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SHORT COMMUNICATION

Separation of Phospholipids from Soybean by NP-HPLC with ELSD

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Abstract−Phospholipids of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) from lecithin were separated on gradient steps by NP-HPLC with ELSD. The ternary mobile phases of hexane, IPA, and water were used, and the optimum mobile phase conditions were experimentally determined. The calibration curves of phospholipids were constructed to analyze quantitatively the amounts of PE and PC. The responses of PE by ELSD showed linearity with the injected amount range but a logarithmic relationship of PC was observed. By quantitative analysis, 14.2% of PE and 36.6% (w/w) of PC were contained in the sample of lecithin. Finally, in the separation of phospholipids, the detection by ELSD was better than that by UV.

Key words: Phospholipids, Mobile Phase Composition, NP-HPLC, ELSD

INTRODUCTION

Phospholipids are usually found in all biological membranes and contain an extremely complex mixture of different classes, such as phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC) [Olsson et al., 1997]. In plant and animal tissue phospholipids commonly serve as structural components in membranes in addition to playing a role in enzyme activation [Eckard et al., 1998]. For this reason, they are widely based in the food and cosmetic industries, as well as industrial manufacturing [Eckard et al., 1998; Munster et al., 2000]. Most of these industrially used phospholipids have been obtained from a by-product of vegetable oil [Sas et al., 1999].

Separations of phospholipids have been studied by many researchers [Miwa et al., 1996; Servillo et al., 1997; Montanari et al., 1999; Row and Lee, 1999] and performed with numerous methods including thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The previous quantification and separation of phospholipids have been almost performed with thin layer chromatography (TLC) [Miwa et al., 1996; Montanari et al., 1999]. This method has several disadvantages, such as quantitative separation of individual species is very difficult [Bunger et al., 1995] and not always accurate. In the case of using a UV detector for analysis of phospholipids, an essential defect was occurring. Because the absorbance of phospholipids arises just from the double bond in fatty acid moieties [Bunger et al., 1995], an extremely poor response is obtained. Accordingly, accurate quantification could not be performed.

In recent years, HPLC with an evaporative light scanning detector (ELSD) was reported as a good analysis tool for phospholipids [Sas et al., 1999; Bunger et al., 1995] because of better reproducibility and less sensitive baseline-shifting during gradient elution, compared to UV detection. The purpose of this work was to separate each of the phospholipids, PE and PC, by normal-phased high performance liquid chromatography with ELSD. The accurate quantification of PE and PC was also considered.

EXPERIMENTAL

1. Reagents

HPLC grade solvents, [chloroform, hexane, and isopropanol (IPA)] were from J. T. Baker (Philipsburg NJ, USA). Water filtered by a Millipore ultra pure water system (Millipore, Bedford, MA, USA). Lecithin, labeled PERCEPT 8140, the major mixture of phospholipids, was supplied from Central Soya (Fort Wayne, Indiana, USA). Phospholipid standards, PC and PE from soybeans, were purchased from Sigma (St. Louis, MO, USA).

2. Sample Preparation

The powder of PERCEPT 8140, 100 mg, was dissolved in chloroform 10 ml, then the concentration of the solution was adjusted to 10,000 μg/ml. The concentrations of PC and PE were 5,000 and 10,000 μg/ml, respectively. A constant injection volume of phospholipid solution, 5 ml, was used throughout.

3. Apparatus and Method

HPLC was performed by using a Waters 600S solvent delivery system (Waters, Milford, MA, U.S.A.) with ELSD (Alltech). Data acquisition system was Autochro 2.0 installed in an HP Vectra 500 PC. The drift tube temperature of ELSD was set at 75 °C and the nebulizer gas flow rate was 1.6 *l*/min. A 2487 UV dual channel detector was used (Waters, Milford, MA, U.S.A.).

The mobile phases were degassed with helium. The flow rate of the mobile phase was fixed at 1.0 ml/min. The mobile phases were hexane, IPA, and water, respectively. Gradient mode of mobile phase was applied to adjust the total run time between 25 and 50 min. The gradient conditions are listed in Table 1. The column was purchased from Alltech Co. The column size was 0.46×15 cm and packed with Allsphere Silica 5 μm. All the experimental runs were done

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Table 1. NP-HPLC gradient conditions

	Gradient conditions			
	Gradient time (min)	Hexane vol%	IPA vol%	Water vol%
Fig. 2	Initial	92	8	0
	4	42	56	2
	9	40	56	4
	13	36	56	8
Figs. 3 and 4	Initial	92	8	0
	3	42	56	2
	11	40	56	4
	13	40	56	4
	15	0	92	8

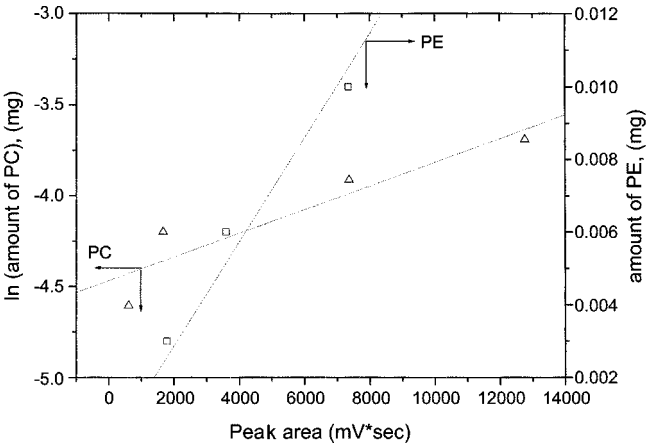

Fig. 1. Calibration curves for phospholipids of PE (□) and PC (△).

at ambient temperature.

RESULTS AND DISCUSSION

Calibration curves were constructed to measure each amount of PC and PE in lecithin. The amount of PC ranged from 0.01 to 0.025 mg, while that of PE from 0.003 to 0.01 mg. The response of PC by ELSD with NP-HPLC was nonlinear and well fitted on the semi-logarithmic scale as shown Fig. 1. However, the response of PE showed linearity in the range of injection amount. It is an interesting result, as the relationship between the amount of injection and peak area was normally linear in a dilute range of UV detector. However, the linearity varied with the compounds injected into ELSD. Therefore, the calibration curve of PC was fitted as $\ln y = 6.53\times10^{-5}x - 4.47$, where y is the amount (μg) of injected PC and x is the peak area (mV*sec). The value of regression coefficient was 0.927. The calibration curve equation of PE was linear and expressed as $y = 1.43\times10^{-6}x$. The regression coefficient was 0.993, and it was enough close to 1.

The gradient-elution mode was employed to separate phospholipids. It is very difficult to resolve PE from PC in isocratic mode, because PE is less polar and PC is more polar. In addition, several gradient steps were necessitated, and four linear-gradient steps were utilized in this work. Although PC could be separated as a single

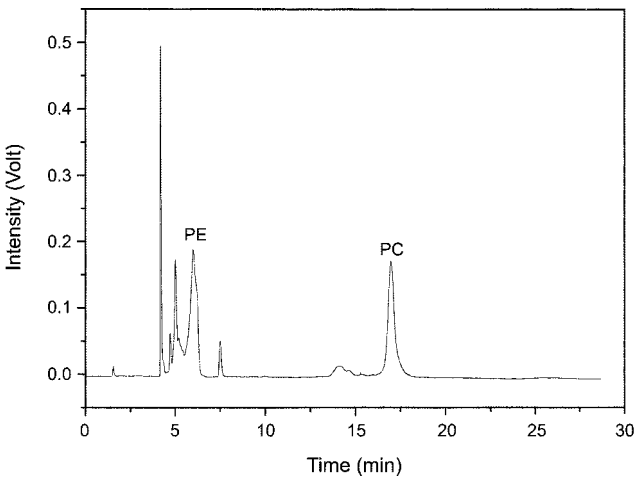

Fig. 2. Separation of phospholipids by NP-HPLC with ELSD (Mobile phase condition in Table 1, concentration 10 mg/ml, injection volume 0.5 μl).

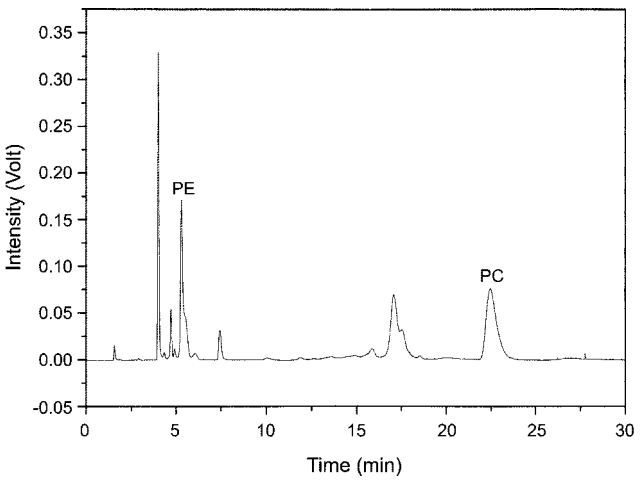

Fig. 3. Separation of phospholipids by NP-HPLC with ELSD (Mobile phase condition in Table 1, concentration 10 mg/ml, injection volume 0.5 μl).

peak, the resolution of PE in Fig. 2 was not good. To adjust the experimental conditions, the mobile phase composition of hexane and the first gradient time was changed from 40 to 42 vol% and from 3 to 4 min. The complete mobile phase condition was listed in Table 1. Since the dead volume used in this chromatographic system was about 3 min, changing the composition of the first step would affect the solutes with the retention time of around 6 min. The resulting chromatogram was shown in Fig. 3. The resolution of PE and PC was improved although the total run time was larger.

The quantifications of PE and PC from lecithin, labeled PERCEPT 8140 from Central Soya, were performed with the calibration curve of a standard chemical. In Fig. 3, the peak areas of PE and PC were 4970.948 and 7198.853 (mV*sec), respectively. The corresponding amounts of PE and PC were determined as 0.0071 and 0.018 mg, respectively. The percentages of PE and PC in the sample of lecithin were 14.2 and 36.7% (w/w).

Fig. 4 shows the separation of phospholipids by NP-HPLC with UV-detector. To separate the substances, linear-gradient mode is

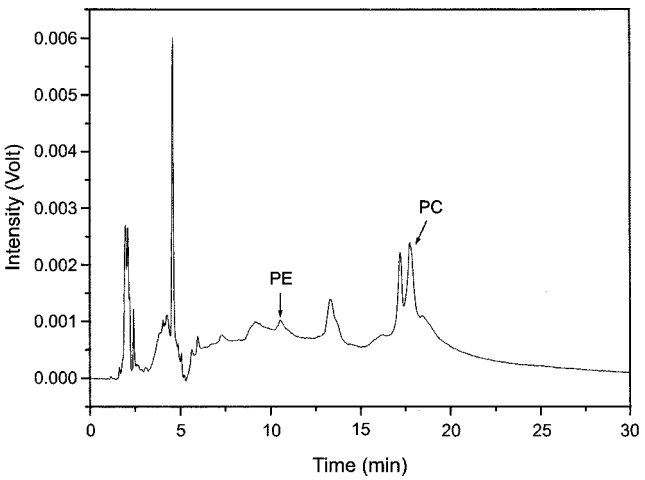

Fig. 4. Separation of phospholipids by NP-HPLC with UV detector (Mobile phase condition in Table 1, concentration 10 mg/ml, injection volume 0.5 μl, 254 nm UV wavelength).

essential, but the baseline might be drifted due to the constant change in mobile phase composition. Compared to isocratic mode, when UV detector was applied, the increase in resolution was offset by unstable baseline. Therefore, in the research of phospholipid, ELSD has been commonly used [Sas et al., 1999; Bunger et al., 1995].

CONCLUSION

Phospholipids were separated on the gradient-elution mode by NP-HPLC with ELSD for more precise quantitative analysis of PE and PC. The operating conditions of mobile phase composition and gradient conditions were experimentally measured. It was found that 14.2 wt% of PE and 36.7 wt%. of PC were contained in the sample of lecithin.

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