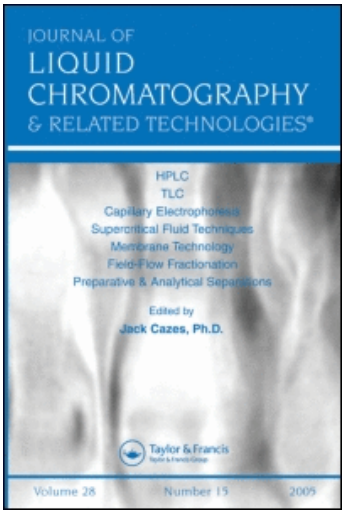

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THE PREPARATIVE HPLC ISOLATION AND IDENTIFICATION OF PHOSPHOLIPIDS FROM SOY LECITHIN

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INTRODUCTION

Lecithins are a constituent of all living membranes. While lecithins are found in both plants and animals, the specific fatty acid subunits vary considerably between plants and animals. The lecithins of plant origin are chiefly derived from soy but other vegetable phospholipids are also used. World consumption of these compounds was estimated @ over 100,000 tons in 1981 (1) with growth expected each year.

The uses of these lecithin compounds in foods and nonfoods are wide spread. Lecithin uses in foods include fat dispersion, viscosity reduction, and product stabilization in foods as diverse as margarine, chocolate and bakery goods. In drug manufacturing, they are used as biological emulsifiers and choline suppliers. A typical composition of soy lecithins free of a fat

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carrier is given in Table I.(2) Figure 1 gives some examples of phospholipid structures.

Various methods exist for the determination of phospholipids in food and nonfood matrices, with TLC and recently HPLC seeing wide use. The phospholipids have little specific ultraviolet (UV) absorbance, and are usually detected by refractive index or by monitoring the UV absorbance of the double bonds in the individual fatty acid moieties.(3) Lecithin types are heterogeneous with fatty acid composition varying depending on the source; the analyst therefore must use a similarly appropriate analytical standard from the species of interest. Using an inappropriate standard for HPLC phospholipid analysis in which the fatty acid composition of the sample differs might yield confusing results.

Some pure standards are available commercially, and those not available have been reported to be prepared by preparative TLC using any number of possible solvent systems.(4) Additionally, the analyst must be aware that standards from commercial sources sometimes are of varying quality and are liable to oxidation when improperly stored.

The study described in this paper outlines the various steps taken to accomplish the successful preparative HPLC isolation and identification of phospholipids from soy lecithin.

EXPERIMENTAL

Instrumentation

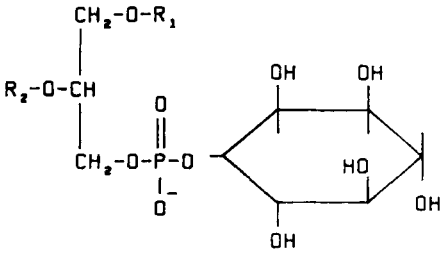
The preparative HPLC system was a Waters Autoprep 500 equipped with computer control and self contained refractive index (RI) detector. The analytical HPLC was comprised of a Model 510 Pump (Waters), Model 7125 Loop Injection Valve (Rheodyne), Model 480 Variable Wavelength Detector (Waters) @ 205 nm and a Houston Omniscribe Recorder (Houston Instruments) attached to a Model E1A Data Unit (Shimadzu Scientific).

The Fourier Transform Infrared (FTIR) instrument was a Model 20SX (Nicolet) equipped with a water cooled globar source and TGS detector.

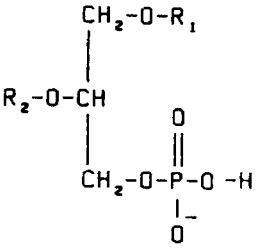
Table I

Typical Natural Lecithin Composition (2)

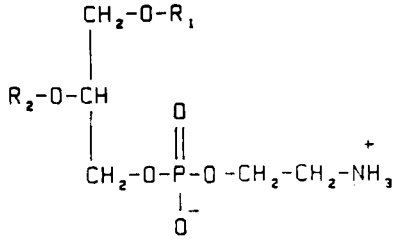
Moiety	%
Phosphatidyl Choline	29.5
Phosphatidyl Ethanolamine	29.5
Phosphatidyl Inositol	31.6
Miscellaneous Phosphatides	6.3



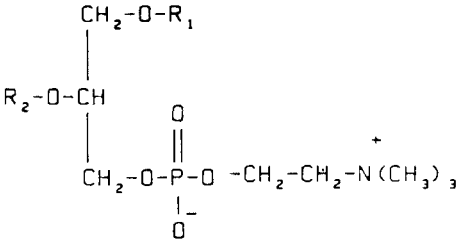
Phosphatidyl Inositol



Phosphatidic Acid



Phosphatidyl Ethanolamine



Phosphatidyl Choline

Figure 1

Phospholipid Structures

Table II

Summary of Analytical and Preparative HPLC Columns

Analytical and Semipreparative Columns

μ Porasil 10 μm (3.9 mm x 30 cm)

Radial Pak Silica 10 μm (8 mm x 10 cm)

Radial Pak Prep Silica 55-105 μm (8 mm x 10 cm)

Preparative Column

Prep Pak 500 Cartridge (55-105 μm, 5.7 cm x 30 cm)

The columns used in the analytical and preparative HPLC are described in Table II.

The mobile phases used in both analytical and preparative separation schemes were mixtures of CH_3CN, CH_3OH and 85% H_3PO_4 in various proportions. The mobile phase used in the analytical HPLC was that reported in earlier studies.(5)

The standards were obtained from Sigma Chemical Co. and Calbiochem; these consisted of phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol. They were made to concentrations of 10 μg/μl in $CHCl_3$ and stored @ 4°C. To ensure correct standard concentrations, standards received in solvent should be dried and made to concentration by weight rather than assuming that the concentration given (10 μg/μl) on the manufacturer's vial is totally correct. Standard solutions were frequently checked by analytical HPLC to monitor for spurious peaks indicative of oxidative degradation.

Analytical Chromatography

The analytical chromatography system used to monitor the preparative HPLC fraction was previously reported (5) and used as outlined therein.

Preparative Chromatography Development

Once analytical chromatography conditions have been established, several steps are necessary in the preparative separation development. Since the

analytical separation was initially developed on an irregular particle silica, the separation was then developed using a spherical silica. The next step was to transfer the separation from the small particle (5 μm) spherical silica to preparative spherical (55 - 105 μm) silica packed in a radial compression module (RCM) cartridge. This transfer step allowed the use of similar column chemistries with the ability to conserve solvent and to evaluate column loading on a smaller scale. The final step in the preparative separation development was a full preparative HPLC trial to refine the separation parameters established in the preliminary studies.

Preparative Chromatography

After the development of the analytical and preparative chromatography, aliquots of commercial soy lecithin dissolved in $CHCl_3$ were injected onto the preparative HPLC with mobile phase flowing at 50 ml/min. Mobile phases used consisted of various percentages of CH_3OH in CH_3CN modified with small amounts of concentrated H_3PO_4. The mobile phases used are listed in Table III.

The three phospholipid fractions were collected based on their characteristic retention times using the programmed functions of the Auto Prep 500.

Table III

Mobile Phases for Preparative Chromatography

Fraction	Mobile Phase	
Phosphatidyl Inositol	95/5/1.2 (V/V/V)	CH_3CN/CH_3OH H_3PO_4
Phosphatidyl Ethanolamine	85/15/1.2 (V/V/V)	CH_3CN/CH_3OH H_3PO_4
Phosphatidyl Choline	70/30/1.2 (V/V/V)	CH_3CN/CH_3OH H_3PO_4

Isolation of Preparative Fractions

After the individual phospholipid fractions were collected, the solvents were removed under vacuum at 30°C. Water was added to the resulting residue, extracted with $CHCl_3$, dried over Na_2SO_4, filtered and transferred to a 100 ml round bottom flask. The $CHCl_3$ was removed under vacuum at 30°C and the resulting phospholipid fraction was then evaluated for purity.

Evaluation of Purity

Each individual phospholipid was checked for purity using HPLC and FTIR. Each fraction was dissolved in $CHCl_3$ and injected onto the analytical HPLC. Figure 2 shows the resulting chromatogram obtained from the purified phosphatidyl inositol fraction. Additionally, each fraction was dissolved in $CHCl_3$, mixed with spectrograde KBr and placed in a conical 3 ml mini-vial to give an approximate concentration of 2 - 5% of the phospholipid in the KBr. The solvent was removed by placing this mixture in a vacuum oven at 60°C for 1 hour. These samples were pressed into 13 mm KBr discs for infrared analysis. These spectra were compared with spectra obtained from pure phospholipid standards.

Results and Discussion

When compared to authentic standards the IR spectra and HPLC analyses of the phospholipids collected from the preparative LC indicated that the fractions were basically pure phospholipid containing very small amounts of impurities.

The loading studies carried out on the Radial Pak Prep Silica indicated a calculated column load of 180 mg was appropriate for the preparative column. The preparative data supported these calculations; when a column load of over 200 mg was used, the resolution (Rs) greatly deteriorated. This experimental data was contrary to the popular premise for preparative chromatography where column loads of several grams are presumed to be possible.

Studies of the possible separation mechanism of the phospholipids indicated that when the appropriate mobile phase was used but contained no

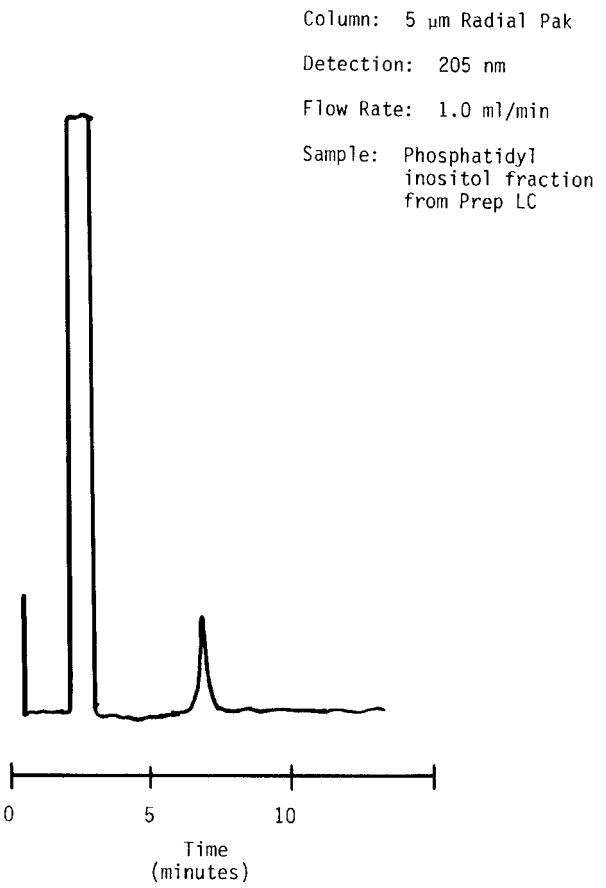


Figure 2. Chromatogram of Phosphatidyl Inositol from Preparative LC.

H_3PO_4, no phospholipid separation was observed. Additionally, when other acids such as H_2SO_4 were substituted for H_3PO_4 no separation of the phospholipids was observed. This would seem to indicate that the possible mechanism of separation might be a solvent generated phosphate column rather than a standard silica separation. This solvent generated phosphate column would therefore be indicative of the limited loading observed on the silica column. Other researchers (6,7) have used hydroxylapatite

$[Ca_{10}/(OH)_2(PO_4)_6]$, a phosphate column, for the separation of polar lipids. They reported that chromatography on silica gel or DEAE-cellulose gave inadequate separation of these lipids.

The results indicate that the column and mobile phase utilized in this preparative separation scheme successfully allowed the fractionation and isolation of pure phosphatidyl inositol, phosphatidyl choline and phosphatidyl ethanolamine; however the limited loading available on this column limits its overall use for preparative purposes.

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