High Performance Liquid Chromatography of Phospholipids with Flame Ionization Detection[1]

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The three major soy phospholipids were determined by normal phase high performance liquid chromatography with flame ionization detection. A Beckman Ultrasphere silica column was utilized with a solvent gradient composed of chloroform and premixed methanol/ammonium hydroxide. This chromatographic system produced excellent resolution of the three major phospholipids and triglyceride component as well as some of the minor constituents in soy oil lecithin. Ammonium hydroxide improved peak shape and resolution but caused the peaks for the phospholipids to appear as doublets. Quantitative analysis was accomplished by the external standard method.

The separation and quantitative analysis of phospholipids is a difficult problem. A recent paper (1) reviews the post-1980 literature on the analysis of glycolipids and phospholipids by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). This paper describes a method of analysis for phospholipids based on HPLC with flame ionization detection (FID).

Most of the separations of phospholipids have been effected on silica columns by using one of two organic solvent based systems. Columns from different manufacturers generally cannot be substituted in these methods because of the different chromatographic properties generated in the silica during the manufacturing process. Acetonitrile/methanol has been used with modifiers such as 85% phosphoric acid (2), sulfuric acid (3), or water (4,5). Solvent modifiers used with hexane/isopropanol were water (6,7) or a combination of phosphate buffer, ethanol and acetic acid (8). The most widely utilized method of detection was monitoring the absorption of ultraviolet radiation in the 200-210 nm wavelength range to take advantage of unsaturation in the fatty acid chains. However, the great variety of fatty acid constituents present in phospholipids results in complex absorption behavior and causes problems in their direct quantitative determination by ultraviolet detection (9). The above mentioned solvent systems did not always produce sharp chromatographic peaks and the major phospholipids were not always resolved. Chen (10) and coworkers reported that phosphatidyl inositol eluted with the solvent front when acetonitrile/methanol/phosphoric acid was used. Scobell (A.E. Staley Mfg. Co., private communication) has observed this same phenomenon. Only one or two of the major phospholipids can be determined by some methods. Chloroform is an excellent normal phase chromatographic solvent for phospholipids, but because of its UV cutoff, it cannot be used in conjunction with UV detection at low wavelengths.

As early as 1973, Erdahl and coworkers (11) utilized a flame ionization detector in the high performance liquid chromatographic analysis of phospholipids in soy lecithin. This detector was the moving wire type and allowed the use of chloroform in a solvent gradient in the chromatography. In 1982 Tracor Instruments developed a rotating disk flame ionization detector for high performance liquid chromatography. This detector has been found to be extremely useful in overcoming the difficulties experienced with ultraviolet detection of phospholipids.

MATERIALS AND METHODS

The HPLC system consisted of a Varian Model 5560 liquid chromatograph, a Dynatech Precision Model 241 autosampler fitted with a 20 microliter sample loop, a Tracor Model 945 flame ionization detector, a Spectrum 1021A filter and amplifier, and a Spectra Physics Model 4100 computing integrator. The design and operation of the flame ionization detector has been described (12). The flame ionizaiton detector was modified by placing a 10 turn potentiometer in the block heating circuit so that the block temperature could be adjusted to within 3°C of the desired temperature. The potentiometer was set so the block temperature was 110°C. The gas flow rates for the detector are detector hydrogen, 160 ml/min; detector air, 550 ml/min; cleaning hydrogen, 300 ml/min; and cleaning oxygen, 150 ml/min.

The separations were done on a 4.6 mm id by 25.0 cm Beckman Ultrasphere Si column (P/N 235341) containing five micron particles. The flow rate was 0.8 ml/min. Solvents utilized in forming the gradient were chloroform and premixed 86/14:methanol/28.7% ammonium hydroxide (v/v). In order to maintain the silica at constant reproducible activity it was necessary to store the column in 100% chloroform when not in use. Prior to the analysis of a sample, a 1 min gradient program to the initial gradient condition was utilized, after which the full gradient was run in order to equilibrate the column. The solvent program began with 10% premixed solvent for 5 min, followed by a 17 min gradient to 30% premixed solvent. This composition was held for 8 min, after which the solvent was returned to the initial condition in 2 min and held for 13 min prior to injection of the next sample.

HPLC grade chloroform and methanol from Baker (Phillipsburg, NJ) or Burdick and Jackson (Muskegon, MI) were used. The concentrated ammonium hydroxide (28.7%) was ACS grade and from Mallinckrodt (Paris, KY). Phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), and phosphatidyl inositol (PI) from soy beans were obtained from Sigma Chemical Company (St. Louis, MO) as chloroform or chloroform/methanol solutions. These standards were diluted to 1 mg/ml with chloroform. Avanti Polar Lipids, Inc. (Birmingham, AL) prepared a custom standard to approximate a soy lecithin. The standard contained 8.75 mg/ml soy oil, 3.75 mg/ml PE, 7.50 mg/ml PC, and 5.00 mg/ml PI in chloroform. This solution was diluted with chloroform before use. Standard solutions were stored in the freezer when not in use.

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Samples of soy lecithin and corn lecithin in oil were obtained from our manufacturing facility and dissolved in chloroform to a final concentration of 20 mg/ml. A commercially available granular soy lecithin was dissolved in chloroform at a final concentration of 10 mg/ml.

RESULTS AND DISCUSSION

Concentrated ammonium hydroxide has been used in a silica column activation procedure to condition the silica prior to the analysis of phospholipids (13). By premixing the concentrated ammonium hydroxide with the methanol it was not necessary to subject the column to the activation procedure. The ammonium hydroxide serves as a solvent modifier and improves peak shape and resolution. Several ratios of methanol/concentrated ammonium hydroxide results in poor peak shapes and too much hastens column deterioration.

Methylene chloride was evaluated as a weak solvent in the gradient for the phospholipid separation. Retention times for PE and PC are nominally unchanged, but the retention time for PI is increased by about 250 seconds. Peak shapes are not quite as good with methylene chloride as with chloroform. The FID performance and response is not affected by the use of methylene chloride.

Quantitative analysis of the lecithin samples was accomplished by the external standard method. The chromatographic system was calibrated prior to the analysis of samples and then checked after the samples were analyzed. Either the Sigma standard phospholipids or the Avanti custom standard was utilized. Figure 1 illustrates the separation of the components in the custom prepared standard. It contained 35% triglycerides, 15% PE, 30% PC, and 20% PI by weight. The chromatogram represents a 20 microliter sample volume containing 10.3 micrograms per microliter of the standard. The broad peak with a retention time of 2100 seconds is due to column deterioration and does not interfere with the analysis of the major phospholipids. The peaks for the lecithins appear as doublets. Shaw (Avanti Polar Lipids, Inc., private communication) has observed the same phenomenon with PC and PE when using ammonium hydroxide as a solvent modifier. According to his findings, the use of ammonium acetate in place of ammonium hydroxide eliminates the double peak effect, but peak shape deteriorates and tailing results. A synthetic PC or PE produced by using one fatty acid gives double peaks when chromatographed with ammonium hydroxide as the modifier. Quantification was not affected by the split peaks.

In addition to PC, PE, and PI, it was possible to determine the triglycerides and the free fatty acids in the samples. Acetylated phosphatidyl ethanolamine and/or lysophosphatidyl choline were detected in some samples. The chromatogram displayed in Figure 2 is that of a typical soy lecithin.

The Spectrum filter and amplifier served two functions. The Tracor flame ionization detector has output connections of 1 millivolt, 1 volt, and 10 volts. Only the 1 millivolt output can be sent through an internal electrical noise filter to aid in achieving a smooth baseline. Since the 1 volt output was utilized in this work, it was necessary to employ an external electrical noise filter in order to

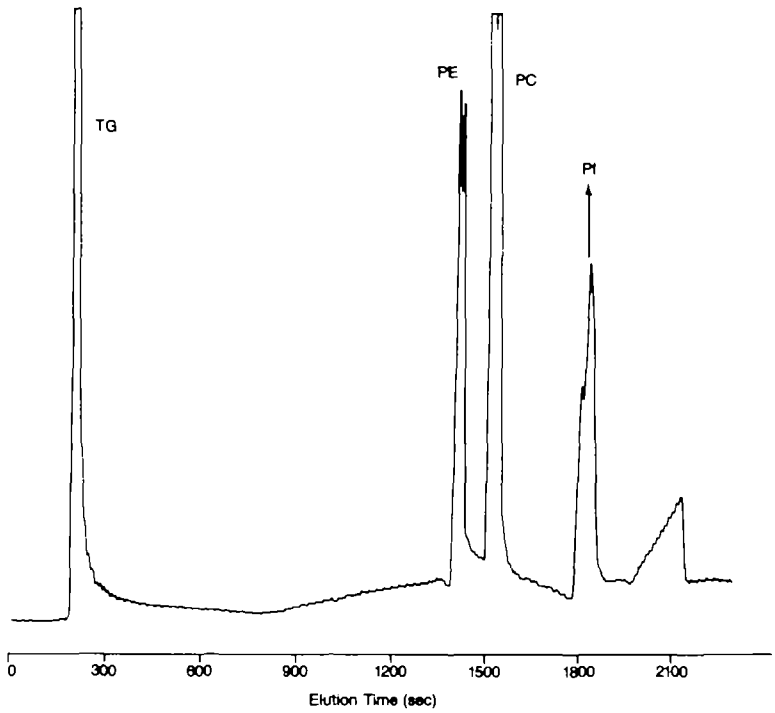

FIG. 1. Chromatogram of Avanti Custom Standard. See text for chromatographic conditions. Peaks: TG, triglycerides; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; PI, phosphatidyl inositol.

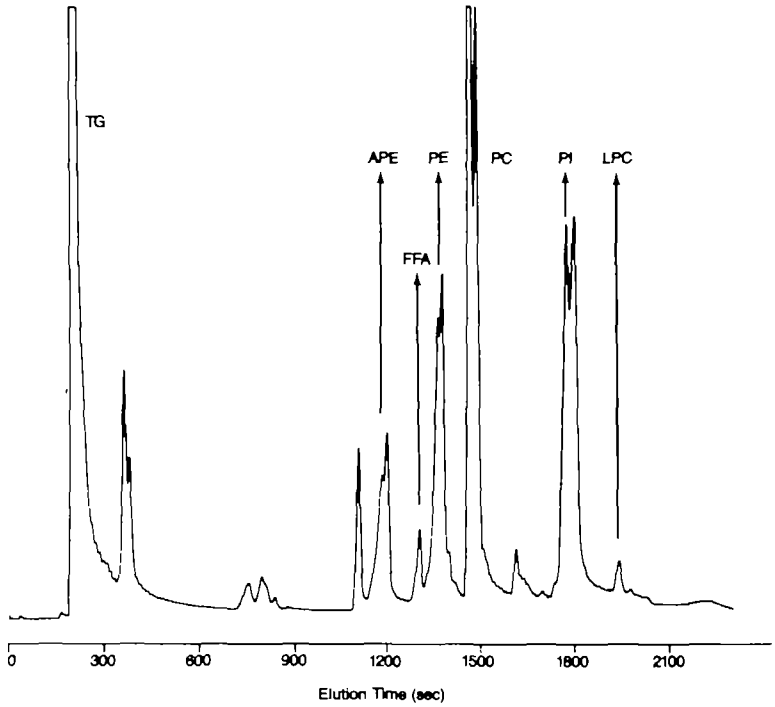

FIG. 2. HPLC separation of phospholipids in a typical soy lecithin. See text for chromatographic conditions. Peaks: TG, trigiycerides; APE, acetylated phosphatidyl ethanolamine; FFA, free fatty acids; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; PI, phosphatidyl inositol; LPC, lyso-phosphatidyl choline.

obtain a smooth baseline. The filter reduced the noise without clipping the tops of the peaks or adversely affecting the integration of peaks. The amplifier was set at 2 so that the signal from the detector was doubled. This feature increased the apparent sensitivity of the detector, although the phospholipid content of the samples was large enough so that detection was not a problem.

One soy lecithin sample was analyzed at the beginning and at the end of a three month period. This sample has served as a typical sample for judging the optimal operation and performance of the chromatographic system. The data presented in Table 1 are mean values and standard deviations obtained at the beginning and at the end of the three month period. The beginning values were obtained after calibration with Sigma standard phospholipids. The Avanti standard was utilized for the later data. The discrepancy in the values for PC may be related to a difference in purity between the two standards. However, no impurities were seen in the chromatogram. Table 2 contains typical values for the three major phospholipids found in samples of different origin.

TABLE 1

HPLC-FID Quantitative Results and Standard Deviations for a Sample of Lecithin in Oil

	n	% PE	% PC	# PI
Beginning	7	3.7 ± 0.2	13.6 ± 0.6	14.2 ± 0.5
End	10	3.5 ± 0.2	11.4 ± 0.4	14.1 ± 0.7

n = No. of observations.

TABLE 2

Phospholipid Results for Three Sample Types

Sample origin	PE wt %	PC wt %	PI wt %
Corn	4.0	17.6	12.6
Soy bean	3.5	11.7	15.2
Commercial granular	18.0	16.0	19.9

The detector produces a linear response over at least a 25-fold concentration range. The coefficient of correlation for the three phospholipids was in the 0.995-0.999 range. Samples were diluted so that the concentration of the phospholipids were within the linear range. Prior to this work, a linear response from the FID was obtained over a 600-fold concentration range for methyl paraben and propyl paraben. The linear range of the detector may well be much greater than 25-fold for phospholipids.

The flame ionization detector for HPLC is a valuable tool in the quantitative analysis of corn and soy lecithins. This detector allows the use of ultraviolet absorbing solvents such as chloroform in forming a solvent gradient to effect the separation of the phospholipids. The detector response is not affected by the variation in unsaturation of the fatty acid constituents in the phospholipids. The chromatographic system provides excellent resolution of the major phospholipids and, coupled with the flame ionization detector, alleviates the problems encountered when utilizing an ultraviolet absorption detector for the analysis.

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