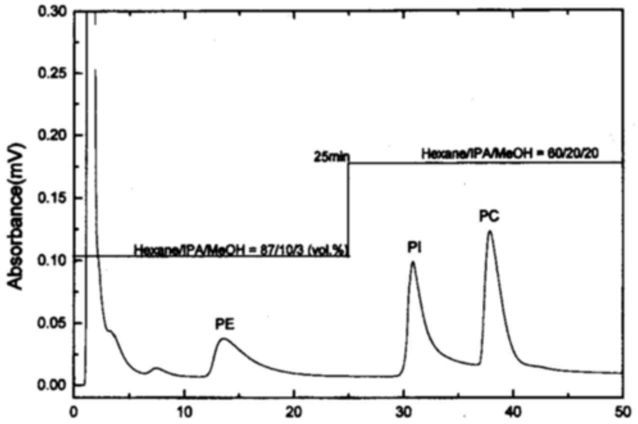

Fig. 4. Gradient chromatogram of phospholipids.

Table 3. Experimental gradient conditions and retention of PI

No.	1st mobile phase (vol%)			Gradient volume, $V_{g,1}$ (ml)	2nd mobile phase (vol%)			Retention volume, V_{Rg} (ml)
	Hexane	Isopropanol	Methanol		Hexane	Isopropanol	Methanol	
1	85	10	5	45	30	30	40	53.28
2	87	10	3	37.5	60	20	20	46.25
3	90	5	5	19.5	50	30	20	28.20
4	85	5	10	22.5	30	40	30	30.26
5	92	5	3	58.5	40	30	30	66.80

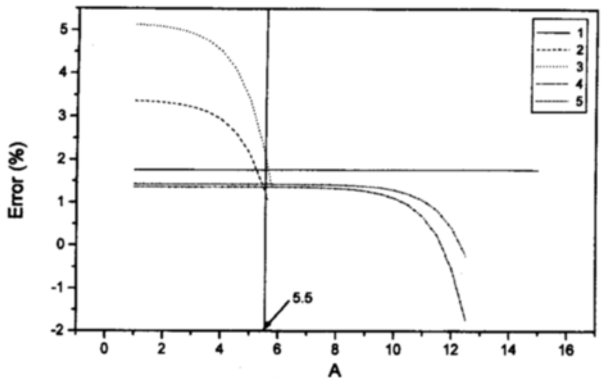

Fig. 5. Error map of the coefficient, A.

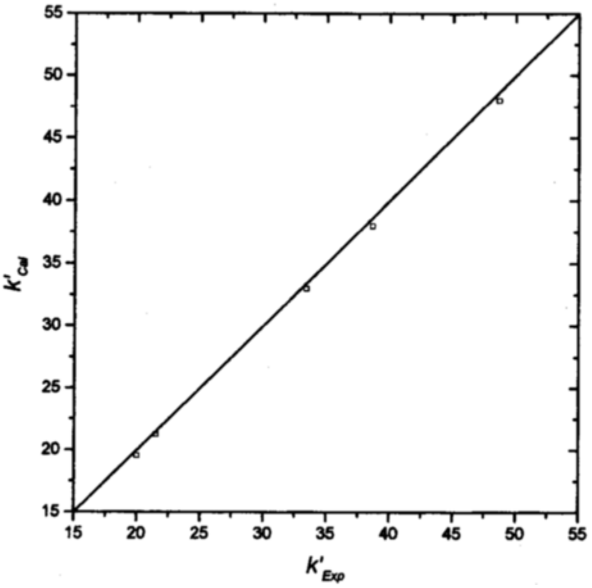

Fig. 6. Comparison of calculated and experimental retention factors of PI (r^2=0.99997).

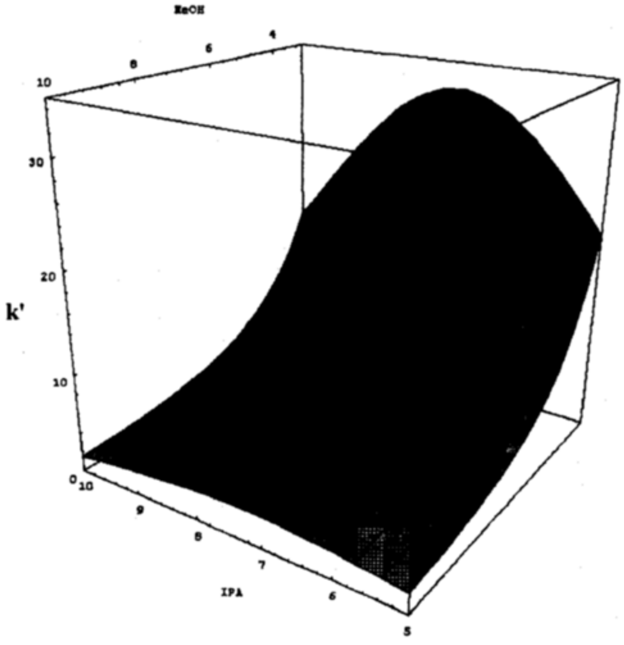

Fig. 7. 3D-plot of retention factors of PE with the mobile phase compositions.

9. As the quantity of methanol increased, the retention factors of PE, PI, and PC decreased. Also, the effect of the amount of isopropanol in mobile phase is shown in Figs. 7 to 9. At the higher amount of methanol, the retention factors are changed negligibly. However, at a very low content of methanol, the retention factors of PE and PC increased with the content of isopropanol. But above a certain point, the retention factors of PE and PC increased with the content of isopropanol. These results indicate that PE and PC are more adsorbed in silica packings at a lower content of isopropanol, while at a higher content of isopropanol, the isopropanol itself is strongly

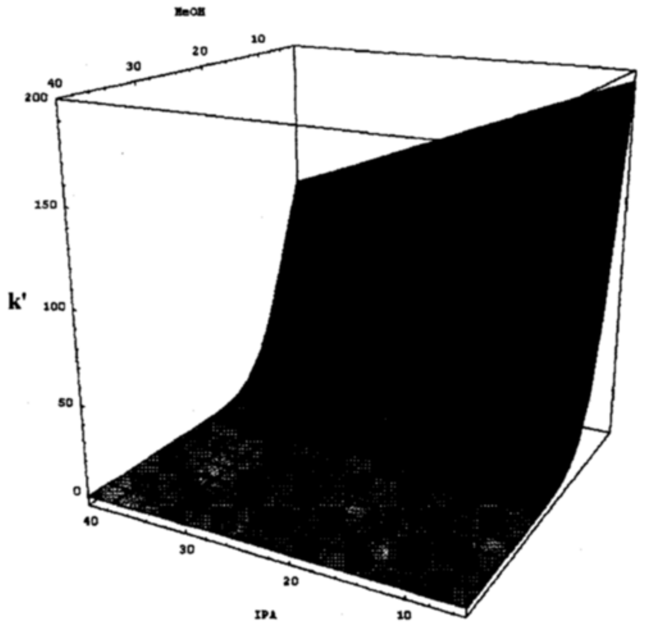

Fig. 8. 3D-plot of retention factors of PI with the mobile phase compositions.

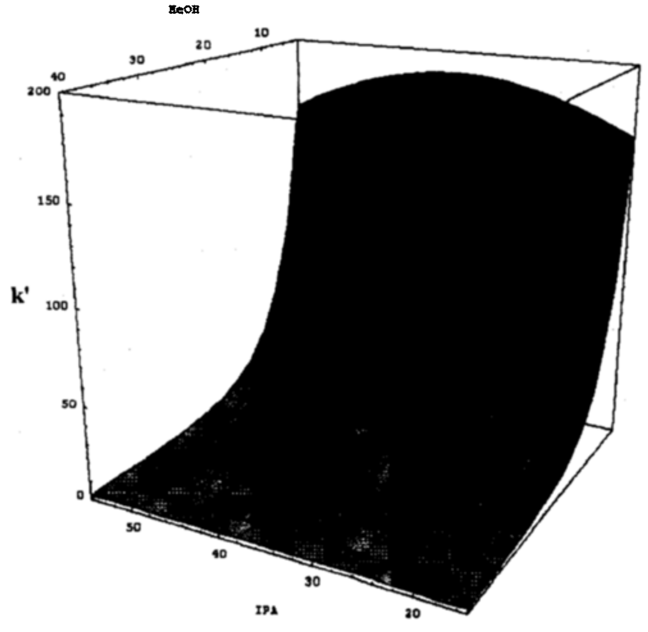

Fig. 9. 3D-plot of retention factors of PC with the mobile phase compositions.

adsorbed, and the retention factors are decreased. The mechanism of adsorption of PE, PC, and PI is discussed according to the solubility of phospholipids dissolved in alcohols [Van der Meeren et al., 1990].

CONCLUSION

It is very difficult to separate phospholipids in an isocratic run, because of the wide range of the lecithin polarities. Gradient elution is usually adopted to overcome the problem. To determine the separation condition in the gradient elution, the gradient time and composition need to be adjusted. The new type of a correlation of retention factor suggested in this work greatly simplifies the optimization for gradient elution. The separation of phospholipids was considered to demonstrate the usefulness of the new correlation. With the polynomial equation, the separation work was already done and will be published elsewhere.

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NOMENCLATURE

A, B, C, D, E : empirical constants
a : slope of the function Y
F, G : volume percentage of isopropanol and methanol in the mobile phase, respectively
k' : retention factor
L : column length [cm]
V : volume of the mobile phase through the column [ml]
V_c, Y_c : initial points of solute migration in second mobile phase
$V_{g,1}$: first gradient volume [ml]
V_m : dead volume [ml]
V_R : retention volume [ml]
Y(V) : migration function of solute in the column
Y_0 : migration function of changing mobile phase zone

Subscripts

1 : first mobile phase
2 : second mobile phase
cal : calculated data
exp : experimental data
g : gradient mode

REFERENCES

Abidi, S. L. and Mounts, T. L., "Reversed Phase Retention Behavior of Fluorescence-Labeled Phospholipids in Ammonium Acetate Buffers," *J. Liquid Chrom.*, **17**, 105 (1994).

Abidi, S. L., Mounts, T. L. and Rennick, K. A., "Separation of Major Soybean Phospholipids on Beta-Cyclodextrin-Bonded Silica," *J. Liquid Chrom.*, **17**, 3705 (1994).

D'Agostino, G., O'Hare, M. J., Mitchell, F., Salomon, T. and Verillon, F., "Optimization of the Mobile Phase Composition in Gradient Elution Reversed-Phase HPLC by Stochastic Prediction," *Chromatographia*, **25**, 343 (1988).

Hathout, Y., Maume, G. and Maume, B. F., "High-Performance Liquid-Chromatographic Study of the Regulation of Phospholipid-Metabolism in Cultured Adrenocortical-Cells," *J. Chromatogr. Biomed. Appl.*, **652**, 1 (1994).

Hurst, W. J., Martin, Jr. R. A. and Sheeley, R. M., "The Preparative HPLC Isolation and Identification of Phospholipids from Soy Lecithin," *J. Liquid Chrom.*, **9**, 2969 (1986).

Kim, J. D., Row, K. H., So, M. S., Polunina, I. A. and Larin, A. V., "Chromatographic Behavior of Deoxyribonucleosides with Respect to Organic Modifier Content in the Mobile-Phase," *J. Liquid Chrom.*, **18**, 3091 (1995).

Klein, B. H. and Dudenhausen, J. W., "Simultaneous Determination of Phospholipid Classes and the Major Molecular-Species of Lecithin in Human Amniotic-Fluid by HPLC," *J. Liquid Chrom.*, **17**, 981 (1994).

Markowski, W. and Golkiewicz, W., "Optimization of Stepwise Gradient Elution in Column Chromatography," *Chromatographia*, **25**, 339 (1988).

Row, K. H. and Lee, J. W., "Preparative Separation of Phsopholipids from Soybean by NP-HPLC," *Korean J. Chem. Eng.*, **14**, 412 (1997).

Snyder, L. R. and Quarry, M. A., "Computer Simulation in HPLC Method Development. Reducing the Error of Predicted Retention Times," *J. Liquid Chrom.*, **10**, 1789 (1987).

Van der Meeren, P., Van der Deelen, J., Huys, M. and Baert, L., "Preparative Scale Purification of Soybean Phospholipids Using High-Performance Liquid Chromatography," *J. Amer. Oil Chemists' Society*, **67**, 815 (1990).

Xie, M., Zhou, Ch., Ren, Zh. and Luo, T., "Experimental Model of Predicting Solute Retention Time as a Function of Mobile Phase Composition in Ternary Reversed-Phase High-Performance Liquid Chromatography," *J. Chromatogr.*, **555**, 33 (1991).