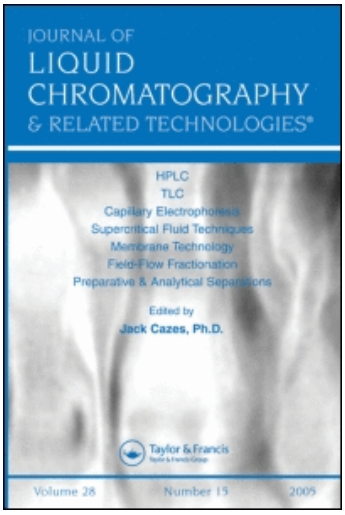

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SEMIPREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF PHOSPHATIDYLCHOLINE MOLECULAR SPECIES FROM SOYBEAN LEAVES

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ABSTRACT

An improved high-performance liquid chromatographic (HPLC) method using UV detection at 205 nm is described for the semipreparative separation of the molecular species of phosphatidylcholine (PC) from soybean leaves. The separations of PC molecular species are achieved isocratically within ca. 75 min on C 18 reversed-phase column using the mobile phase, methanol:0.1 M ammonium acetate, pH 7.4 (95:5, v/v). Five molecular species for soybean PC are identified as 18:3/18:3, 18:2/18:3, 18:2/18:2, 16:0/18:3 and 16:0/18:2.

INTRODUCTION

Analysis of phospholipid molecular species by high-performance liquid chromatography (HPLC) and gas chromatography (GC) has provided much new information on the biosynthesis and metabolism of lipids in animal (1) and plant (2) cell membranes. The need for larger quantities of pure phospholipids and individual molecular species for biochemical studies has led to the

search for more efficient preparative techniques for these lipids.

Recently, Rezanka and Podojil (3) reported the development of a preparative HPLC method for the separation of polar lipids and of individual molecular species from a fresh-water alga lipidic extract. A semipreparative HPLC method was developed in this laboratory for the separation of phosphatidylcholine (PC) from a soybean leaf extract (4). In this earlier investigation, the individual molecular species of PC were separated by an analytical method because there were no preparative methods available for the separation of plant phospholipid molecular species.

The present paper describes an efficient scale-up HPLC method for isolating larger quantities PC molecular species from a soybean lipidic extract.

EXPERIMENTAL SECTION

MATERIALS: Leaves of soybean (Glycine max, Tracy) were harvested from plants grown in the field. HPLC-grade solvents were obtained from EM Science (Cherry Hill, NJ). All other chemicals were of reagent grade and used without further purification.

HPLC PROCEDURES: Phosphatidylcholine (PC) was extracted from soybean leaves and was separated from other lipid components by the procedures described elsewhere (4). The separation of PC in the phospholipid fraction was performed on a semipreparative Excello silica column (R.E. Gourley Co., Laurel, MD), which was 10 x 150 mm and contained 5-μm particles. The mobile phase was isooctane:isopropanol:water (40:51:9, v/v/v) delivered at 2.0 mL/min with a ternary pump (Spectra-Physics, Model SP 8800). Samples were injected on the HPLC with a

syringe loading injector (Rheodyne, Model 7125) fitted with a 5.0-mL loop. PC was detected with a variable wavelength detector (Spectra-Physics, Model SP 8440) set at 205 nm. The elution profiles were recorded and integrated on a Beckman Integrator (Model 427). The PC eluate was collected manually after it passed through the detector and was stored in screw cap vials under nitrogen at 5^0C.

The HPLC separations of PC molecular species were performed on a semipreparative C18 reversed-phase column (Excello Ultra Pac ODS, R. E. Gourley, Laurel, MD), which was 10 x 150 mm and contained 5-um particles. The mobile phase was methanol:0.1 M ammonium acetate , pH 7.4 (95:5, v/v), which was delivered isocractically at 2.0 mL/min. The sample loop had a 5.0 mL capacity. The molecular species were collected manually after passing through the UV detector, which was set at 205 nm.

CHEMICAL ANALYSES: The fatty acid compositions of soybean PC and of the isolated PC molecular species were determined on a Hewlett-Packard Model 5880 gas chromatograph (GC) equipped with a SP 2330 capillary column and a flame ionization detector. The fatty acids were transesterified with boron trifluoride/methanol (Supelco). Lipid phosphorus was determined by the method of Bartlett (5) as modified by Marinetti (6).

RESULTS AND DISCUSSION

HPLC SEPARATION OF PC: The polar lipids were extracted from soybean leaves with organic solvents and were separated subsequently on silica Sep Paks by the procedures described in an earlier report(4). Figure 1 shows the chromatogram for the separation of PC from the polar lipid fraction on a semipreparative HPLC silica

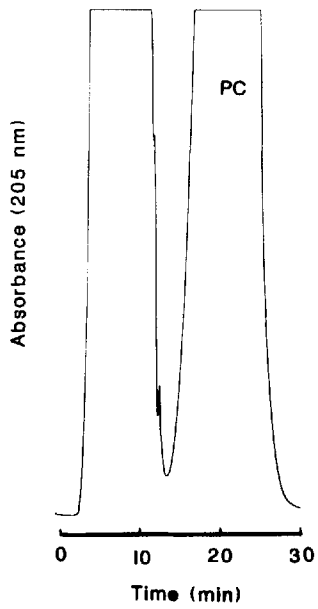

Figure 1. HPLC separation of soybean PC on a semipreparative Excello silica column (150 x 10 mm). UV detection was at 205 nm (0.32 AUFS). The mobile phase was isooctane:isopropanol:water (40:51:9, v/v/v) delivered isocratically at 2.0 mL/min. Injection volume was 2.0 mL; column pressure, 450 psi; chart speed, 0.25 cm/min. PC peak in chromatogram corresponds to approximately 3.6 mg of PC.

column using the isocratic mobile phase, isooctane:isopropanol:water (40:51:9, v/v/v) with a flow rate of 2 mL/min. The peak for PC, which corresponds to approximately 3.6 mg of the phospholipid, was obtained from a single injection of 2 mL of the polar lipid fraction. The optimum column load was found to be approximately 5 mg of soybean PC.

The semipreparative isocratic separation of soybean PC described here is simpler and slightly shorter in time than the preparative separation of algal PC on a silica column reported by Rezanka and Podojil (3). In the present study, the retention time for soybean PC was

ca. 22 min using isocratic elution as compared to ca. 32 min reported for the separation of algal PC using a linear gradient from hexane:isopropanol (6:8) to hexane:isopropanol:water (60:80:14) for 20 min followed by 30 min in the isocratic mode. In our earlier study (4), it was shown that the retention time of PC on the semipreparative silica column was related to the water content of the mobile phase. The retention time witnessed here was decreased by a factor of 2 by using isooctane:isopropanol:water (40:51:9) in place of the isooctane:isopropanol:water (40:53:7) used in the earlier work. Attempts to exceed the 9 parts of water in this mixture were unsuccessful because of the limited solubility of water in isooctane. Two liquid phases formed when the mixture exceeded 9 parts of water.

The fatty acid composition of the PC isolated from soybean leaves (Glycine max, Dekalb) in the present study is compared with the fatty acid composition of PC from another soybean variety (Tracy) used in our earlier study (Table I). The two soybean varieties were grown on the same field plot one year apart under similar conditions. The results for the two fatty acid analyses are in close agreement.

Table I. Fatty acid composition of soybean PC

	%Fatty Acid by weight				
Soybean PC	16:0	18:0	18:1	18:2	18:3
Tracy	18.0	9.0	2.1	40.3	30.6
Dekalb	16.5	7.3	3.4	45.1	27.2

HPLC SEPARATION OF PC INTO ITS MOLECULAR SPECIES: Figure 2 shows the chromatogram for the separation of five PC molecular species obtained from an injection of 2.1 mg of PC onto the C18 column in a 2.0-mL volume. Assignments of the molecular species shown in Table II are based on data obtained from the fatty acid analysis (Table II) and the analytical HPLC chromatograms (Figure 3) of the isolated PC molecular species.

Peaks 1 and 2 in the chromatogram (Figure 2) are assigned to 18:3/18:3 species and 18:2/18:3 species, respectively. The analytical chromatograms of the isolated peaks in Figure 3 show one major peak for 18:3/18:3(A) and 18:2/18:3(B). The separation of these two molecular species on this semiprepartive column are similar to the separation of these species on analytical C18 columns observed in other studies (4,7,8).

As for peak 3, the analytical chromatogram (C) in Figure 3, which shows one major peak, and the high per cent of linoleic acid in the fatty acid composition (Table II) are strong evidence to support the assignment

Table II. Molecular Species found in soybean leaves.

Peak Number	% Fatty Acid of Isolated Molecular Species					Assignments
	16:0	18:0	18:1	18:2	18:3	
1	6.7	5.8	2.6	10.4	72.0	18:3/18:3
2	3.1	6.1	2.0	49.3	36.6	18:2/18:3
3	1.6	4.2	2.4	85.0	5.7	18:2/18:2
4	32.0	2.9	3.0	16.8	43.9	16:0/18:3
5	38.9	2.4	5.8	48.9	2.9	16:0/18:2

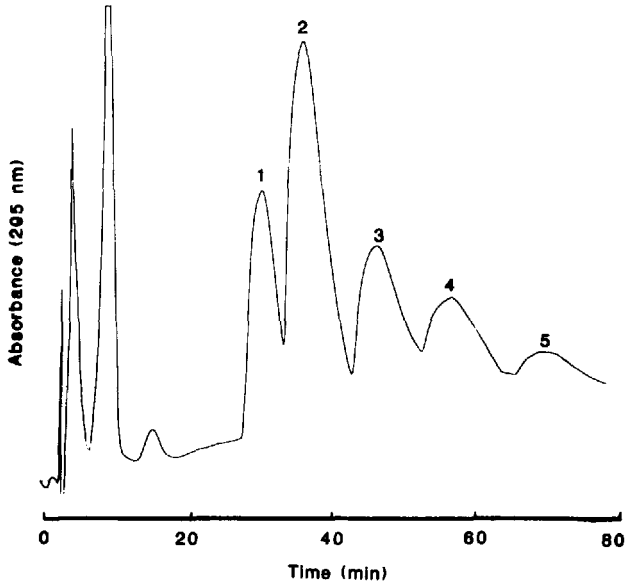

Figure 2. Chromatogram for the separation of soybean PC into its molecular species on a semipreparative Excello Ultra Pac ODS column (150 x 10 mm). UV detection was at 205 nm (0.08 AUFS). The mobile phase was methanol: 0.1 M ammonium acetate, pH 7.4 (95:5, v/v) delivered isocratically at 2.5 mL/min. Column pressure was 785 psi; chart speed, 0.25 cm/min. Chromatogram was obtained from an injection of 1.0 mL of soybean PC (1,000 ug/mL) isolated on the semipreparative silica column.

of 18:2/18:2 as the predominate molecular species in this isolate. This is the first reported evidence for the complete resolution of this 18:2/18:2 molecular species for PC extracted from plant leaves. In related plant studies (4,7,8), 18:2/18:2 species were not separated from 16:0/18:3 PC molecular species using analytical HPLC methods. The difference between the loading capacities of the semipreparative and the analytical columns is offered as an explanation for the observed resolution. The maximum sample capacities per separation on the analytical and semipreparative columns are 3 and 14 mg, respectively (9).

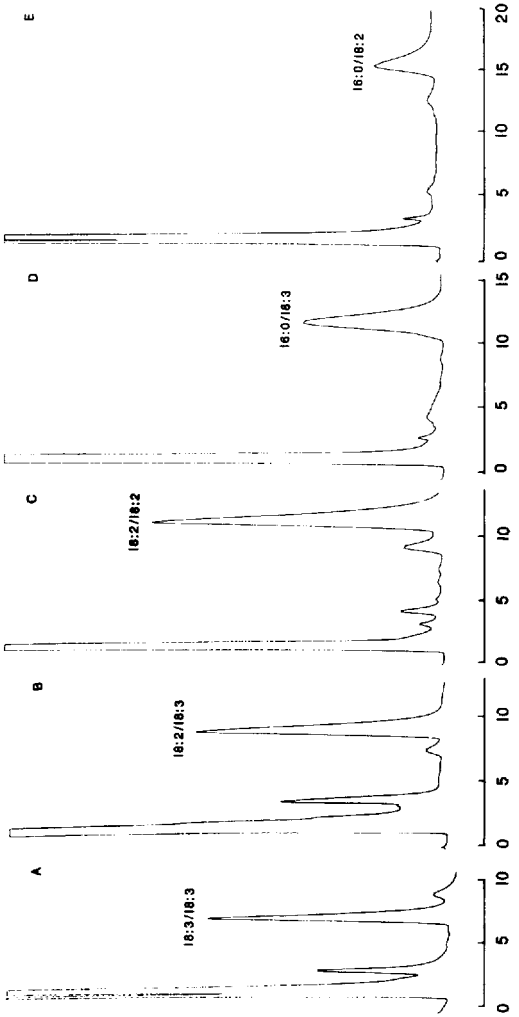

Figure 3. Chromatograms of individual molecular species of soybean PC on an analytical Excello Ultra Pac ODS column (150 x 4.6 mm) eluted with methanol: 0.1 M ammonium acetate, pH 7.4 (95:5). Flow rate was 1.75 mL/min, UV detection at 205 nm (0.04 AUFS); injection volume, 25 uL; column pressure, 2441 psi. Chromatograms: (A) 18:3/18:3 (5 ug), retention time 7.5 min; (B) 18:3/18:2 (15 ug) retention time 9.3 min; (C) 18:2/18:2 (15 ug) retention time 11.5 min; (D) 16:0/18:3 (10 ug) retention time 12.5 min; (E) 16:0/18:2 (7.5 ug) retention time 15.7 min.

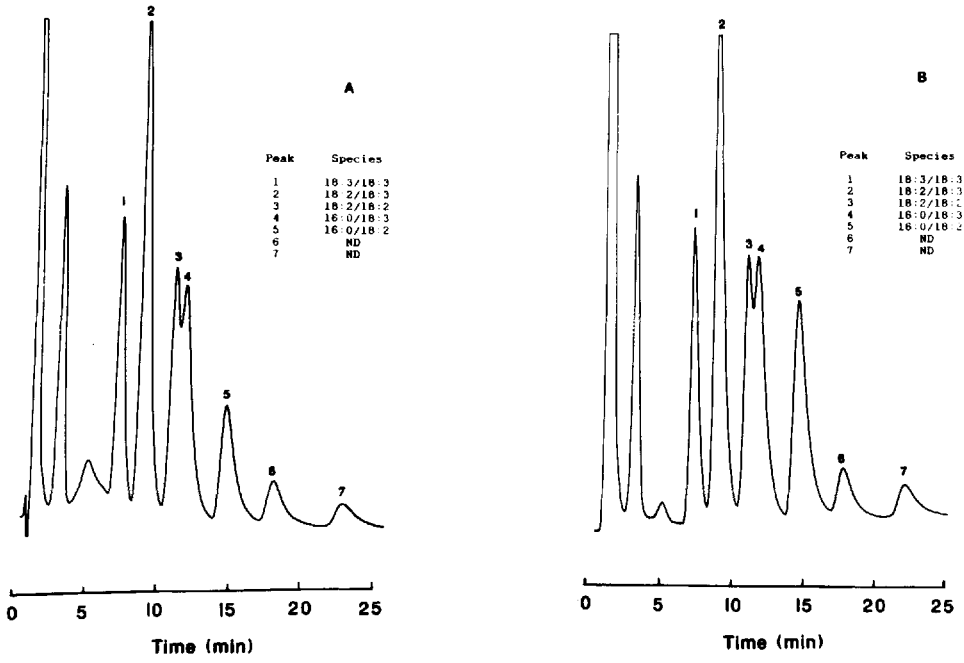

Figure 4. HPLC separation of 50 ug of pure soybean PC into its molecular species on an analytical Excello Ultra Pac ODS column (chromatogram A); separation of 50 ug of pure soybean PC combined with 7.5 ug of isolated 16:0/18:2 PC (chromatogram B). ND in the tables denotes not determined.

The analytical chromatogram (D) for peak 4 in figure 4 suggests that only one molecular species is present but the results of the fatty acid analysis in Table II indicate that traces of 18:2/18:2 species are also present. The interpretation of these data is that 16:0/18:3 is the primary molecular species and that 18:2/18:2 is a contaminant from the preceding peak 3. Data for peak 5 clearly indicate that 16:0/18:2 is the only molecular species. Figure 4 shows the analytical chromatogram (A) for the separation of 50 μg of pure soybean PC into its molecular species. The separation observed here is very similar to the separation of PC molecular species reported in our earlier study (4),

with the exception that 18:2/18:2 (peak 3) and 16:0/18:2 (peak 4) are better resolved here. Although the C18 columns used in the two studies were identical, the C18 column used in this work was recently purchased and was used here for the first time for lipid analysis. However, peaks 3 and 4 became unresolvable after many injections of pure soybean PC onto the C18 column (chromatogram is not shown). Chromatogram B in Figure 4 shows the analytical separation of 50 μg of pure PC spiked with 5 μg of 16:0/18:2 PC, which was collected from the semipreparative column. These results further indicate that the 16:0/18:2 PC isolated here by a semipreparative method is relatively pure.

In conclusion, the semipreparative separation of PC molecular species described here has many advantages over classical thin-layer chromatography and other preparative HPLC methods. This scale-up HPLC method is fast, simple and isocratic.

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Mention of companies or commercial products does not imply recommendation or endorsement by the United States Department of Agriculture over others that are not mentioned.

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