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Isolation and purification of lecithin by preparative high-performance liquid chromatography

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ABSTRACT

Mixed-chain, multispecies, egg yolk-derived lecithin was isolated and purified on a silica column with isocratic elution. A method development column (20 × 0.46 cm I.D.) packed with YMC 15–30 μm, 120 Å spherical silica and a mobile phase consisting of 5 m*M* ammonium acetate in acetonitrile–2-propanol–methanol–water (80:13:5:12) was used to separate the lecithin from other phospholipids. The mobile phase conditions for the method development system was adopted for two types of preparative HPLC systems: a Separations Technology SepTech NovaPrep™ 5000 system with a 20 × 1.93 cm I.D. column and a ST/800A system with a 20 × 5.00 cm I.D. Annular Expansion™ (A/E) column. The maximum load was 50 μl of crude solution (2 mg) for the method development column, 0.90 ml (35 mg) for the 20 × 1.93 cm I.D. column and 6.0 ml (240 mg) for the 20 × 5.00 cm I.D. A/E column. The flow-rates were 2, 35 and 235 ml/min, respectively. The fractions collected from the preparative systems were analyzed for purity by analytical-scale high-performance liquid chromatography and by thin-layer chromatography with selective detection with molybdenum blue for phospholipids and detection of all organic compounds by sulfuric acid. Purity of the recovered lecithin was greater than 99%.

INTRODUCTION

Natural lecithin does not exist as a discrete compound but is composed of a choline polar head and multimolecular species of varying fatty acid chain lengths and degrees of unsaturation[1] (Fig. 1). Besides being a naturally occurring emulsifier and surfactant, lecithin is also of interest as starting material for the synthesis of novel anti-viral and anti-tumor drugs[2–6]. Since purified lecithin was needed for the synthesis

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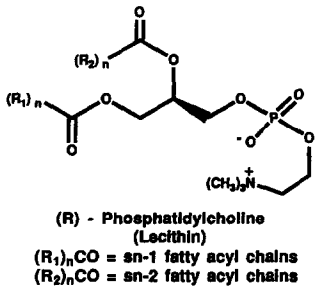

Fig. 1. Structure of lecithin.

of these drugs, we developed a method for the isolation and purification of lecithin from chicken egg yolk, where a large amount of lecithin is present.

Silica has been the sorbent most commonly used in analytical and preparative separations of phospholipids[7–26]. Patel and Sparrow[10] reported a high load when they separated lecithin from egg yolk on a preparative scale using silica packed columns and a mobile phase of chloroform–methanol–water (60:30:4). One disadvantage of this method is that ultraviolet (UV) detection can not be used, since the chloroform has high absorption at low UV wavelengths. In addition, the large volumes of chlorinated solvents required for preparative separations are detrimental to the environment and to the health of laboratory workers. Analytical separations of lecithin from other sources were developed by Jungawala *et al.*[11] with a mobile phase of acetonitrile–methanol–water (65:21:14). In 1987 this mobile phase, to which 2-propanol and trifluoroacetic acid were added, was used for preparative work[20]. Gradient elution with a mobile phase of hexane–isopropanol–water has also been reported for analytical separations[16,17,21,22] and for preparative work[25]. However, isocratic elution is preferable if large quantities of lecithin are to be isolated routinely because of the ease of operation and cost savings. UV detection is preferable to detection by refractive index. Its higher sensitivity allows better monitoring of the separation of lecithin from other phospholipids, which are present in trace amounts relative to the amount of lecithin. Since phospholipids absorb only in the region of 200 to 210 nm the mobile phase had to be transparent at 203 nm, the working wavelength of the separation.

EXPERIMENTAL

Sample preparation

Crude phospholipids were extracted from one dozen, fresh chicken egg yolks by the method of Singleton *et al.*[27]. The combined mass of the yolks was approximately 242 g. The membranous cuticle was removed by placing the yolk on a piece of screening on top of a 2-l beaker containing 600 ml of acetone. The yolk was broken and the fluid forced through the screen with a spatula. When the fluid contacted the acetone, a precipitate formed immediately. The mixture, which was allowed to stand at room temperature in the dark for 1 h, was then vacuum filtered and the solid washed with 300 ml of cold acetone. The acetone extract, which contained most of the neutral fats and pigments, was discarded. The solid was suspended in 400 ml of 95% ethanol and

stored in the dark for 2 h. The mixture was vacuum filtered and the solids washed with 100 ml 95% ethanol. The ethanol extracts were combined and dried at 30–40°C. To dissolve the phospholipids, two (100 ml) portions of petroleum ether were added to the residue. The petroleum ether solutions were combined and reduced in volume with rotary vacuum distillation by a factor of 3 to approximately 70 ml. When the solution was poured into a beaker containing 400 ml of cold acetone, a precipitate formed immediately. The mixture was allowed to stand in the dark at room temperature overnight until the solution cleared. The clear solution was decanted and the yellow solid (11.9 g) was washed with cold acetone. The phospholipids were dissolved in methylene chloride, dried and stored in the freezer under nitrogen.

Materials

All solvents and reagents (Fisher, Pittsburgh, PA, U.S.A.) used for the extraction, method development and analytical analysis were of HPLC grade. The water was doubly distilled and deionized. Each solvent was filtered through a 0.45-μm Nylon-66 filter (AllTech, Deerfield, IL, U.S.A.). For the preparative separations reagent grade solvents also from Fisher were used.

Standard egg yolk phospholipids, *i.e.* lecithin, lysophosphatidylethanolamine (LPE) and sphingomyelin (SPH) were obtained from Sigma (St. Louis, MO, U.S.A.); phosphatidylethanolamine (PE) and lysolecithin from Avanti Polar Lipids (Birmingham, AL, U.S.A.). These standards were used to characterize the phospholipids and determine retention times on the method development and analytical systems. The concentration of each standard was 1 mg/ml.

A stock solution of phospholipids from the egg yolks was prepared: 2.01 g of the egg yolk phospholipids were dissolved in 5 ml of absolute ethanol and 4.9 ml of methanol. To the alcohol solution, 0.1 ml of 10% butylated hydroxytoluene (BHT) in methanol was added as an antioxidant. The stock solution was filtered through a 0.45-μm filter. From the stock solution, 3 ml were removed and diluted with 15 ml of methanol. This working solution contained 40.3 μg of phospholipids/μl.

Method development

The chromatographic system used for the method development studies consisted of a Waters 6000A pump (Waters Division, Millipore, Milford, MA, U.S.A.), a Rheodyne 7125 injector (Rheodyne, Berkeley, CA, U.S.A.), a Knauer variable-wavelength detector set at 203 nm with a sensitivity of 0.64 a.u.f.s. (Sonntek, Woodclift Lake, NJ, U.S.A.), and a method development column 20 × 0.46 cm I.D., packed with YMC 15–30 μm, 120 Å spherical silica (Yamamura, Kyoto, Japan). The column was packed using a Haskel Pump (Haskel, Burbank, CA, U.S.A.). The isocratic mobile phase was 5 m*M* ammonium acetate in acetonitrile–2-propanol–methanol–water (80:13:5:12) at a flow-rate of 2.0 ml/min. The chromatograms were recorded on an HP 3393A integrator (Hewlett-Packard, Avondale, PA, U.S.A.) at 0.2 cm/min, and an Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.) at 0.5 cm/min. All separations were achieved at ambient temperature.

Preparative high-performance liquid chromatography (HPLC)

Two preparative systems were used for the isolation of lecithin: a SepTech NovaPrep™ 5000 (NovaPrep) and a SepTech ST/800A (800A) (Separations Tech-

nology, Wakefield, RI, U.S.A.). With the NovaPrep a ST/2001A column (20 × 1.93 cm I.D.) was used; with the 800A, a ST/3002B Annular Expansion™ (A/E) column (20 × 5.00 cm I.D.). Each column was packed with YMC 15–30 μm, 120 Å spherical silica. The NovaPrep column was packed under high pressure using a Haskel pump, while the A/E column was slurry packed.

The NovaPrep column was equilibrated with the mobile phase at a flow-rate of 35 ml/min. A Knauer variable-wavelength detector set at 203 nm, with a sensitivity of 0.64 a.u.f.s., was used to monitor the separation. The NovaPrep was under computer control, utilizing TurboPrep™ software (Separations Technology). The program was set for an equilibration time of 10 min, injection time of 0.3 min and a run time of 60 min. The working solution was manually injected with a syringe. Data were recorded on both a strip chart recorder and an HP 3393A integrator.

The 800A column was operated under manual control and the A/E column equilibrated with a mobile phase of 5 m*M* ammonium acetate in acetonitrile–2-propanol–methanol–water (80:13:5:12) for 20 min at a flow-rate of 235 ml/min (percent flow-rate 56%). The Knauer detector with the same settings was used.

Analytical analysis

Each fraction was analyzed for purity with a Waters 6000A pump, Rheodyne 7125 injector, a Schoeffel Spectro Flow Monitor SF 770 variable-wavelength detector set at 203 nm, with a sensitivity of 0.1 a.u.f.s. (Schoeffel Instrument Division, Kratos, Westwood, NJ, U.S.A.) and a 20 × 0.46 cm I.D. column containing 8–12 μm Grace silica (W. R. Grace, Baltimore, MD, U.S.A.). The flow-rate was 2.5 ml/min and the mobile phase consisted of acetonitrile–methanol–water (40:9:6).

Prior to HPLC analysis, the fractions were concentrated by rotary vacuum distillation at a temperature range of 30 to 40°C (Table I). Of each fraction 100 μl were analyzed. The fractions containing purified lecithin were pooled and the remaining solvent was removed by lyophilization. A yellowish-white solid was left, which was a mixture of the purified lecithin and ammonium acetate. The lecithin was extracted with methylene chloride. The methylene chloride solution was reduced in volume to less than 10 ml. The final traces of the solvent were removed by a stream of nitrogen. The mass of the lecithin was then determined.

Each fraction was also analyzed with thin-layer chromatography (TLC) since the spots could be detected visually. The mobile phase was composed of chloroform–methanol–water (60:30:4)[11]. The fractions were spotted on silica plates (Fisher) against the phospholipid standards and working solution. The plates were developed

TABLE I

VOLUME OF COLLECTED AND CONCENTRATED FRACTIONS

System	*Volume collected per fraction (ml)*	*Reduced volume (ml)*
Method development	4	0.5
NovaPrep 5000	70	10
ST/800A	230	40

by two methods: molybdenum blue (Sigma, St. Louis, MO, U.S.A.) which is specific for phospholipids, and sulfuric acid which visualizes all organic compounds.

RESULTS AND DISCUSSION

The mobile phase composition was optimized to provide a reasonable capacity factor for the lecithin and the best selectivity for the separation of lecithin from other phospholipids which are present in egg yolk. Since the mobile phase had little low end UV absorption, the separation of phospholipids could be monitored in the 200–206 nm region. A 1-μg/μl mixture of the standards was analyzed using the method development column. The PE and LPE, which had retention times of 4.7 and 7.8 min,

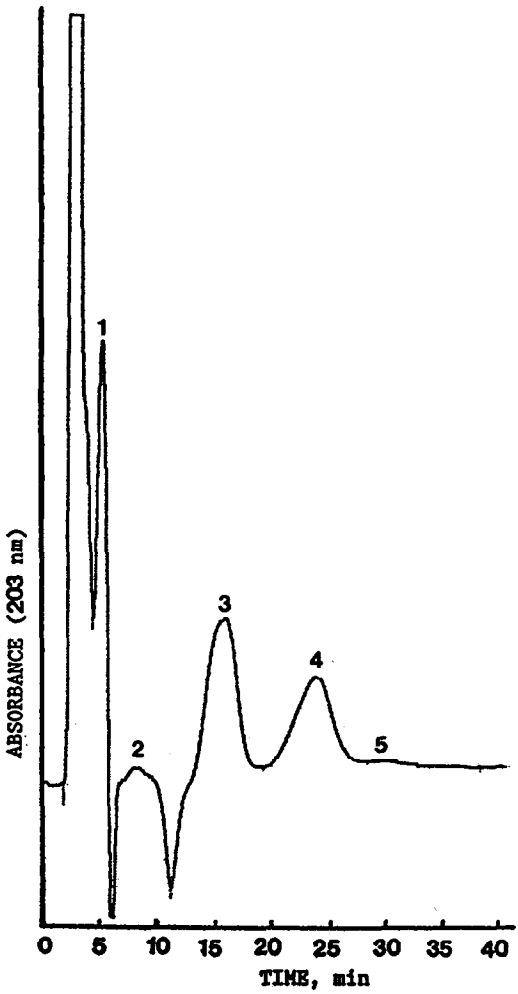

Fig. 2. Method development chromatograms of phospholipid standard mixture, 100 μl of 1 μg of each phospholipid/μl. Peak identification: 1 = PE, 2 = LPE, 3 = lecithin, 4 = SPH and 5 = lysolecithin. Conditions as given in Experimental section.

respectively, were eluted prior to lecithin (retention time of 15.6 min); the SPH was eluted afterwards at 23.8 min, as was the lysolecithin at 28.8 min (Fig. 2).

With the method development column a loading study was performed to determine the injection volume of the working solution which was optimal to obtain adequate selectivity and lecithin purity. Samples of 10, 25, 50, 75 and 100 μl were injected and fractions were collected across the lecithin peak.

The fractions were analyzed by analytical HPLC to determine the purity of the lecithin. Peaks were characterized by co-injection with the standards. The lecithin standard was eluted at 9.4 min (Fig. 3). In addition, two types of blanks were analyzed; the mobile phase before it entered the column (Before Column) and after the column (After Column) for each separation system. The fractions were also characterized by TLC using molybdenum blue spray to visualize selectively the phospholipids and sulfuric acid to detect all organic compounds.

The optimal injection volume was 50 μl (2 mg) of the working solution (Fig. 4A). Nine fractions (2 min each) were collected, concentrated and analyzed. Of the 9 fractions, 3–7 contained only lecithin with the desired purity.

When the method development system was scaled-up for each of the preparative columns, the linear velocity was kept constant. In scaling-up the volumetric flow-rate scales as the square of the column radius, the sample load scales as the column volume, while the run time scales as the column length. The loading scale-up factors for the preparative systems were 17 for the NovaPrep and 118 for the 800A (Table II).

A volume of 900 μl (35 mg) of the working solution were manually injected into the NovaPrep column. The retention time of the lecithin was 17.9 min and the run was terminated at 40 min (Fig. 4B). Seven fractions of 70 ml each were collected and concentrated to 10 ml. Fractions 1–5 contained lecithin of >99% purity. Fraction 6 was contaminated with SPH and fraction 7 contained only SPH. Representative

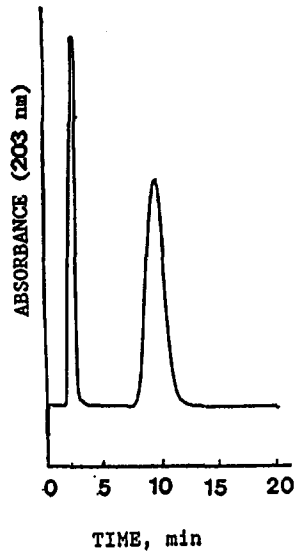

Fig. 3. Analytical chromatogram of 10 μl of 1 μg/μl lecithin standard, retention time 9.4 min. Conditions as given in Experimental section.

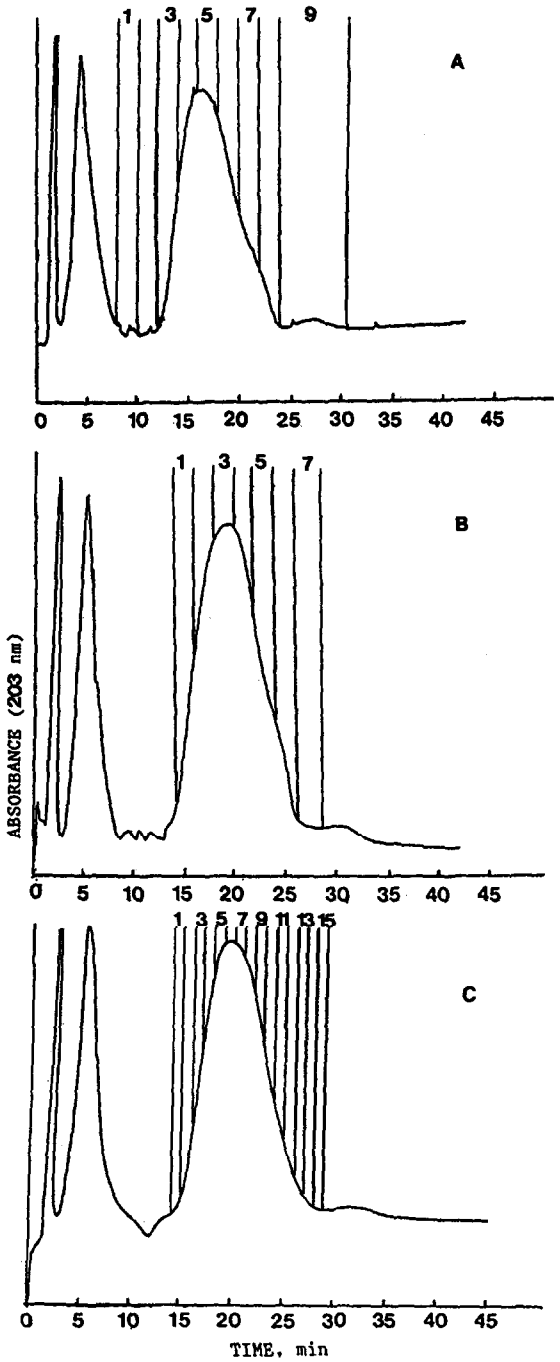

Fig. 4. Chromatograms of crude egg yolk phospholipids: (A) method development chromatogram (2 mg), (B) NovaPrep 5000 separation (35 mg), (C) ST/800A separation (241 mg). Retention time of lecithin; 14.9, 17.9 and 18.0 min, respectively. Conditions as given in Experimental section.

TABLE II

HPLC SCALE-UP

Column	*I.D. (cm)*	*Length (cm)*	*Load (g/run)*	*Flow-rate (ml/min)*	*Run time (h)*	*Solvent usage (l/run)*
ST/2020	0.46	20.00	0.002	2.0	0.83	0.10
ST/2001A	1.93	20.00	0.035	35.2	0.83	1.76
ST/3002B	5.00	20.00	0.236	236.3	0.83	11.81

analytical chromatograms of the fractions are shown in Fig. 5. Confirmation of purity was made by using TLC with two staining methods. For each fraction there was only one spot whose R_F factor corresponded to that of the standard sample of lecithin. In the pooled fractions (1–5) 25 mg of pure lecithin was recovered (72% recovery).

When 6 ml (241 mg) of the working solution were manually injected directly onto the A/E column, the lecithin was eluted at 18.0 min and the run was terminated at 43 min (Fig. 4C). Fifteen fractions of 230 ml each, 1 min in length, were manually collected. Each fraction was concentrated to 40 ml and an aliquot analyzed. Fraction 1 contained a minor component which was eluted prior to the lecithin. Fraction 13 contained only a very small amount of lecithin, whereas 14 and 15 did not have any detectable amounts of lecithin; therefore fractions 1 and 13–15 were not included in the pooled fractions. Representative chromatograms of the analyzed fractions are shown (Fig. 6). The amount of lecithin recovered was 130 mg (54% recovery).

The lecithin from each preparative system was dissolved separately in 1 ml of absolute ethanol and aliquots of 10 μl of each sample were reanalyzed. The lecithin

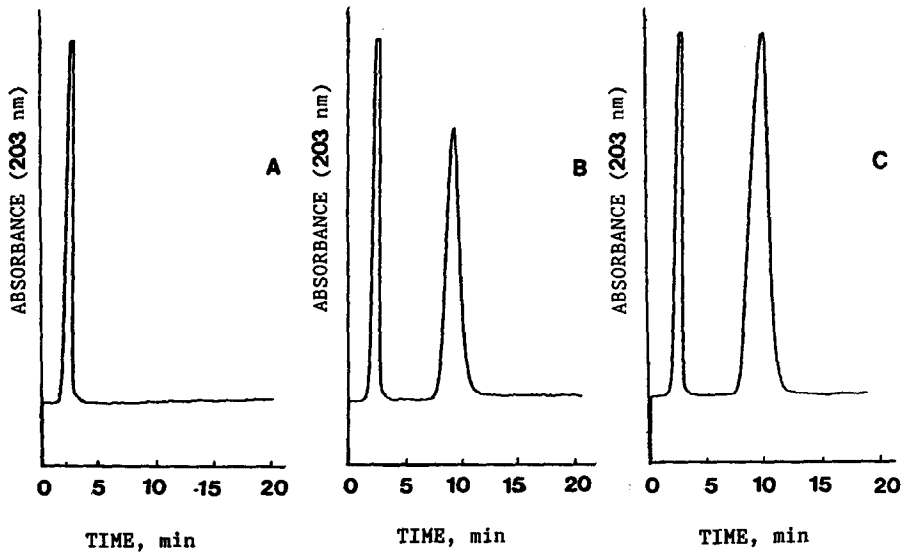

Fig. 5. Analytical HPLC of lecithin containing fractions obtained from the NovaPrep 5000: (A) mobile phase (After Column) blank, (B) fraction 1, (C) fraction 3. For conditions see Experimental section.

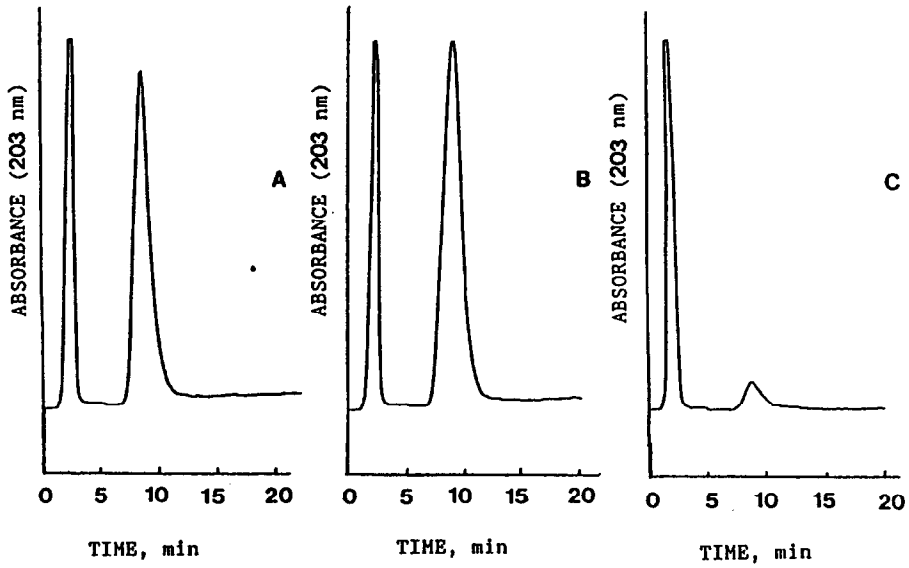

Fig. 6. Analytical HPLC of lecithin containing fractions obtained from the ST/800A: (A) fraction 2, (B) fraction 5, (C) fraction 12. For conditions see Experimental section.

both from the NovaPrep and the 800A had a purity of >99%. Development of the TLC plate with molybdenum blue revealed that only one phospholipid, lecithin, was present; development with sulfuric acid also indicated no organic components other than lecithin.

In conclusion a rapid isocratic preparative HPLC method has been developed for the isolation and purification of natural lecithin. With both preparative systems virtually identical profiles were observed, giving credence to an accurate scale-up. The capacity factors and selectivity values for all three systems are given in Table III. High purity was obtained without the use of chlorinated solvents in the mobile phase. To increase the throughput this method can be automated. In addition, it is possible to adopt these conditions for the isolation and purification of lecithin from other natural and synthetic sources or for the preparative HPLC of other phospholipids.

TABLE III

CAPACITY FACTORS (k') OF LECITHIN AND SPH AND SEPARATION FACTORS (α) OF SPH/LECITHIN

System	k'		α
	Lecithin	*SPH*	
Method development	10.3	19.2	1.9
NovaPrep 5000	12.1	20.7	1.7
ST/800A	12.1	21.5	1.8

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