# Separation and Characterization of Peanut Phospholipid Molecular Species Using High-Performance Liquid Chromatography and Fast Atom Bombardment Mass Spectrometry

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**ABSTRACT:** Total lipid extracts from peanut seed were separated on a silica column into a triacylglycerol fraction and a polar lipid fraction by high-performance liquid chromatography (HPLC). The polar fraction containing the phospholipids was retained on the precolumn, and the triacylglycerol fraction was eluted to a waste flask by a special valve arrangement. Phospholipids were eluted from the precolumn and separated into various classes on a silica analytical column. Each phospholipid class was manually collected and subsequently subjected to reversed-phase HPLC in tandem with a fast atom bombardment mass spectrometer. Phosphatidylethanolamine was separated into five molecular species. Phosphatidylinositol and phosphatidylcholine were each separated into six molecular species.

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**KEY WORDS:** Fast atom bombardment mass spectrometry, HPLC, lipids, molecular species, peanuts, phospholipids, reversed-phase HPLC.

Phospholipids are an extremely important class of lipids because of their biological and chemical properties. This lipid class consists of lipids with a glycerol backbone, a polar head group, and unsaturated fatty acids which are structural components of all living cells (1,2). Phospholipids or membrane lipids have been available for a long time in the form of lecithin materials [primarily, phosphatidylcholine (PC)] as a by-product of fats and oils processing. The demands for various types of phospholipids have increased because of increased human use (3). For example, phospholipids are used in infant formulas, food products, industrial lubricants, bread dough stabilizers, liposomes, mixed micelles, cosmetics, and as excipients and emulsifiers in pharmaceuticals (3,4).

Even though synthetic phospholipids can be used in health care and nutrition, there is a renewed interest in using phospholipids from natural products because of their biocompatibility. Phospholipids, especially PC, were found to be very important in the treatment of neurological diseases, respiratory distress, liver diseases, and many others (5). In the food industry, phospholipids have an important role in the stability of foods. This class of compounds was shown to be synergistic with tocopherols in delaying the onset of lipid oxidation (6–8). Phospholipids participate in the Maillard reaction, providing stability to oils at high temperature as well as participating in the development of flavors (9). In order to understand more about the biological activity and reactions of phospholipids, it is necessary to have a thorough knowledge of the molecular species of each phospholipid class.

This paper reports on a new analytical method that uses a narrow-bore reversed-phase high-performance liquid chromatography (HPLC) scheme for the separation of phospholipid molecular species. Application of the method is shown for the identification of phospholipids extracted from peanuts. Several mass spectrometric techniques were used for the identification of phospholipids (10). The use of soft ionization processes such as field desorption, fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI), thermospray, and electrospray all provides molecular weight information. With field desorption, no structural information from fragmentations is obtained (11). In addition, this technique is not compatible with on-line separations, limiting its application in the identification of complex mixtures of phospholipids. Positive and negative ionization FAB experiments without HPLC separations were used for rapid identification of phospholipid molecular species (12–16). When combined with mass spectrometry (MS)/MS, structural elucidation of the phospholipids is possible (17–19). MALDI-time-of-flight MS is a third soft ionization process that was used for molecular weight identification of phospholipids (20). The MALDI-TOF technique is currently not compatible with on-line HPLC/MS experiments. The results show that the technique provides sensitive measurement of molecular weights but provides no structural information. Additionally, identification is complicated by the presence of sodium and potassium adducts. Electrospray ionization provides sensitive molecular weight information and can be used with or without HPLC separations (21–23). When FAB is used on-line with chromatographic separation, both molecular weight determination and structural elucidation are obtained in a single experiment. On-line HPLC/MS with thermospray, particle beam, and electrospray interfaces were reported (24–27). The particle beam analyses

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failed to provide molecular weight information for the phospholipids. The thermospray analysis generally gave molecular weight, polar headgroup, and fatty acid information, although the structural information regarding the position of the fatty acid substitution cannot be definitively determined since the relative intensities are affected by tip temperature, source temperature, and other instrumental parameters. The electrospray analysis, while more sensitive than other techniques, does not provide structural information.

## MATERIALS AND METHODS

*Materials.* Peanuts (VA NC7) were grown at the NC State Experiment Station, Lewiston, NC, and were dried to approximately 7% moisture at ambient temperature. All solvents used in the lipid extraction were reagent and HPLC-grade (Fisher Scientific, Pittsburgh, PA). Phospholipid standards were obtained from Sigma Chemical Company (St. Louis, MO) or Avanti Polar Lipids (Alabaster, AL).

*Crude lipid extraction.* Lipids were extracted from peanuts using chloroform/methanol ($CHCl_3$/MEOH, 2:1, vol/vol). A 100-g sample was blended with 300 mL of $CHCl_3$/MEOH (2:1, vol/vol) in a Sorvall blender (Ivan Sorvall Inc., Norwalk, CT) for 1 min. The blended material was suction-filtered through filter paper using a Buchner funnel. The resultant cake was reextracted with 300 mL of $CHCl_3$/MEOH (2:1, vol/vol) in a Sorvall blender. Both extracts were combined. A saturated solution of NaCl was added to the filtered solution in a separatory funnel, shaken, and allowed to stand until phase separation occurred. The $CHCl_3$ layer was saved, and the water layer was discarded. The solvent layer was washed twice with saturated NaCl (150 mL), and the $CHCl_3$ removed by flash evaporation. Extracted lipid material was stored in a freezer at −20°C until analyzed.

*HPLC separation of phospholipids from the acylglycerol fraction.* The major fraction of peanut crude lipid is the triglyceride fraction, which approximates 98 to 99%. The phospholipid fraction was separated from bulk triglyceride and concentrated prior to HPLC analysis (28,29). Peanut phospholipids were retained on the silica column (6 μ, 100 × 8 mm i.d.), and the bulk triacylglycerol fraction was eluted to a waste flask (1000 mL Erlenmeyer) using 100% hexane as the eluting solvent (28,29). Valves were automatically switched, and the phospholipid concentrated fraction was eluted from the concentrator column onto the semiprep column (100 × 8 mm i.d.) with 2-propanol/hexane (4:3, vol/vol) (28,29).

*HPLC phospholipid class separation.* Peanut phospholipids eluted from the concentrator column were separated into their various classes on a silica column (100 × 8 mm i.d.) using a combination gradient and isocratic program of mixed solvents and detected at 205 nm with an ultraviolet (UV) detector. Solvent A was a mixed solvent of isopropanol/hexane (4:3, vol/vol), and solvent B was a mixed solvent of isopropanol/hexane/water (8:6:1.5, vol/vol/vol). Phospholipids were separated using a gradient starting at 100% solvent A to 100% solvent B in 20 min, isocratic with 100% solvent B for 15 min, and regeneration of the column for the next analysis with 100% solvent A for 10 min. Phospholipids were identified by retention times by running authentic standards under the same conditions. Individual phospholipids were collected manually and stored at −20°C for further analysis. The injection volume for each sample was 1 mL.

*HPLC separation and FAB MS characterization of molecular species.* A hyphenated technique was used to separate and characterize the phospholipids using a 140A dual syringe pump with a 757 UV detector and 236 microflow cell (Applied Biosystems) attached to the inlet of a JEOL JMS-HX 110 double-focusing FAB mass spectrometer with a pneumatic splitter (Tokyo, Japan) (30–32). The phospholipid molecular species were separated on a C8 reversed-phase column (2.1 × 150 mm; MacMod Analytical, Inc., Chadds Ford, PA). A mobile phase consisting of $CH_3OH$/hexane/0.05 M $NH_4OAc$/glycerol (84/5.5 to 7/8/0.5 to 0.7) at a flow rate of 300 μL/min was used to achieve baseline separation. An isocratic run time of 25 min was required for separation of the molecular species. Molecular and fragment ions were characterized by FAB MS. Full-scan data were obtained for every analysis. The scan ranges used were either 200 to 1000 daltons for negative ions or 400 to 1000 daltons for positive ions.
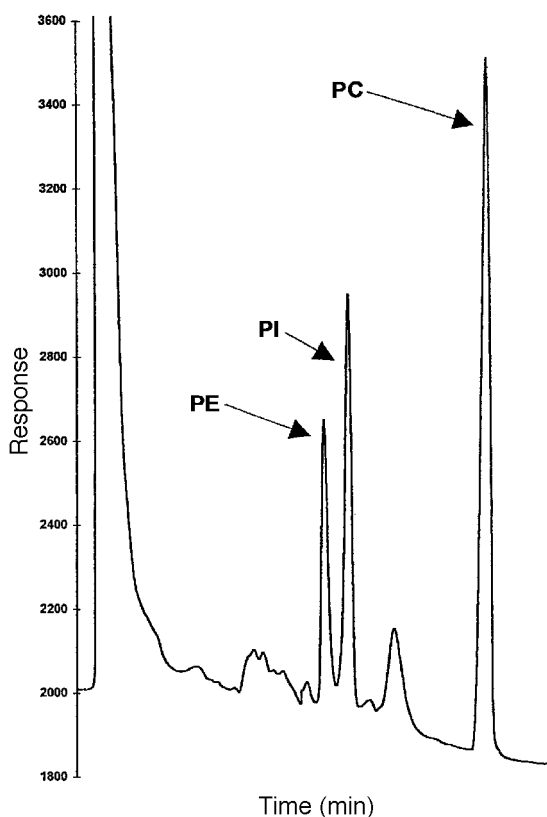


**FIG. 1.** Peanut phospholipid class separation on a silica high-performance liquid chromatography (HPLC) column. PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine.
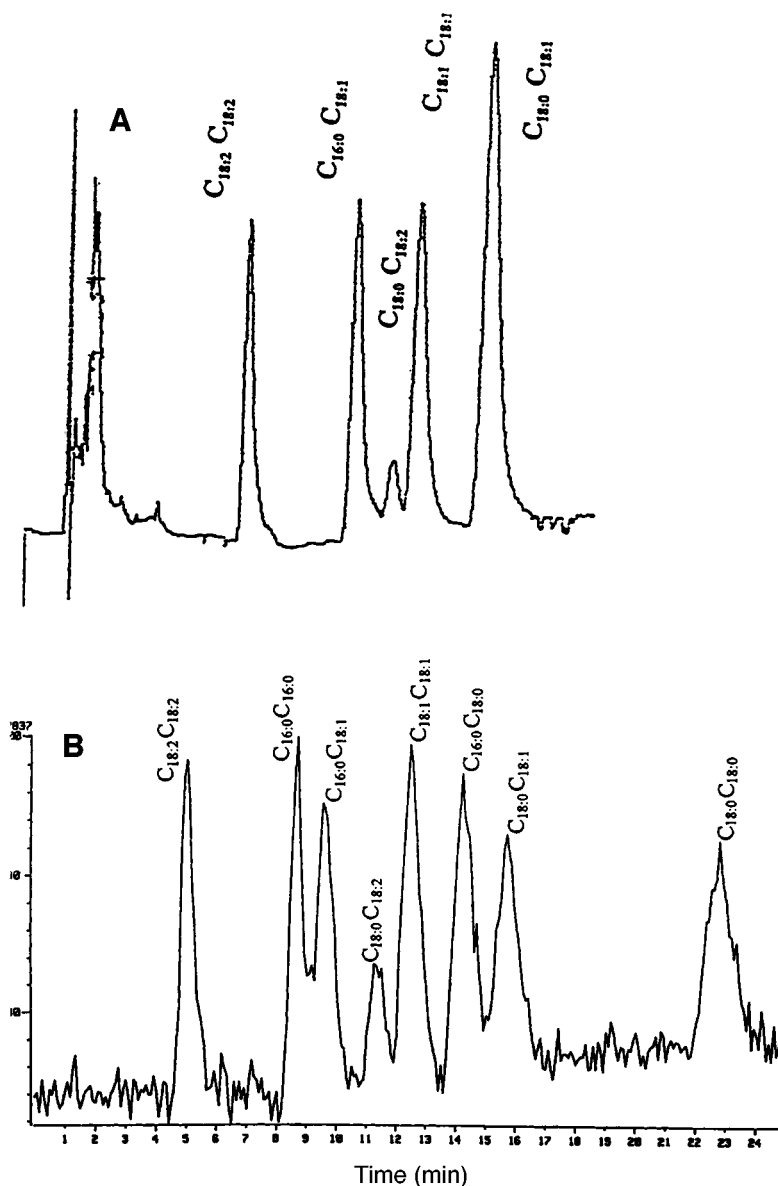
**FIG. 2.** HPLC/ultraviolet (UV) chromatogram of a phosphatidylcholine (PC) standard separated into molecular species and flow-fast atom bombardment (FAB) detection. (A) HPLC/UV chromatogram of PC molecular species at 210 nm, (B) flow-FAB data of summed ion currents for the PC molecular species. See Figure 1 for other abbreviation.

## RESULTS AND DISCUSSION

*Phospholipid class separation.* Phospholipid class was achieved using normal-phase HPLC. Figure 1 shows a typical phospholipid class separation, which was based on the different adsorption characteristics of the polar headgroup on a silica stationary phase. Phosphatidylethanolamine (PE) eluted first, followed by phosphatidylinositol (PI) and PC.

*HPLC separation of a PC standard into molecular species.* A PC standard was separated into its molecular species by HPLC and detected with a UV detector at 210 nm (Fig. 2A) and by flow-FAB MS (Fig. 2B). Only the unsaturated PC

molecular species was detected with the UV detector at 210 nm, whereas both saturated and unsaturated molecular species were detected with flow-FAB MS.

*Separation and characterization of PE molecular species.* Five PE species were identified by FAB. They are, in order of elution, $C_{18:2}C_{18:2}$, $C_{16:0}C_{18:2}$, $C_{18:1}C_{18:2}$, $C_{16:0}C_{18:1}$, and $C_{18:1}C_{18:1}$. A reconstructed HPLC trace of the M + H ions for each species is shown in Figure 3A. On the reversed-phase column, the most polar species eluted first. The molecular species of PE was analyzed both in the positive and negative modes, since both modes are equally sensitive for this phospholipid class and they give complementary information. The
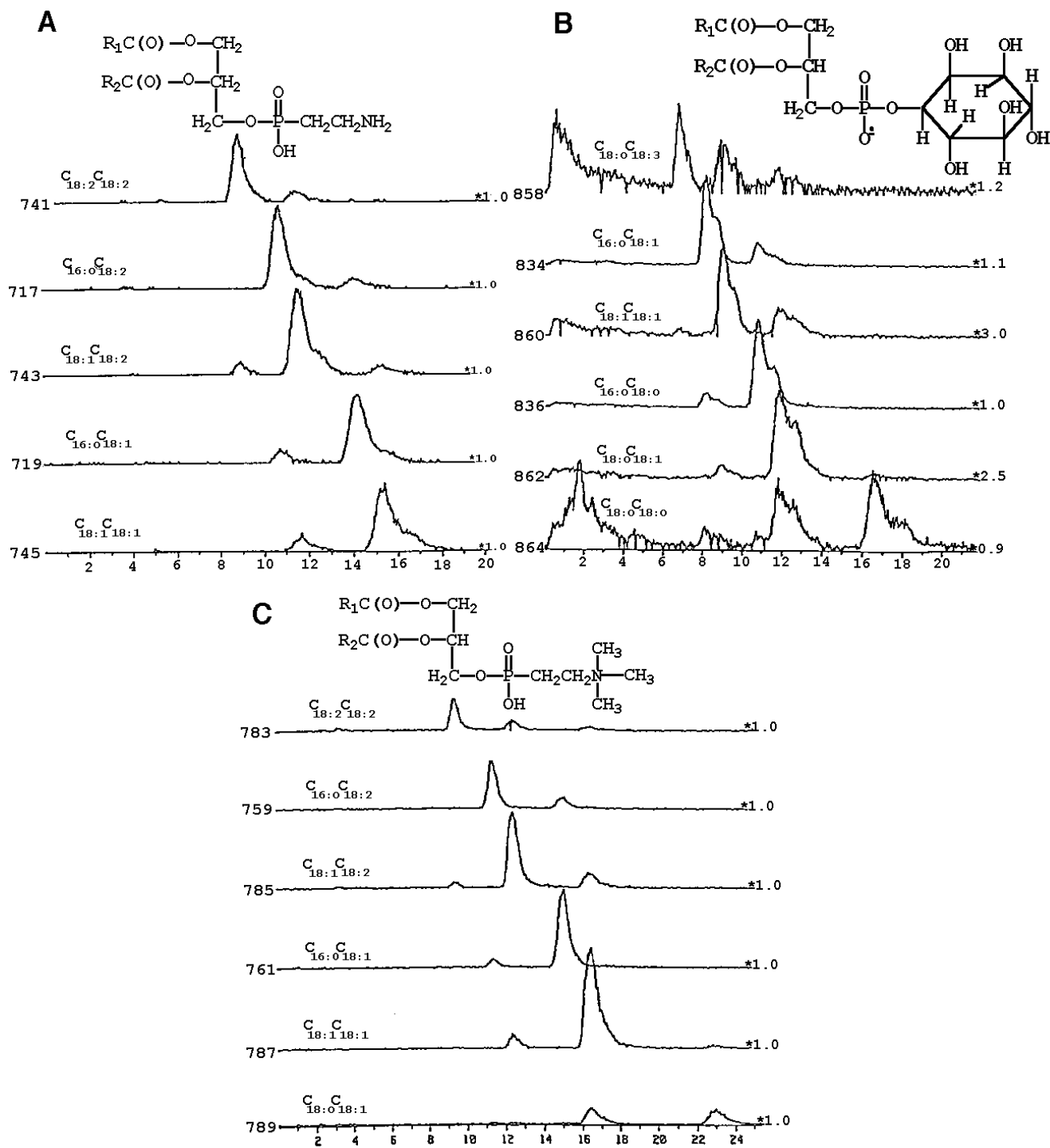
**FIG. 3.** HPLC separation of phospholipid molecular species detected by full-scan analysis with FAB–mass spectrometry (MS). (A) PE molecular species, (B) PI molecular species, (C) PC molecular species. See Figures 1 and 2 for other abbreviations.

positive-ion FAB mass spectrum for PE molecular species ($C_{16:0}C_{18:2}$) is shown in Figure 4. The ion at *m/z* 716 represents the protonated molecular ion, while the fragment ion at *m/z* 576 represents the molecular ion minus both the polar headgroup and the phosphate ($HOP(O)3CH_2CH_2NH_2$) at the *sn*-3 position of the molecule. The fragment ion at 454 repre-

sents the pronated molecular minus $C_{18}H_{31}O_2$ of the fatty acid. The $C_{16:0}$ fatty acid moiety was found to be in the *sn*-1 position with $C_{18:2}$ fatty acid moiety in the *sn*-2 position, since the fragmentation of the fatty acid in the *sn*-2 position is more facile than the fragmentation of the *sn*-1 fatty acid (10,13,15,18). Two other positive-ion FAB spectra are shown
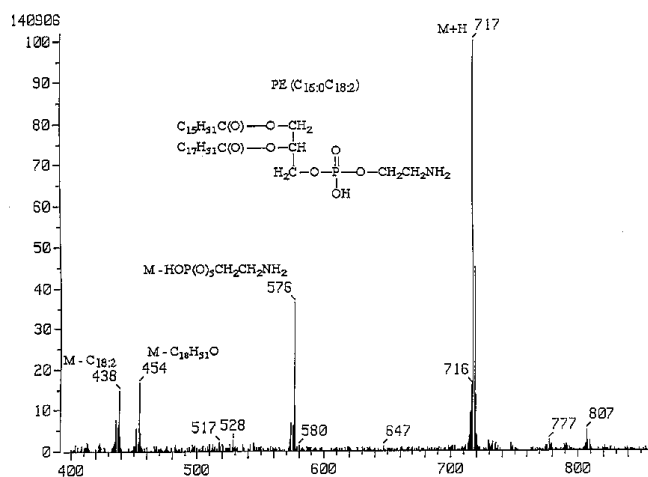
**FIG. 4.** FAB mass spectra of PE molecular species $C_{16:0}C_{18:2}$. See Figures 1 and 3 for abbreviations.



**FIG. 6.** FAB mass spectra of PE molecular species $C_{16:0}C_{18:1}$. See Figures 1 and 3 for abbreviations.

for PE $C_{18:2}C_{18:2}$ and $C_{16:0}C_{18:1}$ (Figs. 5 and 6, respectively). The fragment ions observed follow the same pattern as described for PE $C_{16:0}C_{18:2}$. The pronated molecular ions are observed at $m/z$ 741 and 719. The loss of the polar headgroup and the phosphate from the pronated molecular ions is observed at $m/z$ 600 and 578. For PE $C_{18:2}C_{18:2}$, the fatty acid losses from the pronated molecular ion of $C_{18}H_{31}O$ and $C_{18}H_{31}O_2$ are observed at $m/z$ 478 and 462. For PE $C_{16:0}C_{18:1}$ the typical fatty acid losses for both substituents are observed: $M + H - C_{16}H_{31}O$ ($m/z$ 480), $M + H - C_{16}H_{31}O_2$ ($m/z$ 464), $M + H - C_{18}H_{33}O$ ($m/z$ 454), and $M + H - C_{18}H_{33}O_2$ ($m/z$ 438). The ions from $C_{18:1}$ fatty acid are dominant and can therefore be used to assign the *sn*-2 position to that species. The mass spectra for the other two PE molecular species would be interpreted similarly (data not shown).

*Separation and characterization of PI molecular species.* Six PI species were identified by negative-ion FAB. Negative-ion FAB was used since PI are difficult to protonate but readily form M − H anions under the analytical conditions.
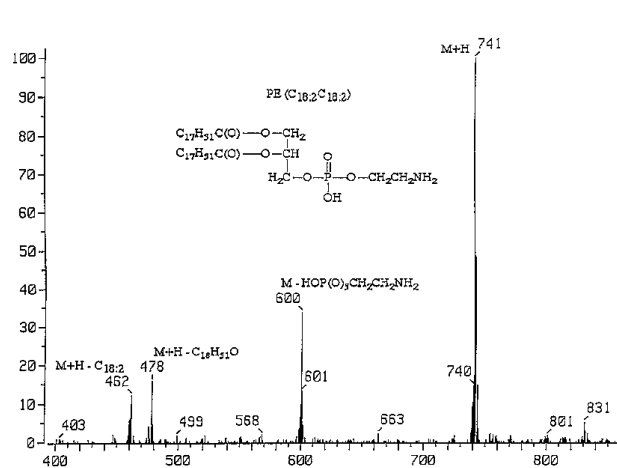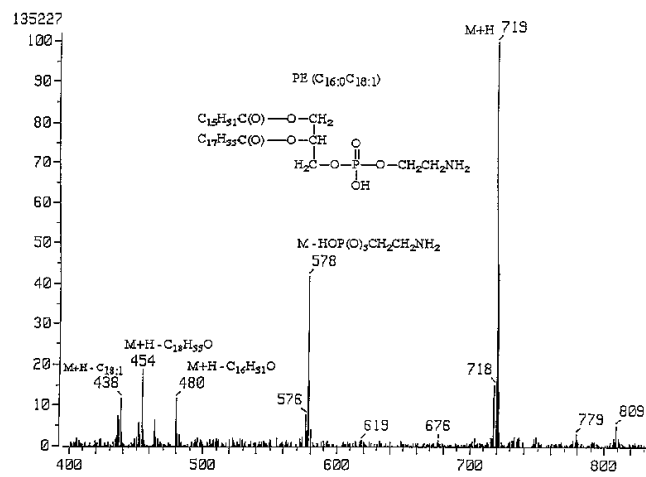
The PI identified are in order of elution, $C_{18:0}C_{18:3}$, $C_{16:0}C_{18:1}$, $C_{18:1}C_{18:1}$, $C_{16:0}C_{18:0}$, $C_{18:0}C_{18:1}$, and $C_{18:0}C_{18:0}$. A reconstructed HPLC trace of the M − H ions for each species is shown in Figure 3B. The FAB mass spectrum for PI molecular species ($C_{16:0}C_{18:0}$) is shown in Figure 7. Diagnostic ions in the spectrum include $m/z$ 836 (M − H)⁻ and $m/z$ 674 (M − inositol)⁻. Ions related to the fatty acid constituents are also observed: The molecular ion minus $C_{16}H_{31}O$ ($m/z$ 598), M − $C_{18}H_{34}O$ ($m/z$ 571), and M − H − $C_{18}H_{35}O_2$ ($m/z$ 533). As before, the dominant ions in this region indicate that the $C_{18:0}$ is substituted in the *sn*-2 position. Other important ions observed in the negative ion spectra for $C_{16:0}C_{18:0}$ PI are: M − $C_{18:0}$ − inositol ($m/z$ 391), M − $C_{16:0}C_{18:0}$ ($m/z$ 299), and $C_{16:0}$ fatty acid anion ($m/z$ 255). Two other negative-ion FAB spectra are shown for PI $C_{18:0}C_{18:1}$ and $C_{16:0}C_{18:1}$ (Figs. 8 and 9, respectively). The fragment ions observed follow the same pattern as described for PI $C_{16:0}C_{18:0}$. The molecular ions minus H are observed at $m/z$ 862 and 834, respectively. The loss of the inositol polar headgroups is observed at $m/z$ 700



**FIG. 5.** FAB mass spectra of PE molecular species $C_{18:2}C_{18:2}$. See Figures 1 and 3 for abbreviations.
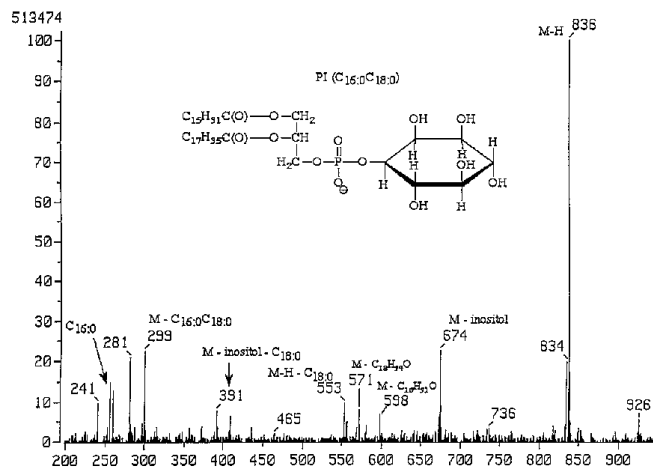


**FIG. 7.** FAB mass spectra of PI molecular species $C_{16:0}C_{18:0}$. See Figures 1 and 3 for abbreviation.

and 672. The FAB mass spectrum for PI $C_{18:0}C_{18:1}$ shows that the substituent is located at the *sn*-2 position due to the dominant ions at $M - C_{18}H_{33}O$ (*m/z* 598) and $M - H - C_{18}H_{33}O_2$ (*m/z* 579). Likewise, for $C_{16:0}C_{18:1}$ the substituent at *sn*-2 is readily identified as the $C_{18:1}$ fatty acid by the presence of ions at $M - C_{18}H_{33}O$ (*m/z* 571) and $M - H - C_{18}H_{33}O_2$ (*m/z* 533) in Figure 9. The other three PI compounds are identified using the same criteria (data not shown).

*Separation and characterization of PC molecular species.* Six PC species were identified by both positive and negative ion FAB. The PC identified are in order of elution, $C_{18:2}C_{18:2}$, $C_{16:0}C_{18:2}$, $C_{18:1}C_{18:2}$, $C_{16:0}C_{18:1}$, $C_{18:1}C_{18:1}$, and $C_{18:0}C_{18:1}$. A reconstructed HPLC trace of the M − H ions for each species is shown in Figure 3C. The greatest sensitivity was achieved in the positive FAB mode, since PC is inherently positively charged under the conditions of analysis. However, in the positive-ion mode there is insufficient fragmentation to definitely characterize the polar headgroup. In the negative mode, no molecular ions are observed; however, $(M - CH_3)^-$ and $(M - choline)^-$ are diagnostic for this class. The positive-ion FAB spectrum for PC $C_{18:1}C_{18:1}$ is shown in Figure 10. The pronated molecular ion is observed at *m/z* 787. Characteristic ions for the fatty acid substituents are observed for $M + H - C_{18}H_{33}O$ (*m/z* 523), and $M + H - C_{18:1}H_{33}O_2$ (*m/z* 507). Other characteristic ions for this class correspond to the loss of both fatty acids, $M - C_{18:1}C_{18:1}$ (*m/z* 224). The spectrum for PC molecular species $C_{18:1}C_{18:2}$ is shown in Figure 11. The pronated molecular ion is observed at *m/z* 785. The fatty acid substituent located in the *sn*-2 position is identified as $C_{18:1}$ based on the abundant ions corresponding to $M + H - C_{18}H_{33}O$ (*m/z* 520) and $M + H - C_{18}H_{33}O_2$ (*m/z* 504). The loss of the other fatty acid is also observed at only slightly reduced sensitivity, $M + H - C_{18}H_{31}O$ (*m/z* 522) and $M + H - C_{18}H_{31}O_2$ (*m/z* 506). Again, the species-specific ion at *m/z* 224 is observed, indicating the loss of both fatty acids. Similar interpretations would be made for the remaining PC molecular species including the $C_{18:2}C_{18:2}$ species shown in Figure 12.
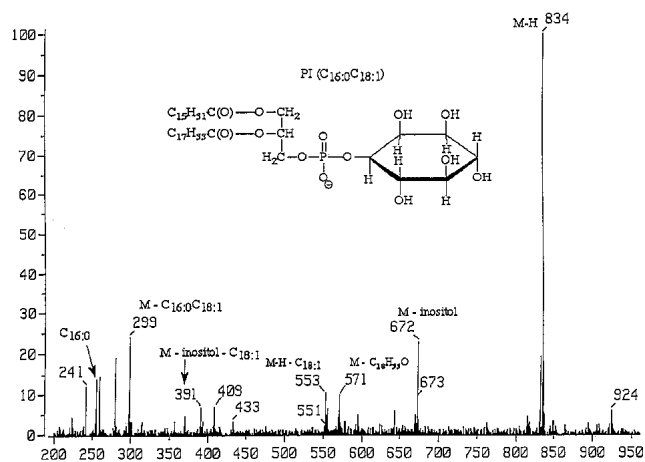


**FIG. 9.** FAB mass spectra of PI molecular species $C_{16:0}C_{18:1}$. See Figures 1 and 3 for abbreviations.

Phospholipids were characterized by MS using a variety of interfaces including particle beam, thermospray, FAB, and electrospray (10–27). With the exception of particle beam, these interfaces work well for the identification of molecular species. Neither particle beam nor electrospray is as sensitive as the FAB approach presented here. The electrospray interface was shown to have superior sensitivity for PE, PC, and phosphatidylserine; however, electrospray does not provide sufficient fragmentation for structural elucidation. FAB, particularly when used in the negative-ion mode, has the advantage of providing definitive structural information without the use of tandem mass spectrometer compared to the other liquid chromatography/MS interfaces. FAB has the disadvantage of significant ion interference from matrix and solvent up to 300 daltons. Both thermospray and electrospray exhibit similar background at low mass. Overall, the flow-FAB technique presented here using a reversed-phase HPLC column and glycerol matrix is both sensitive and specific. The molecular weight, polar headgroup, and fatty acid substituents can
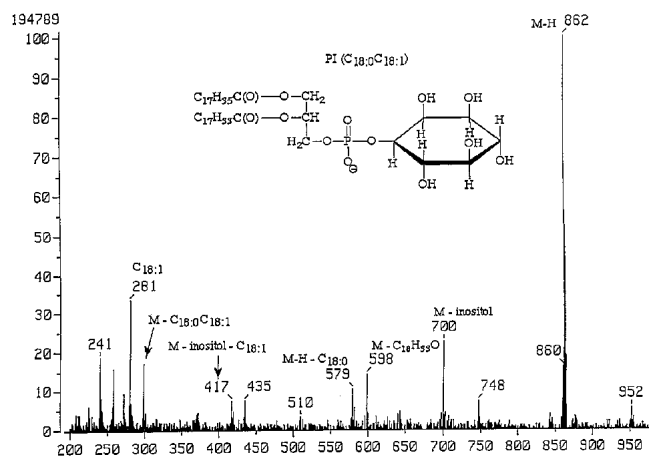


**FIG. 8.** FAB mass spectra of PI molecular species $C_{18:0}C_{18:1}$. See Figures 1 and 3 for abbreviations.
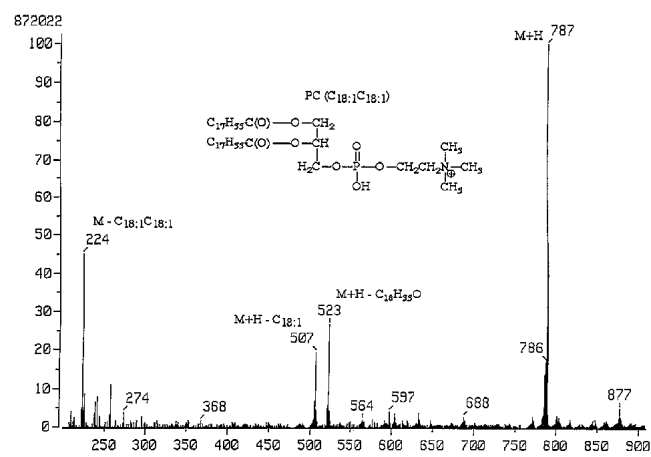


**FIG. 10.** FAB mass spectra of PC molecular species $C_{18:1}C_{18:1}$. See Figures 1 and 3 for abbreviations.
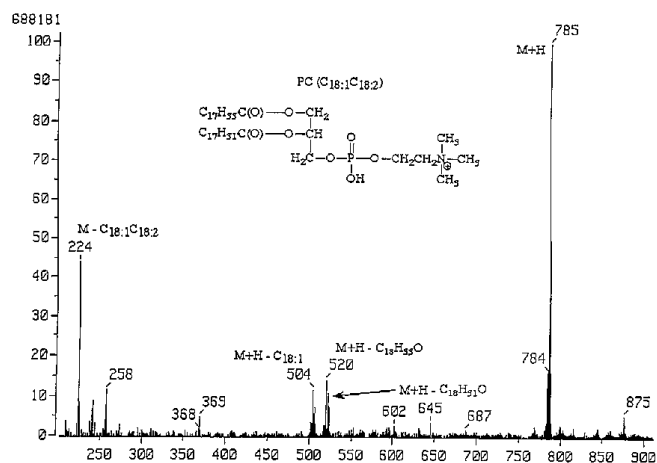
**FIG. 11**. FAB mass spectra of PC molecular species $C_{18:1}C_{18:2}$. See Figures 1 and 3 for abbreviations.
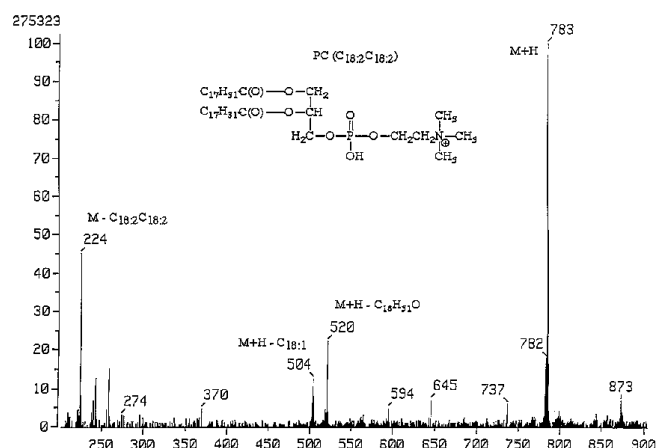


**FIG. 12.** FAB mass spectra of PC molecular species $C_{18:2}C_{18:2}$. See Figures 1 and 3 for abbreviations.

be characterized definitively. This technique, in combination with a silica column class-specific separation, was shown to be applicable to the identification of phospholipids isolated from peanuts.

## ACKNOWLEDGMENT

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