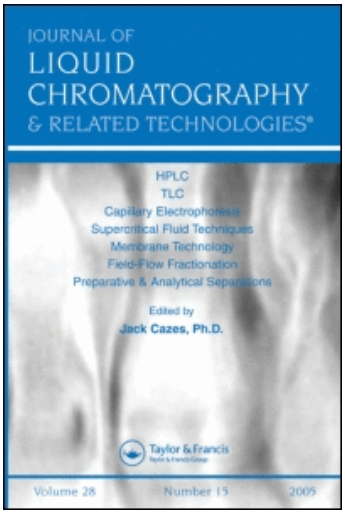

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SEPARATIONS OF MAJOR SOYBEAN PHOSPHOLIPIDS ON β-CYCLODEXTRIN-BONDED SILICA

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ABSTRACT

The four major phospholipids (PL) phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA) found in soybean oil were separated by normal-phase high-performance liquid chromatography (HPLC) on β-cyclodextrin-bonded silica (CDS) with UV detection. Adequate base-line separations of the PL components were achieved by isocratic elution with mobile phases containing hexane, isopropanol, ethanol and water/tetramethylammonium phosphate (TMAP). The presence of TMAP in the mobile phases was critical to improve component resolution and enhance peak symmetry. Analyte retention and component separations were dramatically influenced by a small change in mobile phase compositions. Under the HPLC conditions employed, the elution order appeared to follow the order of increasing PL polarity with increasing retention times (PE < PC < PI < PA). The HPLC method was used in the qualitative analyses of selected

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commercial lecithin samples. Potential applicability of the HPLC-UV-CDS technique for the quantitative analysis of several crude oil samples derived from genetically modified soybean oil is demonstrated.

INTRODUCTION

Phospholipids (PL) are important physiological substances naturally occurring in animal and plant cell membranes. Our on-going research directed toward assessment of the impact of genetic engineering on quality of vegetable oils, required continuous development of new chromatographic techniques for the analysis of PL in oil samples. Accurate analysis of the polar lipid compositions and molecular species distributions in modified oils provides useful information on the stability and quality of the oil. Although there are many publications in the literature on analytical methodology for the quantification of PL in a wide variety of tissue samples derived from plants and animals, high-performance liquid chromatographic (HPLC) separations of PL mixtures are heavily dependent on experimental conditions employed, specific sample matrices used, and the nature and complexity of PL structures in the mixtures for particular studies. Therefore, methodological alternatives to published HPLC procedures continue to be of much interest to lipid scientists.

Mixtures containing various PL classes have been separated by normal-phase HPLC (1-23). Most PL

separations have been carried out on silica (SI) columns. To a lesser extent, some researchers have used columns of silica-based polar phases such as amino- (15, 19-21), diol- (22, 23) and benzene sulphonate-bonded silica (7) for PL analyses. In recent years, another type of polar stationary phase, cyclodextrin-bonded silica (CDS) columns have become available commercially and have been used extensively in chiral separations. However, the CDS phases have not been utilized for the separation of polar lipids. In view of the wide applicability of the CDS column for the analysis of a broad spectrum of compounds (24) including tocopherols (25), the normal-phase HPLC behavior of soybean PL components on CDS was studied under mobile phase conditions similar to the hexane-isopropanol-water solvent systems conventionally used in conjunction with a SI column. The results are reported in this paper.

EXPERIMENTAL

Materials:

Reference soybean PL standards phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), lyso-PC, lyso-PE, and egg phosphatidic acid (PA) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Commercial soybean lecithin samples were supplied by Lucas Meyer Inc. (Decatur, IL, USA). Soybean oil samples derived from

genetically modified soybeans (supplied by North Carolina State University, NC, USA) were subjected to silica gel column chromatography following a published procedure (26) to give analytical samples for soybean PL assays. Tetramethyl ammonium phosphate (TMAP) was prepared from a 25% aqueous solution of tetramethyl hydroxide (Aldrich Chemical Co., Milwaukee, WI, USA) by titration with 85% phosphoric acid (Fisher Chemicals, Fair Lawn, NJ, USA) until the solution reached Ph 6.3. Chromatography-grade hexane and isopropyl alcohol (ISP) were obtained from Fisher Chemicals. Ethyl alcohol (ETOH) was the product of U.S. Industrial Chemical Co. (Cincinnati, OH, USA). HPLC-quality water was obtained by purification of distilled water through a Milli Q water purifier (Millipore Corp., Bedford, MA, USA). All other reagents and solvents were purchased from J. T. Baker, Inc. (Phillipsburg, NJ, USA).

Methods:

In all HPLC experiments, a Spectra-Physics (San Jose, CA., USA) liquid chromatograph equipped with a Model SP8700 solvent delivery system was used. The LC instrument was interfaced with an LDC Analytical (Riviera Beach, FL, USA) SpectroMonitor D variable wavelength UV detector which was set at 208 nm for monitoring column effluents. Mobile phases employed

three solvent reservoirs containing (1) hexane, (2) ISP-ETOH, and (3) water/5 Mm TMAP (Ph 6.3). Solvent proportions were controlled by a built-in solvent programmer and a dynamic mixer of the LC instrument. Two ISP-ETOH solutions in ratios of (67:33, v/v) and (55:45, v/v) were used in this study to obtain reasonable separations of the PL components. Throughout the HPLC experiments, the mobile phase eluents were filtered through a 0.2 μm filter, degassed with a helium sparge, and pumped isocratically through a β-CDS column at a flow rate of 1 ml/min.

The β-CDS stationary phase was prepacked commercially with β-CDS of 5 μm spherical particles in a stainless steel column (250 x 4.6 mm ID) (Advanced Separation Technologies, Inc. Whippany, NJ, USA). Aliquots (0.5-1 μl) of samples in chloroform solutions (1-3 mg /ml) were injected onto the column via a Rheodyne (Cotati, CA, USA) Model 7125 injector fitted with a 10-μl loop. Capacity factors (k') were determined as $k' = t/t_0 - 1$, where t and t_0 represent respective retention times of a PL analyte and an unretained solute. Separation factors (α) for adjacent HPLC peaks were determined as $\alpha = k'_{c+1}/k'_c$, where subscript "c" represents a PL component.

Calibration curves for soybean PE, PI, and PC were constructed separately by plotting amounts (μg) of the

individual polar lipids against peak areas (cm^2). For PA quantitation, fractions containing pure soybean PA were collected from column effluents. The isolated PA samples were then subjected to phosphorus analysis following a published procedure (27). A linear calibration plot for PA was then prepared from the HPLC peak areas and the amounts of analytes as determined by phosphorimetry for phosphorus contents.

A mixture of PL standards and a genetically modified soybean oil sample were analyzed by normal phase-HPLC on an EM Separations (Gibbstown, NJ, USA) LiChrospher Si 60/II column (250 x 4 mm ID, 3μm spherical particles). The HPLC experiment was conducted using a linear gradient with mobile phase solvents (A) chloroform-t-butyl methyl ether (75:15) and (B) methanol-chloroform-ammonium hydroxide (92:1:7). The gradient solvent program was run as follows: 100% solvent (A) was gradually changed to 100% solvent (B) over a 30 min linear gradient, and was then held at solvent (B) for 10 min before returning to 100% solvent (A) in a 10 min linear gradient. The mobile phase flow rate was 0.5 ml/min. A Varex (Burtonsville, MD, USA) evaporative laser light scattering detector Model ELSD II was used for all gradient HPLC work. The ELSD instrument parameters used in this study were the same as those set for previous work (26).

RESULTS AND DISCUSSION

In the early phase of this work, attempts to separate the four major soybean PL (FIGURE 1) on a CDS column with a wide range of mobile phase systems were unsatisfactory. First, HPLC-UV of PL inevitably suffers from limitations of required low UV-absorbing solvents for mobile phases. As common practice, the UV detector is normally set at 208 nm in most HPLC-UV work on polar lipids. Accordingly, mobile phase solvents chosen initially for the present study were (1) a hexane-ISP-water system and, (2) an acetonitrile-methanol-water system because both were nearly transparent at the low UV range. HPLC of mixtures of standard PL samples with different combinations of hexane, ISP, and water without mobile phase additives led to either ill-defined peaks or unresolved broad bands. In addition, acetonitrile-methanol-water based solvent mixtures were too polar for the PL components to be adsorbed on the CDS phase. However, addition of TMAP to the hexane-ISP-water mobile phases significantly improved the separation of the polar lipids, although component peaks remained broad. The undesirable peak shape problem was solved by incorporation of ETOH into the hexane-ISP-water/TMAP system, which markedly sharpened the chromatographic peaks.

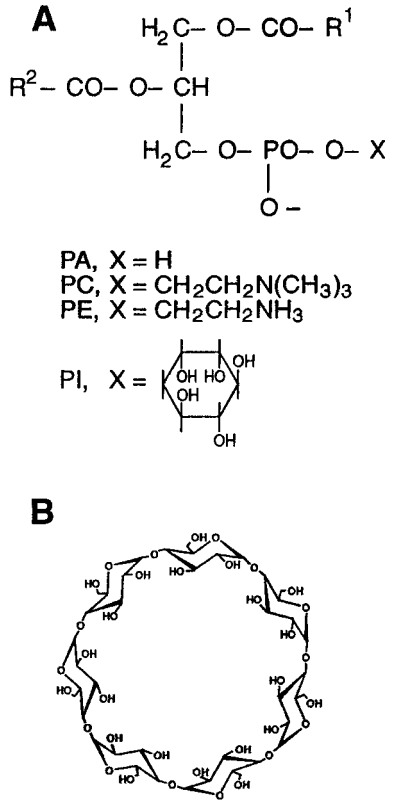

FIGURE 1. (A) Structures of soybean phospholipids PE, PC, PI and PA. (B) Structure of β-CD.

Unlike component separations in the reverse-phase mode where solutes interact with the CDS via inclusion complex formation (28), the retention behavior of the PL solutes in normal phase HPLC is believed to be due to solute adsorption to the hydroxyl-surface of the β-cyclodextrin entity (29) whose structure is depicted in FIGURE 1B. Accordingly, in HPLC of a standard soybean

PL mixture with a mobile phase of hexane-ISP-ETOH-water/5 Mm TMAP(Ph 6.3) (35 : 32.7 : 26.8 : 5.5), PI was found to be more strongly adsorbed to the CDS phase than PC (FIGURE 2A). Under the HPLC conditions employed, the PL components eluted from the CDS column in the order of increasing polarity and retention times: k'-PE < k'-PC < k'-PI < k'-PA (FIGURE 2A).

On the other hand, normal-phase HPLC-ELSD detection of a standard mixture of soybean PL on a SI column in the gradient mode with a mobile phase consisting of (i) chloroform/t-butyl methyl ether, and (ii) methanol/chloroform/ammonium hydroxide (see EXPERIMENTAL for details of the gradient elution program) led to an elution order (FIGURE 2B) that was different from that found in the HPLC-UV-CDS work described above. The PL components eluted from the SI column in accordance with the following order of increasing retention times: k'-PE < k'-PI < k'-PA < k'-PC. In this situation, PC was apparently more strongly adsorbed to the SI phase than both PI and PA.

TABLE 1 summarizes results obtained from HPLC-UV-CDS separations of a standard PL mixture studied under various mobile phase conditions. Examination of the retention data indicated that both k' and α values were sensitive to changes in the ratio of ISP-ETOH-water/5 Mm TMAP(Ph 6.3) solvents even though the hexane content

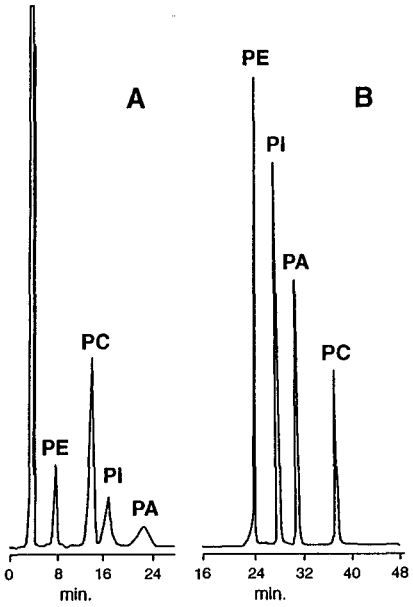

FIGURE 2. Separations of mixtures of PL standards (A) by HPLC-UV under isocratic elution on a β-CDS column; (B) by HPLC-ELSD under gradient elution on a SI column. Mobile phases: (A) [hexane-ISP-ETOH-water/5 Mm TMAP(Ph 6.3)] (35: 32.7: 26.8: 5.5); (B) See EXPERIMENTAL for gradient solvent systems.

was kept at a constant value of 35%. HPLC with a lower water content in the mobile phase [experiment (v), TABLE 1] tended to give more evenly dispersed component peaks with a smaller range of α values (1.42-2.01). However, the retention times (higher k' values) of the PL components were too long to be of any analytical value.

FIGURES 3A-D present typical examples of normal-phase HPLC-UV-CDS separations of PL in selected oil

TABLE 1

Effects of HPLC Mobile Phase Solvent Compositions [hexane-ISP-ETOH-water/5 Mm TMAP (35:x:y:z) on Capacity Factors, k', and Separation Factors, α, of The PL Components of Interest (UV detector at 208 nm; CDS column)

Mobile phase solvent ratio[a]	k'[b]						
	Component						
x : y : z	PE	(α)	PC	(α)	PI	(α)	PA
(i). 32.5:26.5:6.0	1.21	(2.36)	2.86	(1.52)	4.36	(1.11)	4.86
(ii). 32.7:26.8:5.5	1.71	(2.30)	3.93	(1.27)	5.00	(1.42)	7.09
(iii). 39.9:19.6:5.5	2.07	(1.79)	3.57	(1.27)	4.53	(2.05)	9.29
(iv). 40.5:20.0:4.5	3.14	(1.87)	5.86	(1.17)	6.86	(2.22)	15.2
(v). 40.9:20.1:4.0	4.86	(2.01)	9.79	(1.87)	18.3	(1.42)	26.0

[a]Values under the (x:y:z) column represent solvent ratios of ISP-ETOH-water/5 Mm TMAP(Ph 6.3) with hexane at a constant value of 35% for the mobile phases evaluated.

[b]Values in parentheses are separation factors, α, for adjacent component peaks. For definition of k' and α values, see EXPERIMENTAL.

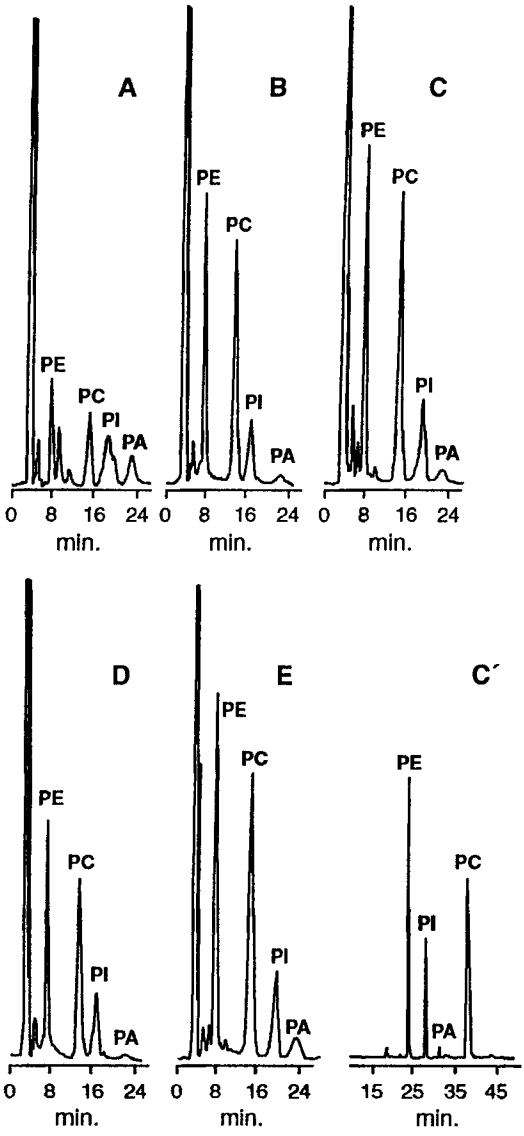

FIGURE 3. HPLC-UV-CDS separations of PL components in selected samples derived from genetically modified soybean oils (A, B, C,and D) along with a control unmodified soybean oil sample (E); mobile phase conditions are same as in FIGURE 2A. HPLC-ELSD-SI separation (C′) of PL components was obtained from sample (C); mobile phase conditions are same as in FIGURE 2B.

samples derived from genetically modified soybeans. Along with the modified oil samples, an unmodified soybean oil sample was also analyzed (FIGURE 3E) by the same HPLC method for comparison. Depending on the nature of samples, variable degrees of separations of the sample components were obtained with a mobile phase of hexane-ISP-ETOH-water/5 Mm TMAP(Ph 6.3) (35: 32.7: 26.8: 5.50). Traditionally, hexane-ISP-water solvent mixtures have proved successful in gradient HPLC analyses of PL classes on SI columns. Nonetheless, the HPLC chromatograms shown in Fig's. 3A-E indicate that, in general, isocratic HPLC-UV-CDS experiments with soybean PL mixtures led to satisfactory separations of PL components within reasonable elution times of about 25 min. For the purpose of comparing the HPLC peak profiles obtained from the two detection methods with different stationary phases, the same sample was analyzed by the HPLC-UV-CDS and HPLC-ELSD-SI procedures to give clearly different patterns of component separations as shown in FIGURE 3C and FIGURE 3C', respectively. It must be pointed out here that the isocratic mobile phases employed in the present HPLC-UV-CDS work can not be used with the ELSD because of incompatibility of TMAP with the detector.

To demonstrate the general applicability of the HPLC-UV-CDS technique in practical qualitative

analyses of commercial samples, a standard reference soybean lecithin sample together with three experimental soybean lecithin samples were chromatographed (FIGURE 4). The experimentally modified lecithin samples derived from industrial sources were obtained by partially treating the reference standard lecithin with hydroxylating, acetylating, or hydrolyzing agents. Generally, the PL separations of the standard sample (FIGURE 4A) were similar to those of the three experimental samples (FIGURES 4B-D) with the exception of minor peaks present in the chromatograms of the latter samples. Thus, hydroxylation of standard lecithin led to few notable changes in HPLC peak pattern within the 25 min elution time of the product (FIGURE 4B). HPLC of the acetylated lecithin sample produced a diminished PE peak shouldered by a new unresolved peak (FIGURE 4C). An enzymatically hydrolyzed product of standard lecithin contained significant amounts of lyso-PE and Lyso-PC (FIGURE 4D). When the enzyme-treated lecithin sample was analyzed under different mobile phase conditions with somewhat lower hexane and water contents, PI was found to elute from the CDS column before PC (FIGURE 4D').

Because UV absorptions vary among the different PL classes within a sample as well as in samples derived

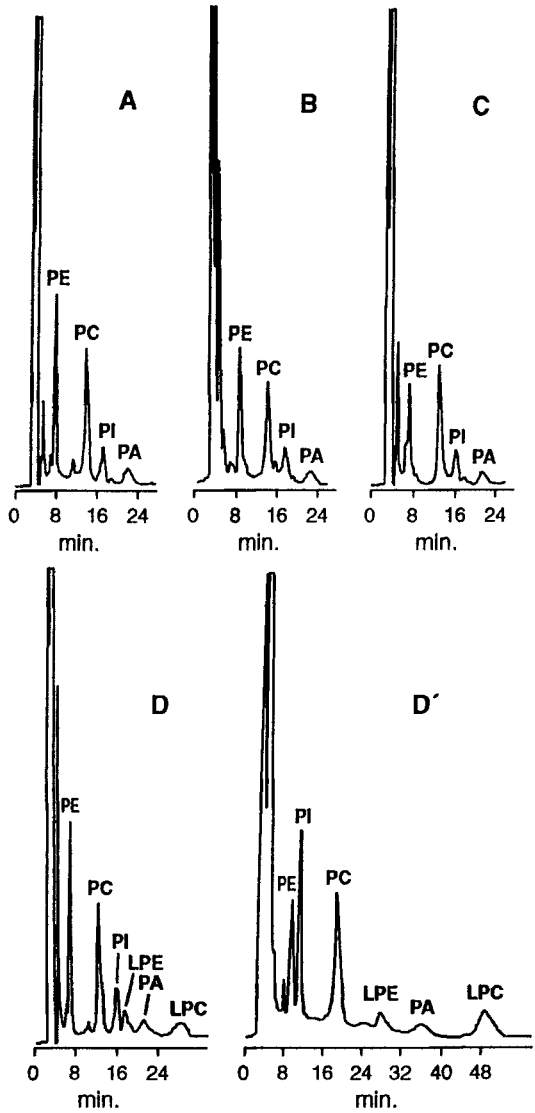

FIGURE 4. HPLC-UV-CDS separations of PL components in a commercial soybean lecithin sample (A) and experimental soybean lecithin samples (B, C, and D). HPLC-UV-CDS separation (D') of PL components was obtained from sample (D). Mobile phases: (A, B, C, and D) same as in FIGURE 2A. ; (D'), [hexane-ISP-ETOH-water/5 Mm TMAP(Ph 6.3)] (25: 38.5: 31.5: 5.0).

from different sources (plants or animals), HPLC-UV detector responses of samples require calibration with standards of the same origin as the sample prior to the quantitative estimation of the PL components present in a sample. Direct determinations of PL compositions can be accomplished by the HPLC-ELSD-SI. It was apparently fortuitous that the HPLC-UV-CDS peak intensities shown in FIGURE 3C are visually similar to those obtained by HPLC-ELSD-SI (FIGURE 3C') of the same sample.

A correlation study established linear relation between the injected amounts of soybean PL standards PE, PC, and PI and the corresponding HPLC-UV-CDS peak areas (TABLE 2). Because soybean PA standards are not commercially available, phosphorimetric PA analyses were necessary in order to eliminate intrinsic analysis errors caused by differences in molecular species distributions between soybean PA and egg PA. A calibration curve for PA analyses was constructed by plotting peak areas with amounts of PA obtained by phosphorimetry (TABLE 2). Inspection of the calibration data in the table showed that the slope (b) values of the correlation lines increase with decreasing retention times m (PE) > m (PC) > m (PI) > m (PA).

The results of quantitative HPLC-UV-CDS analyses of major soybean PL in four samples of genetically

TABLE 2
Calibration Data for Quantitative Analyses of Major Soybean PL By HPLC-UV Under HPLC Conditions Employed[a] (UV detector at 208 nm; CDS column)

PL	Slope (m) $cm^2/\mu g$	Coefficient of variation (CV)[c]	Correlation coefficient (r)
PE	8.752	2.5	0.9955
PC	5.375	2.2	0.9969
PI	3.333	3.0	0.9941
PA[b]	0.232	3.2	0.9921

[a]See Experimental section for mobile phase conditions.
[b]Data obtained from phosphorimetry.
[c]CV values are based on mean values of three determinations of peak areas.

modified oil and a control unmodified oil sample are summarized in TABLE 3. The numerical values given in the table are mean values of triplicate determinations. Coefficients of variation of the sample analyses ranged between 1.8 - 6.9%. Since the UV absorbance of the polar lipids is dependent on the fatty acid composition, quantitative analyses were carried out with the assumption that the fatty acid composition of each PL class is nearly identical to that of the PL standard. As shown by the analytical data (TABLE 3), the percent compositions of individual PL classes in the modified oils decrease in the order % PC > % PE > % PI > % PA, which is same as that observed in the

TABLE 3
Quantitative Analyses of PL in Selected Samples of Soybean Oil By HPLC-UV With a CDS Column[a]

Sample No.[b]	Soybean PL component							
	PE		PC		PI		PA	
	A	B	A	B	A	B	A	B
#1	17.9	30.3	26.0	44.0	11.8	20.0	3.40	5.70
(CV)	(3.5)	(4.0)	(2.3)	(3.3)	(3.7)	(3.9)	(5.8)	(6.3)
#2	19.8	28.9	32.0	46.7	13.5	19.7	3.15	4.61
(CV)	(4.1)	(3.7)	(1.9)	(1.8)	(3.3)	(5.3)	(5.0)	(6.5)
#3	31.9	28.7	47.8	42.9	25.5	22.9	6.10	5.50
(CV)	(3.6)	(4.5)	(2.0)	(3.0)	(2.7)	(2.3)	(4.9)	(5.8)
#4	31.9	35.2	32.3	35.6	22.0	24.2	4.53	5.00
(CV)	(4.5)	(4.4)	(2.9)	(3.1)	(4.3)	(3.6)	(6.0)	(6.7)
#5	39.7	27.0	72.1	48.9	32.3	21.9	3.20	2.20
(CV)	(4.0)	(3.9)	(2.3)	(1.9)	(5.2)	(1.9)	(6.6)	(5.5)

[a]A = amount (mg) of a PL component present in 10 g of each soybean oil sample. B = percent composition of a PL component in each oil sample. These values are mean values of three determinations.
[b]Samples #1-4 represent genetically modified soybean oil; sample #5 represents control unmodified soybean oil. CV= coefficients of variation.

control oil sample. The PA levels in the modified oils (TABLE 3, samples # 1-4) appeared to be at least two times greater than that in the control sample (TABLE 3, sample #5). The analytical results obtained by HPLC-UV-CDS are generally in good agreement with those obtained by HPLC-ELSD-SI (30).

In conclusion, the results of this study represent the first report on the use of a CDS phase for the

separation of phospholipids. The four major PL (PE, PC, PI, and PA) found in soybean oil can be separated by normal-phase HPLC on β-CDS. The two minor PL (LPE and LPC) found in enzymatically treated soybean oil can also be separated from the major PL components. The HPLC-UV-CDS technique coupled with reference calibration and phosphorimetry can be used for the quantitative analysis of PL classes in soybean oil samples. The HPLC-UV-CDS method can be utilized for the qualitative analysis of industrial lecithin products. The method may be applicable to the separation of a wide range of PL by optimization of HPLC mobile phases. The HPLC technique may be used as a viable alternative to PL analysis in various sample matrices.

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