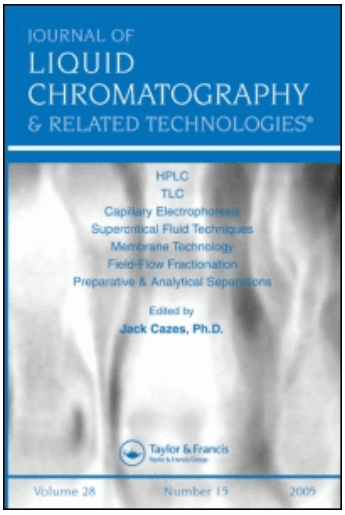

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A RAPID METHOD FOR PHOSPHOLIPID CLASS SEPARATION BY HPLC USING AN EVAPORATIVE LIGHT-SCATTERING DETECTOR

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ABSTRACT

A high-performance liquid chromatographic method for the separation and quantification of major natural phospholipid classes using an evaporative light-scattering detector is described. Separation and detection of cholesterol, palmitic acid, phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine and sphingomyelin was achieved in less than 15 minutes using a Spherisorb 10 cm long silica column. A simplified ternary mobile phase gradient of hexane, isopropanol and water was used to separate these six standards reproducibly and easily. A crude sample of egg yolk was also analyzed using this method and found to contain mostly cholesterol with small amounts of phosphatidylethanolamine, phosphatidylcholine and sphingomyelin. The evaporative detector gave a stable flat baseline with detection limits for most compounds in the low nanogram range.

INTRODUCTION

A variety of methods have been published describing phospholipid class separation utilizing high-performance

liquid chromatography. The separation of the major classes has been accomplished mostly through the use of normal phase methods incorporating a variety of detection methods. UV (205 - 220 nm) (1,2,3), flame ionization (4) and evaporative light scattering detectors (ELSD) (5,6,7) have been the mostly widely used. A book written by William Christie (8) was published in 1987, detailing many of these detection techniques. Some of these methods require elaborate ternary gradient systems consisting of complex mixtures of several solvents as well as analysis times ranging from 20 to 60 minutes (4,5,7). More recently an isocratic method was published which separated some of the more popular phospholipid classes in as little as 10 minutes. The method, while quick, failed to resolve cholesterol from the other lipids and also used high concentrations of phosphoric acid which could be detrimental to the column (1). Many of the UV methods fail to resolve all compounds of interest since a chromophore is not always present in the sample. Gradient methods using either a flame ionization or UV detector also contribute to baseline stability problems. Because of these weaknesses the evaporative light-scattering detector was chosen because it offered the greatest advantages for this analysis. Even though the response from the evaporative light-scattering detector is sometimes not linear it's extremely stable baseline

and lack of response to "solvent fronts" enable it to easily detect most phospholipids without the need for derivatization or other tedious and less quantitative forms of sample preparation. It does require pure, volatile mobile phases and can not be used with non-volatile buffers. This apparent drawback is not a disadvantage in phospholipid analysis since many of the solvents used are highly volatile and pure. An excellent paper on the evaporative light-scattering detectors operating principle and responses has already been described elsewhere (9).

A simpler gradient method which could be used to identify many phospholipid classes which did not require the use of mobile phase modifiers or result in long analysis times was sought. The method developed is able to resolve several animal tissue phospholipid classes ranging from cholesterol to sphingomyelin as a single peak aiding in quantitation. Such a method was developed and is described in the text that follows.

MATERIALS & METHODS

Chemicals

Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SPH) were all greater than 97 % purity from chicken egg yolk sources. Phosphatidylserine (PS) was from bovine brain source. Cholesterol (C), dried

egg yolk and palmitic acid (P) were also purchased with the above mentioned samples from Sigma Chemical Company (St. Louis, MO, USA) and were all used as standards in this work. Each of the standards and samples were stored frozen, away from any sources of light and mixed up fresh the day of use. All of the solvents used were purchased as HPLC grade from Burdick and Jackson (Muskegon, MI, USA) and thoroughly degassed with helium before use. Nitrogen gas for the evaporative detector was ultra high purity grade and purchased from Matheson Gas Products (Secaucus, NJ, USA).

Instrumentation

A quaternary HPLC pump, model 1050, from Hewlett -Packard (Avondale, PA, USA) was used for all of the analytical separations. The HPLC system consisted of a Rheodyne model 7125 injector valve fitted with a 20 ul loop (Berkeley, CA, USA), 'AT' style computer with MS-DOS ChemStation data acquisition, control and analysis software, A/D interface and Evaporative Light-Scattering Detector, model ELSD IIA, from VAREX Corporation (Burtonsville, MD, USA). The ELSD IIA incorporates an improved drift-tube and 780 nm, 20 mW laser over earlier designs for detection of sample particles.

Chromatographic Conditions

A Spherisorb 80 Å, 100 mm x 4.6 mm I.D. analytical column purchased from Alltech Associates (Deerfield, IL,

TABLE I

Gradient System For Lipid Class Separation

	Composition of Mobile Phase		
Time (min.)	% A	% B	% C
0.00	2	40	58
7.00	8	40	52
15.00	8	40	52
18.00	2	40	58
20.00	2	40	58

A = Water , B = Hexane & C = Isopropanol

USA) packed with 3 micron spherical particles was used to separate the phospholipid classes. No guard column was used.

The mobile phase consisted of isopropanol, hexane and water run as a ternary gradient described in Table I. Isopropanol was used to mediate the change from 2 to 8 % water against hexane in 7 minutes. The solvent flow rate was held constant at 1.25 mL / min at ambient temperature throughout the separation.

Nebulization in the ELSD was set to 105°C drift-tube temperature (68°C Exhaust Temperature) and 50 mm of nitrogen gas flow to the nebulizer (which corresponds to 2.5 L / minute gas flow).

RESULTS & DISCUSSION

This method detects various phospholipid classes without the need to derivatize the sample or use special

mobile phase modifiers. The evaporative light-scattering detector enables the operator to detect nanogram amounts of many phospholipids with great ease and without interference from baseline fluctuations and solvent peaks normally associated with other types of detection methods. This method uses a simple ternary mobile phase relying on a change in solvent polarity to separate the simple and complex individual animal tissue phospholipid classes from each another.

The method allows for a change from 2 % water to 8 % water in 7 minutes causing the backpressure on the column to rise from 1300 to 1700 psi. Throughout the method the concentration of hexane remained constant while the amount of isopropanol was changed to compensate for the increase in water. The water concentration was then held constant for 8 more minutes after which time the solvent program is reversed to restore the column to the proper starting conditions. The column is rapidly restored in 5 minutes and is ready to accept the next sample. As a precaution, the column was flushed with 100 % methanol every 10 to 20 runs to rinse off any bound matter that may have accumulated from injection of the crude samples. Reproducibility with this system was found to be excellent with over 120 injections of crude samples and standards. Analysis of three different standards over the course of one day resulted in minimal changes in

TABLE II

Retention Time Reproducibility of Standards

Phospholipid Class Std.	Retention Times In Minutes	Mean +/- S.D.
PC	11.251, 11.276, 11.248, 11.297	11.268 +/- 0.020
PE	7.376, 7.338, 7.425, 7.407	7.387 +/- 0.033
SPH	13.541, 13.752, 13.662, 13.804	13.690 +/- 0.010

retention time. Examples of the retention time variation of these three standards are shown in Table II. The column's performance did not deteriorate and the retention times of several standard peaks were within 1 to 2 % of each other.

A mixture containing PE, PC, PS, C, sphingomyelin and palmitic acid, dissolved in equal parts of mobile phase and chloroform, each at a concentration of 7 ug / 20 uL were injected onto the column as standards. The resulting standards are shown in the chromatogram labeled Figure 1. All six standards were clearly baseline resolved in under 15 minutes. Even though the concentrations of each standard were identical the peak heights and areas for each standard were different due to the fact that each compound has a different particle size

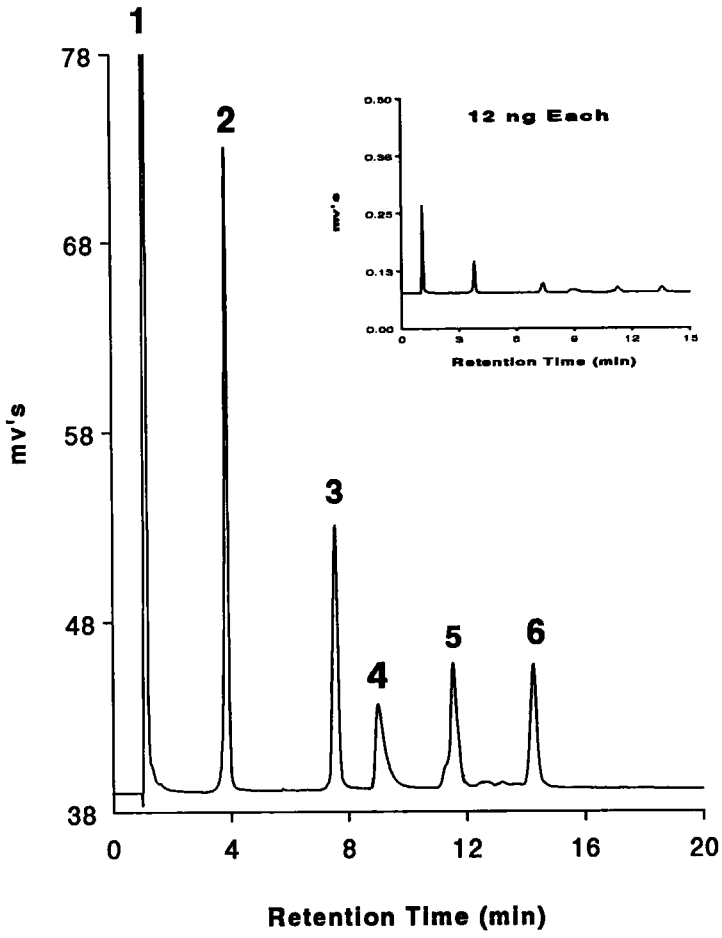

Figure 1. A Chromatogram of six phospholipid class standards injected onto the silica column described in the text. 1) Cholesterol, 2) Palmitic Acid, 3) Phosphatidylethanolamine, 4) Phosphatidylserine, 5) Phosphatidylcholine and 6) Sphingomyelin each 7 ug / 20 uL by evaporative light-scattering detection.

when it passes by the laser in the detector. This difference in response factors is due mainly to several factors regarding the amount of light the particle scatters inside the flow cell of the detector. An excellent description of this phenomenon and an explanation of it can be found in a paper by Charlesworth (9) that is well worth reading. The variation of response factors was not a problem with this system as the use of a modern computer to precisely integrate the peak areas and the construction of a calibration curve still allowed quantitative results to be obtained.

A 5 mg sample of crude egg yolks was weighed out and dissolved into one mL of liquid containing equal parts of mobile phase and chloroform. The solution was filtered through a 0.2 micron syringe filter and a 20 uL volume was injected into the analytical system for analysis. Figure 2 depicts the resulting chromatogram of the analysis. The main peak that eluted at 1 minute has been identified as cholesterol and was the major constituent. Three other peaks were also present and identified as phosphatidylethanolamine, phosphatidylcholine and a trace peak of sphingomyelin respectively. All peak identities were assigned by comparing the retention times of the sample peaks to that of the phospholipid class standards run earlier. Other techniques such as LC-Mass spectroscopy were not available for conformation of these peak assignments.

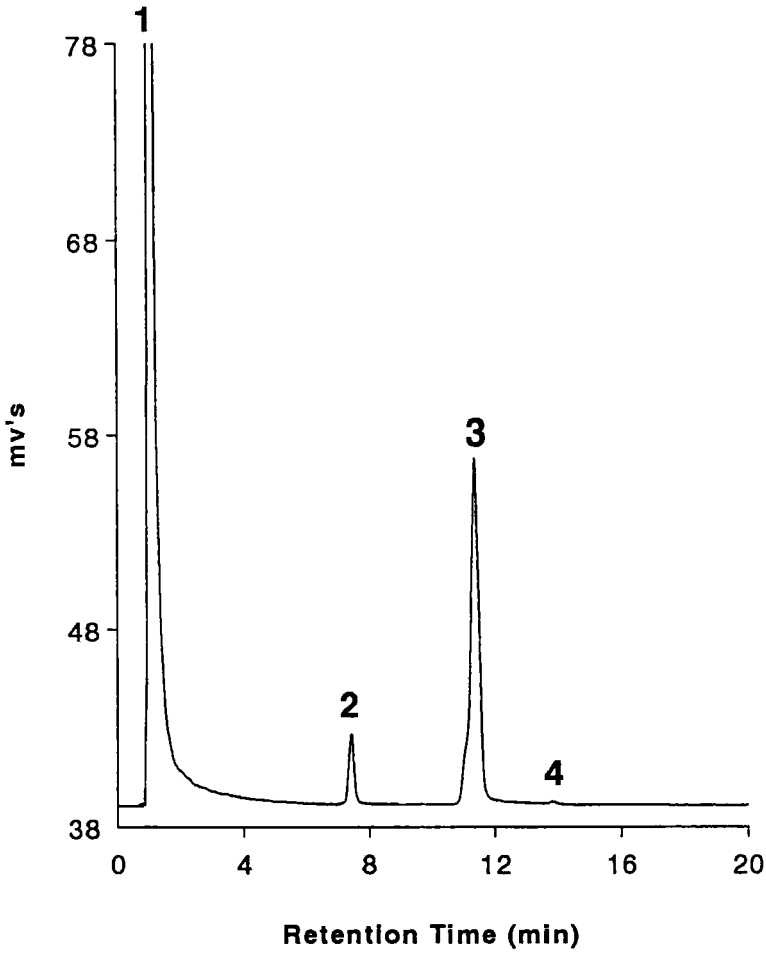

Figure 2. A sample chromatogram of crude egg yolk phospholipid classes using evaporative light-scattering detection. 1) Cholesterol, 2) Phosphatidylethanolamine, 3) Phosphatidylcholine and 4) Sphingomyelin were all detected using the gradient system described in the text.

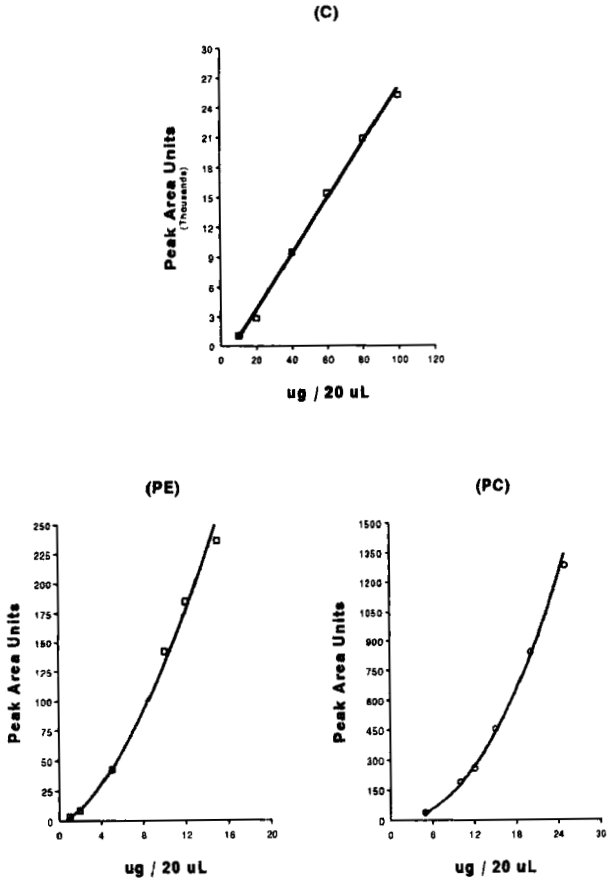

Figure 3. Calibration plots of cholesterol (C), phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Each point was the mean of three areas. The curve fit for (C) was found to be linear in the range tested with r^2 = 0.999 while the curve fits for (PE) and (PC) were found to be power curves with r^2 = 0.986 and 0.997 respectively.

To quantitate the amount of each of the three major components 3 standards of varying concentrations were injected and the areas recorded. Calibration curves of each standard were made and can be seen in Figure 3. High concentrations (> 10 ug / 20 uL) resulted in linear curves while lower concentrations resulted in non-linear curves. From these three curves the amount of cholesterol, phosphatidylethanolamine and phosphatidylcholine was determined as 1.50, 0.30 and 0.65 mg / mL respectfully with the remainder being attributed to insoluble matter. Sphingomyelin was found in trace amounts and was not quantitated.

Since all of the peaks in the standard mix were baseline resolved some improvements to the method are certainly possible. The flow rate could probably be raised from 1.25 to 1.50 mL / min. A different silica particle size and shape could be tested to determine if there is any selective advantage of one size over another in this analysis. If desired, the rate at which the water is increased in the gradient could be varied to selectively separate one component or class into more species. The method could also be converted to a binary system thereby simplifying it even further.

CONCLUSION

In this study an attempt was made to present a new method for the separation of natural animal tissue

phospholipid classes. Its simplicity, absence of baseline drift and short analysis time make it a convenient alternative over earlier methods. Quantitation is accomplished with the aid of individual class peaks, baseline resolution of all peaks and the use of a modern computer data station for integration of areas. Reproducibility was found to be excellent and the limit of detection for most of the standards was estimated to be in the low nanogram range. The method was used to successfully analyze four phospholipid classes in crude egg yolk in less than 15 minutes. It's application to plant tissues is presently unknown but will be explored at a later date.

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