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Review

Separation procedures for phosphatidylserines

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Abstract

This paper reviews working procedures for the separation of phosphatidylserines (PS) in complex sample matrices. It begins with an introductory overview of important aspects of PS involvement in cellular lipid biochemistry. The main body of the review describes various procedures for the extraction, isolation, purification, and separation of the PS class and its molecular species in tissue samples. Published high-performance liquid chromatographic methods are summarized to demonstrate the variability and versatility of separation techniques. Factors influencing normal-phase and reversed-phase separations are delineated. The last section covers selected chemical derivatization procedures useful for enhancing the separation efficiency and detection sensitivity and specificity.

Keywords: Reviews; Phosphatidylserines

Contents

1. Introduction

Phosphatidylserines (PSs), 1,2-diacyl-*sn*-glycerol-3-phospho-L-serine, are ubiquitous substances occurring in small amounts in animal tissues. The compounds consist of molecular species that differ in diacyl fatty acid chain structures (Fig. 1). The PS complex represents a class of the only amino acid

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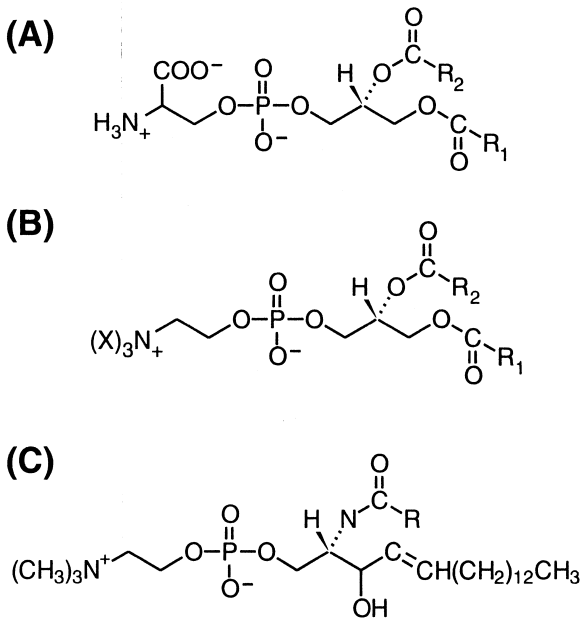

Fig. 1. General structures of nitrogenous GPLs. (A) phosphatidylserine (PS), (B) phosphatidylethanolamine (PE), X=H; phosphatidylcholine (PC), X=CH_3, (C) sphingomyelin (SP). R_1 and R_2 represent respective alkyl and alkenyl groups of the fatty acid chains.

containing glycerophospholipid (GPL) found in mammalian cell membranes. They are important metabolic intermediates [1–4] and are known to be involved in a number of cellular biochemical processes [5–8]. As one of the key constituents in the membrane bilayer, the acidic polar lipid is believed to play a crucial role in the immune response [9–11].

Biosynthesis of PS is generally dependent on cell substrates. In one of the known pathways for GPL metabolism, PS can be formed from serine and cytidine diphosphate (CDP) diacylglyceride in cell tissues [12]. The latter CDP-diglyceride intermediate is derived from phosphatidic acid (PA). Upon enzymatic decarboxylation, PS is transformed to phosphatidylethanolamine (PE) which is then converted to phosphatidylcholine (PC) by reaction with a methyl donor, *S*-adenosylmethionine. Hence, PS not only serves as an essential precursor to the two major polar lipids PE and PC, but also coexists with other GPLs (e.g. phosphatidylinositol (PI), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG) owing to their concurrent formation from the CDP-diacylglyceride intermediate. Alternatively, PS can also be synthesized by an enzymatic exchange reaction in which a base group in a GPL is replaced by serine [13].

Since its first isolation in the brain [14], PS has been the subject of many biochemical and methodological studies. It is known that various PS species are present in most animal tissues (~5–25% of total GPLs) and the distribution of the PS complex and individual molecular species in cellular systems varies among different tissue types. Unlike other GPLs, PS complexes are believed to be devoid of alkylacyl- or alkenylacyl species. To date, only the diacyl compounds have been identified. As the GPLs isolated from brain tissues contain fair amounts (20–25%) of PS, it has not been easy to purify PS from the GPL mixtures. Samples of animal lecithins always contain PS as a minor component or as a contaminant. However, PS is absent in commercial lecithin products obtained from soybeans because of its low abundance in plants. Occasionally, it is necessary to separate PS from the rest of phosphatides in animal lecithins to mimic plant lecithin preparations.

In view of the fact that the carboxyl-containing polar lipid is capable of participating in numerous physicochemical interactions in biological systems, analysis of PS can provide useful information for specific biochemical studies such as blood coagulation processes and protein kinase C activities, and for other general biomedical investigations. Furthermore, evaluation of the polar lipid distributions in tissues is particularly meaningful because chemical and physical properties of cell membranes as well as lipid functions can be affected by variation in PS compositions.

Analogous to the prototype analysis of natural products, isolation of PS compounds from complex tissue matrices entails various chromatographic procedures preceded by elaborate solvent extraction of total lipids. Utilization of an array of separation techniques, i.e. solid–liquid adsorption chromatography, thin-layer chromatography (TLC), and normal-phase high-performance liquid chromatography (HPLC), enables the purification and separation of PS from other GPL classes. Then, subcomponents or molecular species of PS can further be resolved by reversed-phase HPLC. With the advent of modern separation technology in recent years (ca. two de-

cades), simultaneous separations and quantitation of PS can be achieved with a high degree of separation efficiency and detection sensitivity.

A literature search of chromatographic separations of lipids showed that a considerable body of research has been centered around analytical methodologies of GPLs in animals or plants. Nonetheless, owing to its low abundance in nature, published reports on the analysis of PS have been sporadic in contrast to the vast volume of research papers available for the more abundant glycerophosphatides (e.g. PC, PE, and PI). The presence of a carboxyl group and an amino group together with ionic charges in the PS molecule inevitably poses problems in the separation and quantitation of the subject analytes. The difficulties become more serious when dealing with trace levels of PS in samples. This paper follows a comprehensive approach to provide a critical review on separation methodologies for the analysis of PS and molecular species in various sample origins.

2. Isolation techniques

The general procedures for the isolation of PS from cells and tissue materials are virtually the same as for all GPL compounds. In cell membranes, the amino acid containing PS is associated with proteins as a complex characterized by dipole or hydrogen-bonding interactions involving the polar and nonpolar moieties of the lipid–protein complexes. To effectively disrupt the noncovalent bonding requires the use of a combination of polar and nonpolar solvents for complete recovery of the polar lipid from cellular matrices.

2.1. Lipid extraction procedures

2.1.1. Chloroform–methanol–water method [15]

To a suspension of tissue homogenate containing a tissue sample (10 g), chloroform (10 ml), methanol (20 ml), and water (8 ml), an additional portion of chloroform (10 ml) is added. This is followed by blending the mixture for 30 s. Water (10 ml) is then added and the mixture is blended for 30 s. The chloroform–methanol–water solvent ratios for the initial and final stages of homogenation should be maintained at 1:2:0.8 and 2:2:1.8, respectively. After filtration by vacuum suction through a Buchner funnel, the filtrate is collected and the chloroform layer is separated. To ensure quantitative recovery of lipids, the extraction procedure is repeated with the tissue residue left in the filter paper by the addition of a fresh aliquot of the extracting solvent, chloroform (10 ml). A small amount of the same solvent (5 ml) is also used to rinse the residual matters. The chloroform layers are combined and shaken with 10 ml methanol–water (10:9, v/v) to remove nonlipid impurities. Evaporation of the chloroform extract under a stream of nitrogen yields the lipid residue.

2.1.2. Hexane–isopropanol method [16]

This solvent system is less expensive and environmentally more acceptable than the method described above. A mixture of the test tissue (1 g) and a solution of 18 ml of hexane–isopropanol (3:2, v/v) is homogenized for 30 s. After filtration, all vessels including tissue residue are thoroughly rinsed three times with the extraction solvents. The combined filtrated are then stirred for 1 min with a solution of 12 ml of sodium sulfate (1 g) in water (15 ml). The layers are then separated. Removal of solvent from the upper layer gives the crude lipid residue.

2.2. Purification procedures

2.2.1. TLC methods [17]

2.2.1.1. *Analytical-scale two-dimensional TLC.* A sample (up to 2 mg) of total lipids are spotted on a silica gel H plate (20×20 cm, 250 μm) and developed in the first dimension with chloroform–methanol–ammonium hydroxide (65:35:7, v/v). The plate is then developed in the second dimension with acetone–chloroform–methanol–acetic acid–water (48:36:12:12:5, v/v) and the PS spot is visualized and quantitated by spraying with a solution of 6-*p*-toluidine-2-naphthalene sulfonic acid (1 ml) in 50 m*M* Tris–hydrochloric acid [18]. The TLC procedure is repeated for standards on another plate run under the same conditions. Recovery of PS can be done by extraction of the scrapped material that has the R_F value of a PS standard with chloroform–

methanol–water (1:2:0.8). The extract is then shaken with additional portions of chloroform and water to separate the desired polar lipid in the bottom layer. Evaporation of the solvent (chloroform) yields purified PS.

2.2.1.2. *Preparative TLC*. A sample of the lipid extract (5–10 mg) is streaked on a preparative silica gel H plate (10×20 cm, 1000 μm) and developed in single dimension with solvents described above.

2.2.2. Alumina column chromatography method [17,19]

A sample of a lipid extract dissolved in a minimum amount of chloroform–methanol (1:1, v/v) is chromatographed on a neutral alumina column (1 mg lipid phosphorus/1 g alumina). The column is eluted with the solution solvent system until all neutral lipids and PC-type compounds have been removed as indicated by phosphorus and sulfuric acid spot tests. Further elution of the column with ethanol–chloroform–water (5:2:1, v/v) produces a mixture of PE and PS from which the latter can be separated by preparative TLC.

2.2.3. Silica gel column chromatography methods

2.2.3.1. *Solid-phase extraction for total lipid separations* [20]. For small scale isolation of GPLs from lipid extracts, a crude lipid sample (100 mg) is dissolved in a minimal volume of chloroform–methanol (1:1, v/v) and loaded onto a silica gel cartridge. Elution in sequence with chloroform (40 ml), acetone–methanol (160 ml), and chloroform–methanol (80 ml) affords the total GPL fraction in the last eluent. The GPL concentrate is then subjected to class separations by TLC or HPLC.

2.2.3.2. *Silica gel chromatography* [21]. A 10-g silica gel column loaded with a 5-g sample of the lipid extract is eluted sequentially with chloroform (200 ml), acetone (100 ml), methanol (100 ml), and 0.1% phosphoric acid in methanol (100 ml). The methanol fractions (~200 ml) are combined and evaporated to dryness under reduced pressure to leave GPL residues which is redissolved in chloroform. After successive neutralization with bicarbonate solutions and removal of the solvent, the recovered total GPL sample is further separated into individual GPL classes by solid-phase extraction (SPE) or normal-phase HPLC.

2.2.4. Aminopropylsilica solid-phase extraction method for GPL class separations [22–24]

A GPL sample in chloroform (100 μl) is charged onto a SPE 100-mg cartridge preconditioned with hexane (1 ml). The solid-phase is then eluted sequentially with three solvent systems 2.4 ml acetonitrile–*n*-propanol (2:1, v/v), 1.2 ml methanol and 0.9 ml isopropanol–methanolic hydrochloric acid (4:1, v/v). A TLC analysis of the last fraction developed in solvents chloroform–methanol–acetic acid–water (100:75:7:4, v/v) normally reveals only one PS spot with R_F=0.3. The spot can be seen by spraying with a solution (10%) of molybdatophosphoric acid hydrate in ethanol and heated at 120°C for 5 min. However, the procedure is not suitable for the separation of a mixture of PS and PA because they coelute under the conditions described.

3. Normal-phase HPLC separation techniques

Purified GPL isolates can be separated and analyzed for the composition of PS along with other polar lipid classes by analytical-scale normal-phase HPLC. To obtain sufficient amounts of pure PS for subsequent molecular species determinations or biochemical studies, the GPL mixtures are preferably separated by preparative procedures coupled with automatic fraction collection. In general, successful separations of PS from other GPLs depend not only on sample matrices, but also on chromatographic variables such as mobile phases, stationary phases, and elution methods. In the normal-phase mode, the PS analytes interact with polar sites of a stationary phase predominantly via adsorption processes. Therefore, the degree of lipid separations is basically governed by the difference in their ionic and polar characteristics. A survey of the elution methods commonly employed in normal-phase HPLC of PS indicated that gradient elution methods have been more frequently used than isocratic procedures for the separation of multiple-component mixtures.

However, the latter elution methods are meritorious in the operational simplicity and easy control of solvent equilibration. Furthermore, the availability of suitable detectors and mobile phase solvents is often the pragmatic element to be considered prior to the selection of elution methods.

Table 1 summarizes selected literature methods (methods 1–8) [25–31,58] for the isocratic normal-phase HPLC separation of PS in various GPL mixtures. The reported retention times obtained with these methods are within the range 2.9–55 min. With a silica column, a good baseline separation of PS from other GPLs can be achieved using an acetonitrile–methanol–water mobile phase containing a small percentage of acid as in methods 4–6 [27–29]. In this system, the PS peak appeared relatively early on the GPL chromatogram (Fig. 2) in less than 10 min. However, when eluted under similar acidic mobile phase conditions, PS emerged much later from an amino-bonded silica phase (Fig. 3) as the last peak in the GPL profile [30]. Isocratic elution of PS in a hexane–isopropanol–acetate buffer system fails to give a clear separation of PS from PA and PI [31].

For detection of column effluents, three different techniques have been reported for the isocratic normal-phase HPLC separation of PS. Five of the eight methods outlined in Table 1 utilize ultraviolet (UV) spectrophotometric detectors for monitoring analyte elution in mobile phases transparent to UV absorption (methods 27–31). If one elects to use UV absorbing eluents for separations, HPLC of PS must

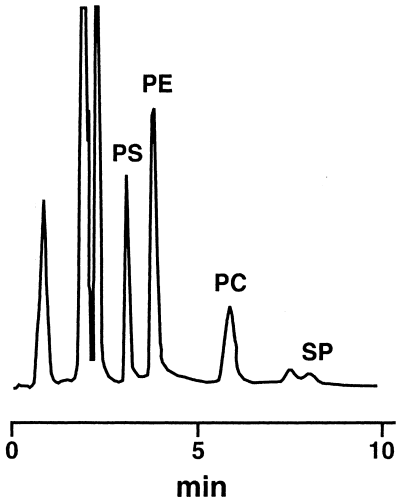

Fig. 2. Normal-phase isocratic HPLC–UV detection of GPLs in human erythrocyte ghost membranes. Column, Ultrasphere Si (250×4.6 mm I.D., 5 μm). Mobile phase, acetonitrile–methanol–85% phosphoric acid (100:10:1.8, v/v). Flow-rate, 1.5 ml/min. UV 203 nm (from [29] with permission).

Table 1
Isocratic normal-phase HPLC methods for the separation of PS

(Method no.) Column	Mobile phase solvent (v/v) Elution order	t_R (min) (detector)	References
(1) Corasil II	Chloroform–methanol–ammonia (50:35.9:7) PE>PS>PI>PC>PA>SP>LPC	6.7 (FI)	[25]
(2) μBondapak-NH_2 10 μm	Chloroform–[methanol–water (25:1)] (20:8) PA>PG>PS>PC>PE	5.0 (FI)	[26]
(3) MicroPak Si-10 10 μm	Acetonitrile–methanol–85% H_3PO_4 (130:5:1.5) PI>PS>PE>PC>LPC>SP	5.8 (UV)	[27]
(4) Ultrasphere-Si 5 μm	Acetonitrile–methanol–H_2SO_4 (100:3:0.05, v/v) PI>PS>PE>PC>LPC>SP	8.12 (UV)	[28]
(5) Ultrasphere-Si 5 μm	Acetonitrile–methanol–85% H_3PO_4 (100:10:1.8) PS>PE>PC>SP	3.11 (UV)	[29]
(6) Nucleosil 5-NH_2 5 μm	Acetonitrile–methanol–water–50% MPA (146:50:3:0.06) PC>SP>LPC>PG>PE>PI>PS	55 (UV)	[30]
(7) Nucleosil 50-5 10 μm	Hexane–isopropanol–acetate buffer (8:8:1) PE>PA>PS>PI>PC	2.9 (UV)	[31]
(8) Partisil PXS 5 μm	Isopropanol–methanol–hexane–0.1 *M* ammonium acetate–acetic acid (348:261:6:6:0.2) PI>PS>PE>PC	3.5 (MS)	[58]

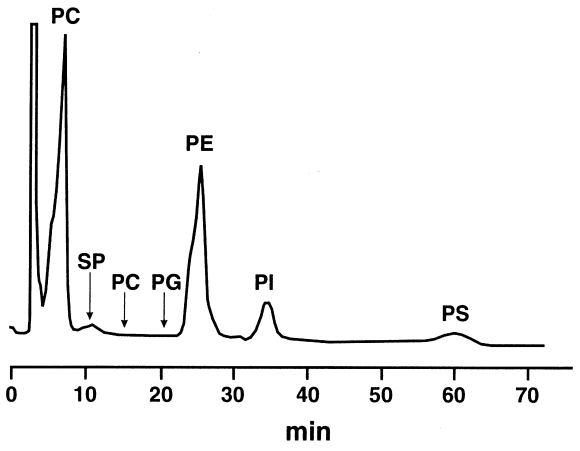

Fig. 3. Normal-phase isocratic HPLC–UV detection of GPLs in porcine liver. Column, Nucleosil 5 NH_2 (175×4.6 mm I.D., 5 μm). Mobile phase, acetonitrile–methanol–water–50% methylphosphonic acid (1460:500:30:0.6, v/v). Flow-rate, 1 ml/min. UV 205 nm (from [30] with permission).

resort to other detectors such as a flame ionization (FI) detector (methods 1 and 2) or a mass spectrometer (MS) (method 8). Examination of the GPL elution sequences shown in the table under methods 3–5 indicates that isocratic elution of a GPL sample on a silica column with a acetonitrile–methanol–water–acid mobile phase led to a common pattern of analyte elution: PI>PS>PE>PC>sphingomyelin (SP). In consideration of the basic structures in the nitrogenous GPL series (PS, PE, PC, SP) (Fig. 1), the presence of a carboxyl functionality in the PS head group appeared to somewhat diminish adsorptive interactions of PS with the silica stationary phase under acidic mobile phase conditions. Under these HPLC conditions, the trimethylamino-containing GPLs (i.e. PC and SP) were apparently more polar than the other two polar lipid classes in the series.

Table 2 summarizes selected literature methods (methods 9–28) [32–49,59,60] for the gradient normal-phase HPLC separation of PS in various lipid mixtures. These HPLC procedures have employed different modes of elution including flow-rate gradients [34], composition gradients [32,33,35,38,40,47,48,59], and solvent gradients [36,37,39,41–46,49,60]. The documented retention times for PS were in the range of 2.5–36 min (Table 2). There are three general types of mobile phase solvent systems recorded in the gradient methods: (1) chloroform–methanol–water/ammonia (seven methods), (2) hexane–isopropanol–water (ten methods), and (3) acetonitrile–methanol–water (two methods). The majority of researchers have preferred the type 2 (hexane–isopropanol–water) mobile phases to other solvent systems for the gradient HPLC separation of PS in GPL samples. The advantages of using type (2) mobile phase solvents lie in the simplified UV detector systems, low solvent volatility, and economical instrumentation.

As gathered from the published information (Table 2), many of the mobile phases used in gradient normal-phase HPLC separations of PS in lipid samples contain acids, bases or phosphate buffers. The mobile phase additives are intended to alleviate peak broadening and peak tailing commonly observed in HPLC of PS and other acidic, negatively-charged GPLs. Generally, with mobile phases of chloroform–methanol–ammonium hydroxide, gradient HPLC of GPL mixtures tends to yield sharper chromatographic peaks and separations of PS from other lipid components (Figs. 4 and 5) are normally more efficient than those obtained with other mobile phase systems listed in Table 2. On the other hand, a good baseline separation of PS from other GPLs in a lipid sample can be achieved by using a mobile phase of hexane–isopropanol–water containing electrolyte additives, but the GPL peaks are broader and the analysis times are usually longer to complete the separation of all GPL classes in the sample (Fig. 6). In routine HPLC work, analysts always face problems associated with disposing an enormous volume of waste solvents. To cope with such solvent predicaments, method 26 [49] demonstrated an example of using a narrow-bore column to reduce solvent consumption for the simultaneous separation and quantitative determination of PS along with six other GPL components (Fig. 5).

Inspection of the gradient normal-phase HPLC methods presented in Table 2 showed that in many instances, lipid scientists used either UV- (nine methods) or evaporative light scattering-detection techniques (ELS) (seven methods) for the quantification of the underivatized PS. Fluorescent derivatives of PS have been analyzed by interfacing a HPLC system with a fluorescence (FL) detector (methods 19, 27). The parent PS can also be separated and

Table 2
Gradient normal-phase HPLC methods for the separation of PS

(Method No.) Column	Mobile phase solvent (v/v) Elution order	t_R (min) (detector)	References
(9) LiChrosorb Si-60 10 μm	Hexane–isopropanol–water (6:8:0.75)→(6:8:1.4) PA>PE>LPE>PI>PS>PC>LPC	25 (UV)	[32]
(10) MicroPak Si-5 5 μm	Hexane–isopropanol–water–H_2SO_4 (97:3:0:0.02)→ (75:24:0.9:0.1) PA>DPG>PG>PS>PI>PE>LPE>PC>LPC	36 (UV)	[33]
(11) LiChrospher Si-100 10 μm	Hexane–isopropanol–25 m*M* phosphate–ethanol–acetic acid (367:490:62:100:0.6, v/v), 0.5→1 ml/min PE>PA>PI>PS>PC>LPC	34 (UV)	[34]
(12) LiChrosorb Si-100 5 μm	Hexane–isopropanol–water (60:80:7)→(60:80:14) PE>PI>PS>PA>PC	27.5 (UV)	[35]
(13) Bio-Sil HP 10 10 μm	Acetonitrile→acetonitrile–water (80:20) PA>PG>PI>PE>PS>PC>LPC	22 (UV)	[36]
(14) Spherisorb Si 3 μm (C)	(A) Hexane–THF (99:1) (B) chloroform–isopropanol (1:4) isopropanol–water (1:1); (A)→[(A)–(B)](4:1)→ [(A)–(B)–(C)] (42:52:6)→[(A)–(B)–(C)] (32:52:16) DPG>PE>PI>PS>PC>SP	15 (ELS)	[37]
(15) LiChrosorb Si-60 5 μm	Hexane–isopropanol–water (54:41:5)→(52:32:9) DPG>PE>PI>PS>PC>SP>LPC	17 (UV)	[38]
(16) Zorbax Sil 5 μm	Hexane–isopropanol (3:2)→ Hexane–isopropanol–water (283.5:189:27.5) PE>DPG>PI>LPE>PS>PC	21 (UV)	[39]
(17) LiChrosorb Si-60 7 μm	Hexane–isopropanol–water (6:8:0.75)→(6:8:1.55) PE>PI>PS>PC>SP	27 (UV)	[40]
(18) LiChrosorb Si 10 μm	Chloroform→chloroform–methnaol–ammonia (1:92:7) DPG>PA>PG>PE>PI>PC>SP>PS>LPC	23 (ELS)	[41]
(19) RadialPak Si 10 μm	Isooctane–tetrahydrofuran (99:1)→isopropanol–dichloromethane (4:1)→isopropanol–water (1:1) PG>PE>PA>PS>PC>LPC	30 (FL) (PRD-PS)	[42]
(20) Nucleosil-100 3 μm	Chloroform–trifluoroacetic acid (400:5)→chloroform–methanol–heptane–water–trifluoroacetic acid (100:400:50:15:5) PG>PI>PS>PE>PC	20 (ELS)	[43]
(21) LiChrocard Si-60 5 μm	Acetonitrile→acetonitrile–85% H_3PO_4 (99.8:0.2)→ methanol–85% H_3PO_4 (99.8:0.2) DPG>PI>PS>PE>LPE>PC>SP	28 (UV)	[44]
(22) LiChrospher Si-60 5 μm	Chloroform–methanol–30% ammonia (80:19.5:0.5)→ Chloroform–methanol–water–30% ammonia (60:34:5.5:0.5) PE>PI>PS>PC>PA>SP>LPC	11 (ELS)	[45]
(23) LiChrosorb Si 5 μm	Chloroform–tetrahydrofuran (1:1)→chloroform–methanol–$N(Et)_3$ (4:92:4) PE>PI>PS>PA>PC	27 (ELS)	[46]
(24) Spherisorb 3 μm	Hexane–isopropanol–water (2:40:58)→(8:40:52) PE>PS>PC>SP	9 (ELS)	[47]
(25) LiChrosphere-Si 5 μm	Hexane–methanol–0.5% ammonia (0:88:12)→(12:88:0) PS>PI>PA>PE>PC	2.5 (MS) 48	
(26) Inertsil Si 5 μm	(A) chloroform–methanol (8:2),(B) chloroform–methanol–water–20% ammonia (60:34:6:0.25);(B)→(A)→(B) PI>PE>PS>PC>SP>LPC	10 (ELS)	[49]
(27) Mikro-Pak Si 10 μm	Dichloromethane–methanol–ammonia (91:9:1)→(70:20:5) PE>LPE>PS>LPS	25 (FL) DN-PS	[59]
(28) Spheri-Si 5 μm	(A) Dichloromethane–methanol–water (93:6.5:0.5), (B) Dichloromethane–methanol–ammonia (65:31:4:0.2); A–B(88:12)→A–B(55:45)→B PE>PC>PI>SP>PS	15 (MS)	[60]

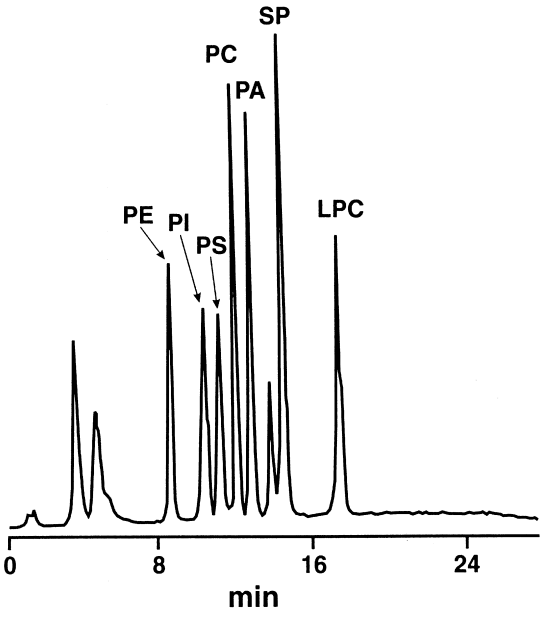

Fig. 4. Normal-phase gradient HPLC–ELS detection of GPL standards. Column, LiChrospher Si 60 (125×4.6 mm I.D., 5 μm). Mobile phase, solvent A=chloroform–methanol–30% ammonium hydroxide (80:19.5:0.5, v/v), solvent B=chloroform–methanol–water–30% ammonium hydroxide (60:34:5.5:0.5, v/v); gradient, A→B→B→A→A (14→7→8→5 min). Flow-rate, 1 ml/min (from [45] with permission).

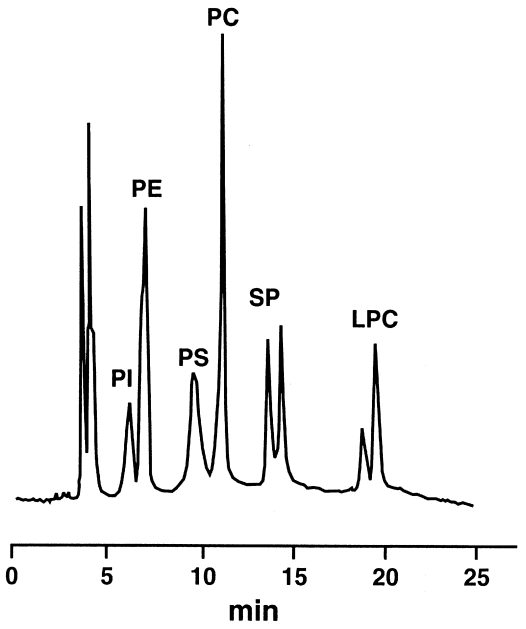

Fig. 5. Normal-phase gradient HPLC–ELS detection of GPL standards. Column, Inertsil Si (250×2.1 mm I.D., 5 μm). Mobile phase, solvent A=chloroform–methanol (8:2, v/v), solvent B=chloroform–methanol–water–20% ammonium hydroxide (60:34:6:0.25, v/v); gradient, B→B→A→A→B (12→5→8→10 min). Flow-rate, 0.22 ml/min (from [49] with permission).

detected by an on-line LC–MS technique (methods 25 and 28) based on the reported observation of characteristic head group ions in MS spectra of GPLs [48,58]. Although the LC–MS procedures involve relatively sophisticated and expensive instruments, qualitative and quantitative assays including structural analyses of PS in GPL mixture can be achieved with high reliability and sensitivity. With the advent of the ELS detection technology, limitations of UV detection methods to only mobile phase solvents void of UV chromophores can be compensated by applying the HPLC–ELS detection techniques for PS analyses in many useful organic solvents. Unfortunately, it is well recognized that HPLC–ELS detection systems are not compatible with mobile phases containing nonvolatile buffer salts. The HPLC–UV detection method in conjunction with a chemical derivatization procedure often provides greater detection sensitivity than the ELS-detection method.

The HPLC data in Table 2 show that the elution sequence observed in gradient normal-phase HPLC of PS in GPL samples was dramatically affected by the variation in mobile phases and stationary phases

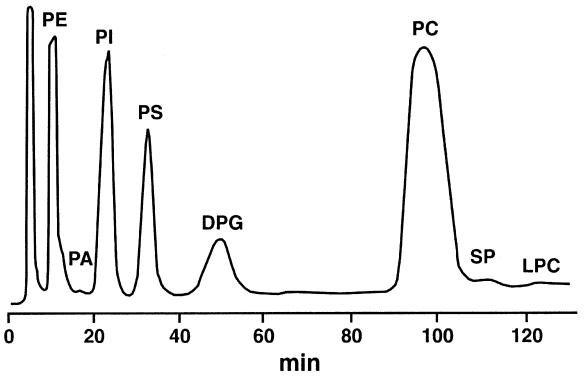

Fig. 6. Normal-phase gradient HPLC–UV detection of GPLs in rat liver. Column, LiChrospher Si-100 (250×4.6 mm I.D., 10 μm). Mobile phase, hexane–isopropanol–25 m*M* phosphate buffer–ethanol–acetic acid (367:490:62:100:0.6, v/v), gradient, flow-rate 0.5→0.5→1.0→1 ml/min (60→0→70 min), UV 205 nm (from [34] with permission).

employed. When chloroform in a mobile phase of chloroform–methanol–ammonia was replaced with hexane, elution of PS along with other GPLs on silica was changed from relatively strong adsorption on the stationary phase to little retention by the column (methods 18 vs. 25). Except for methods 10 and 25, HPLC results from all other reported methods in Table 2 indicated that, irrespective of the mobile phase pH value, PS had a longer retention time than PI under gradient HPLC conditions despite the obvious polar inositol moiety in the PI molecule. The acidic and basic mobile phases used in respective methods 10 and 25 seemed to have little influence on the elution order PS>PI (i.e. PS eluted ahead of PI). Normal-phase gradient HPLC with very similar chloroform–methanol–ammonia mobile phases but different solvent gradients (methods 22 vs. 26) produced two different elution patterns for the trio PE, PI and PS. This disparity in elution behavior of the GPLs of interest might be, in addition to the differential gradient consequence, attributed to the size effect of silica columns of different dimensions (Fig. 4 vs. Fig. 5). Further, under the mobile phase conditions specified in method 23, separations of PS from PI were found very sensitive to gradient variations [46].

Interestingly, regardless of the mode of elution (isocratic or gradient), HPLC in an acetonitrile–methanol–water–acid system has been shown to give the same elution order PI>PS>PE>PC>SP (methods 3–5, Table 1 vs. method 21, Table 2). As depicted in Fig. 1, PS is structurally related to PE by the extra carboxyl group that substitutes a hydrogen atom of the α-carbon of the PE molecule, it is worthwhile to probe the elution order of PS in relation to PE. Thus, in an acidic mobile phase, the acidic PS has been noted to elute earlier from a silica column than PE as expected based on its weaker adsorption on the silica phase in the acid mobile phase medium. Conversely, HPLC of PS in a neutral or a basic mobile phase tended to yield a elution pattern in which PS emerged from a column later than PE. An exception to this generalization can be seen in method 25, Table 2, where a rather unusual combination of solvents (hexane–methanol–ammonia) was used.

With regard to the selection of stationary phases, investigators have generally used various commercial silica columns for the normal-phase HPLC separation of PS from other GPLs in lipid mixtures mainly derived from animal sources (Tables 1 and 2). In rare cases, alkylamino-bonded silica columns (methods 2 and 6, Table 1) have also been used for the isocratic normal-phase HPLC analysis of PS in GPL samples. In a typical analysis of a lipid sample containing PS, HPLC with a deactivated silica-based column of small particle size (3–5 μm) would result in efficient separations of analyte components with desirable peak characteristics. In light of the continuous demand for the new stationary phases to keep abreast with the advancement of HPLC methodology, it is highly plausible in the near future to perform rapid routine analyses of PS in complex sample matrices by normal-phase HPLC with high detection sensitivity and separation selectivity.

4. Reversed-phase HPLC separation techniques

As shown in Fig. 1, the structure of PS contains two diacyl tail groups derived from variable fatty acid chains. Consequently, there are a number of molecular species present as subclass components in a PS class. Since the PS molecular species differ principally in the carbon-chain length of diacyl groups, reversed-phase HPLC techniques have been utilized for their separations and quantification. Table 3 compiles selected literature methods (methods 29 to 35) [34,50–57] for the reversed-phase HPLC separation of PS molecular species in a variety of animal tissue samples. All but two investigators chose an Ultrasphere octadecylsilica (ODS) column for the speciation of PS species for its relatively high efficiency and stability in buffered mobile phases. It is a common practice in reversed-phase HPLC to select stationary phases of good surface coverage and endcapping for improving the separation of ionic compounds. Also, in principle, highly deactivated alkyl-bonded silica columns with no exposure of the silica surface are recommended to use with mobile phases containing counter-ion electrolytes for reversed-phase ion-pair HPLC separations of charged analytes.

An example of the application of a reversed-phase ion-pair HPLC technique to the separation of PS

Table 3
Reversed-phase HPLC methods for the separation of PS molecular species

(Method no.) Column	Mobile phase solvent (elution mode)	Detector	References
(29) Ultrasphere ODS 5 μm	Methanol–water–30 m*M* phosphate–acetonitrile–acetic acid (90.5:7:2.5:0.8) containing 30 m*M* choline chloride (isocratic)	UV	[34]
(30) Ultrasphere ODS 3 μm	Methanol–hexane–0.1 *M* ammonium acetate (50:3:2.5) (isocratic)	MS	[50]
(31) Axxi-chrom ODS 5 μm	Methanol–water–10 m*M* ammonium acetate (84:16)→(87:13) (gradient)	UV (TNP-PS)	[51,52]
(32) Ultrasphere ODS 5 μm	Acetonitrile–isopropanol (8:2) (isocratic)	UV (DNB-PS)	[53]
(33) NovaPak C_{18} 4 μm	Acetonitrile–methanol–5 m*M* TEAP (70:22:8) (isocratic)	UV	[54]
(34) Ultrasphere ODS 5 μm	(A) Water–sodium acetate–tetrahydrofuran (95.5:0.5) (B) 100 m*M* sodium acetate–acetonitrile (2:8); A→A–B (7:3)→A–B (1:1) (gradient)	FL (PHA-PS)	[55,56]
(35) Ultrasphere ODS 5 μm	(A) Water–10 m*M* ammonium acetate (B) acetonitrile–methanol (1:1); A–B (8:92)→(6:94)→(3:97) (gradient)	UV (TNP-PE)	[57]

molecular species is shown in Table 3 (method 33 [54]). In this method, tetraethylammonium phosphate (TEAP) dissolved in a mobile phase of acetonitrile–methanol–water served as an ion pairing reagent for the negatively charged PS species. Without the addition of TEAP to the mobile phase, the polar PS species passed through an ODS column with no retention on the stationary phase nor the separation of its subcomponents as demonstrated in Fig. 7A. Quite often, some ill-defined peaks appeared in HPLC with mobile phases of high water content in the absence of any ion pairing reagent. Upon incorporation of the positively charged TEAP counter-ion into the mobile phase, distinct separations of molecular species were attained (Fig. 7B). Under the reversed-phase ion pair HPLC conditions employed, retention times of PS analytes increased with an increase in the concentration and hydrocarbon chain length of the quaternary alkyl ammonium phosphate (QAAP) counter-ion used [54,61,62]. Linear correlation between capacity factors (k') and the total number of QAAP provides a unique means to predict the type of unknown QAAP or unknown k' values of analyte species. An important benefit of ion-pairing techniques is that it is possible to manipulate the QAP concentration and the size of its alkyl groups to bring about reasonable separations within optimal retention times. The observed counter-ion effects on the analyte retention behavior were illustrative of an ion-pair retention mechanism via which analyte

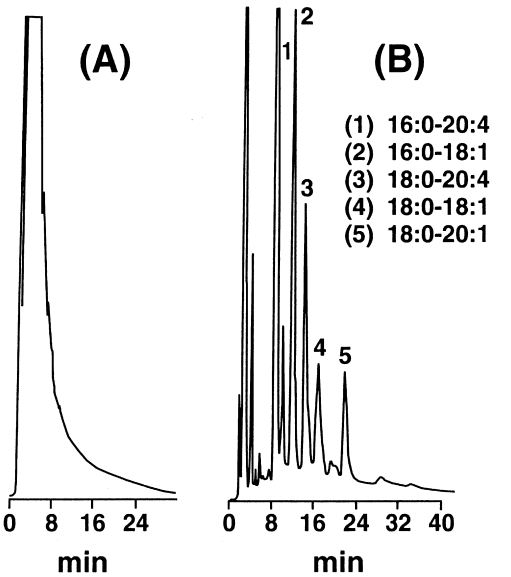

Fig. 7. Reversed-phase isocratic HPLC–UV detection of PS molecular species in bovine brain. Column, NovaPak C_{18} (300× 3.9 mm I.D., 4 μm). Mobile phase, acetonitrile–methanol–water (70:22:8, v/v) containing 5 m*M* TEAP at pH 7. Flow-rate, 1 ml/min. UV 208 nm (from [54] with permission).

solutes interacted with both stationary and mobile phase during chromatographic separation processes. As result, the apparent reduction in ionic character of PS analytes enhanced solvophobic interactions to produce well-dispersed PS subcomponent peaks without peak tailing (Fig. 7B).

Unlike the situation in normal-phase HPLC of GPL class separations where gradient elution methods are prevalent, lipid researchers have employed both isocratic (four methods) and gradient elution methods (three methods) for the separation of PS molecular species (Table 3). It is logical to conduct the reversed-phase HPLC analysis under gradient elution if the PS complex comprises molecular species having a wide range of diacyl chains. Selected mobile phases used in reversed-phase separations of PS molecular species can be categorized into three types: (1) methanol–water (three methods), (2) acetonitrile–alcohol–water (two methods) and (3) acetonitrile–water (one method). In analyses of underivatized intact molecular species of PS, it was mandatory for investigators to add electrolyte buffers (phosphates or acetates) to each of the above three types of mobile phases as shown in Table 3 (method 29 to 31, 33 to 35). Evidently, separations of PS molecular species can also be effected by using a buffered mobile phase (e.g. ammonium acetate) other than conventional ion-pairing reagents [63]. The observed PS retention behavior in electrolyte media might be rationalized in terms of the analyte participation in ion interactions, ion-exchanges, or ion-pairing with ODS in the electrolyte-modified mobile phase [63,64].

For detection of intact PS molecular species in low UV absorbing mobile phases, many investigators have opted for reversed-phase HPLC–UV detection techniques with the detector set at a wavelength ranging from 205 to 210 nm (Table 3). On the other hand, derivatized PS molecular species have been separated and detected at the λ_{max} of analyte species containing UV chromophores (methods 31, 32, 34, 35) (Fig. 8). Using a solvent system (method 30) compatible with a mass-specific detector, Kim's group separated and analyzed bovine brain samples for PS molecular species by reversed-phase HPLC–thermospray MS [50]. As shown in Fig. 9, six molecular species of the bovine brain PS were identified and quantitated by selected ion monitoring

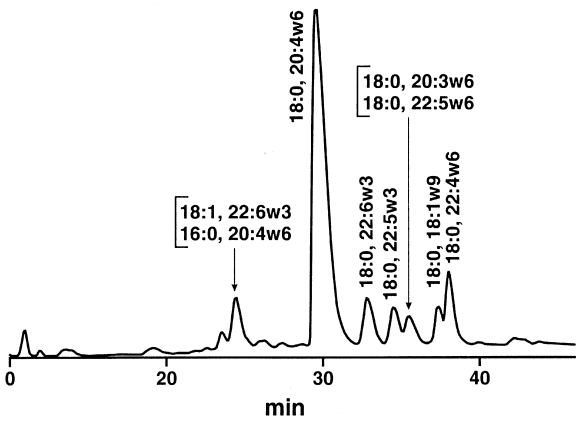

Fig. 8. Reversed-phase gradient HPLC–UV detection of human red blood cell PS molecular species as trinitrophenyl (TNP) derivatives. Column, Axxi-chrom ODS (250×4.6 mm I.D., 5 μm). Mobile phase, solvent A=methanol–10 m*M* ammonium acetate (84:16, v/v), solvent B=methanol–10 m*M* ammonium acetate (87:13, v/v); gradient, A→A→B→B (5→15→40 min). Flow-rate, 1 ml/min. UV 342 nm (from [52] with permission).

of their diglyceryl fragment ions (base peaks) and calibration with synthetic standards. In addition, reversed-phase separations of fluorescent PS molecular species have been monitored by HPLC–FL detection (method 34, Table 3).

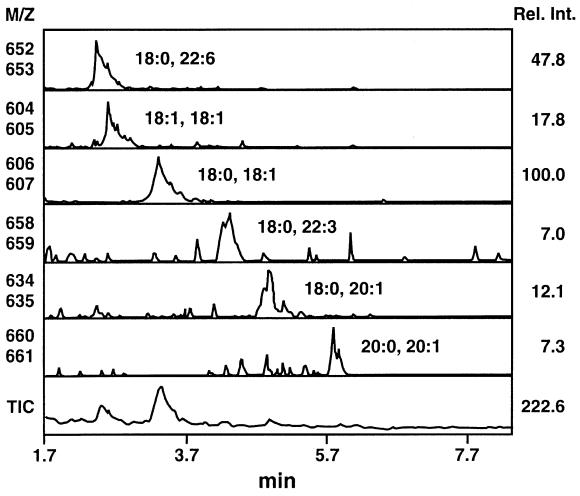

Fig. 9. Reversed-phase isocratic HPLC–thermospray MS detection of PS molecular species in bovine brain. Column, Ultrasphere-ODS (75×4.6 mm I.D., 3 μm). Mobile phase, methanol–hexane–water–0.1 *M* ammonium acetate (50:3:2.5, v/v). Flow-rate, 1 ml/min (from [50] with permission).

5. Separations of PS derivatives

In order to minimize the adverse effects of the polar amino- and carboxyl-moieties (Fig. 1) on PS separations, it is prudent to modify the PS molecular structure with a chromophore or a fluorophore to form a less polar derivative. HPLC of PS derivatives can afford not only superior separation characteristics but also enhanced detection sensitivity. Hence, modifications of both analyte structures and mobile phases are the critical factors affecting PS separations. In a recent reversed-phase HPLC paper [54], the author noted that in the absence of any electrolyte in a mobile phase, solvophobic interactions between an alkylsilica phase and the polar head group of PS solutes were apparently weaker than those between solvents and silanol sites causing little analyte retention (Fig. 7A). Since PS is present in small amounts in most animal tissues, separations and detection of the polar lipid at trace levels in complex sample matrices can be a formidable task. Therefore, chemical derivatization techniques are indispensable assay procedures for the normal- (method 19) and reversed-phase (methods 31, 32, 34, 35) separation and quantification of PS compounds.

A few published derivatization techniques (Fig. 10) for the analysis of PS are described as follows: (1) a pyrene derivative [42] is prepared by reacting lyso-PC with 1-pyrenyl dodecanoyl (PRD) anhydride in the presence of pyrrolidinopyridine to form PC labeled at the 2-position with the pyrene fluorophore. The fluorescent PC is then converted to the corresponding PRD-PS via transphosphatidylation with serine catalyzed by phospholipase D; (2) a densyl derivative [59] is prepared (Scheme 1) by treating the parent PS in triethylamine (Et_3N) with 1-dimethylaminonaphthalene-5-sulfonyl (DAN) chloride to give DAN-PS; (3) a nitrophenyl derivative [51,52] is prepared (Scheme 2) by incubating the parent PS with a fresh solution of 2,4,6-trinitrobenzenesulfonic acid (TNBS) in an aqueous medium of sodium bicarbonate, sodium chloride and glucose at 0°C to give the trinitrophenyl (TNP) derivative of PS, TNP-PS; (4) a dinitrobenzoyl derivative [53] originated from PS is prepared (Scheme 3) by hydrolyzing the parent PS with a mixture of phospholipase C, ethyl ether and sodium borate buffer to give the diacylglycerol (DAG) product, which is then treated with 3,5-dinitrobenzoyl (DNB) chloride in pyridine to yield the dinitrobenzoate, DNB-DAG; (5) a phthalate derivative [55,56] is prepared (Scheme 4) by treating the parent PS with an *o*-phthalatealdehyde (OPA) and an organic thiol in a solution of borate buffer to give the isoindole (IID) product,

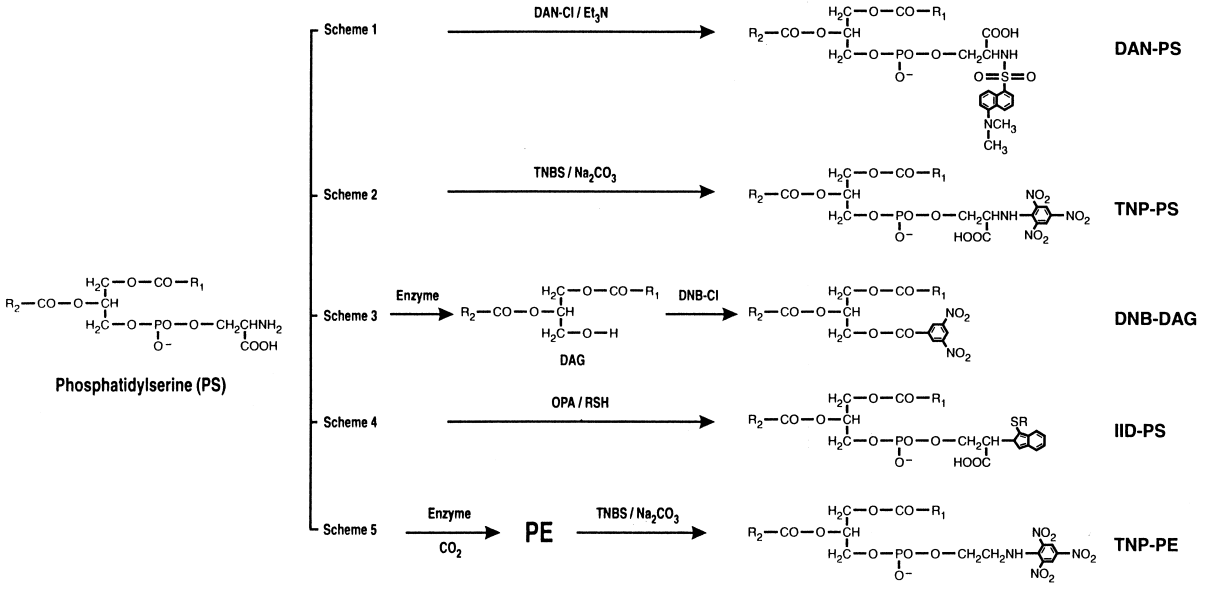

Fig. 10. Conversion of PS to its derivatives.

IID-PS; (6) a fluorescent PE derivative [57] is prepared (Scheme 5) by initial decarboxylation of PS with a purified mitochondrial fraction [65] and then with mitochondrial protein to yield PE, which is then converted to TNP-PE as shown in Scheme 2.

Cursory scrutiny of the HPLC data in Tables 2 and 3 revealed that partial modification of the PS head group at either the amino or carboxyl functionality via chemical derivatization appeared to be inadequate for PS separations unless electrolyte buffers are added to mobile phase solvents (methods 27, 31, 34, 35). The author has found that addition of triethylamine to a buffered mobile phase system significantly reduces peak tailing phenomena and improves the reversed-phase HPLC separation of DAN-PS [63]. In a unique circumstance where a large hydrocarbonaceous pyrenyl fluorophore was introduced into the PS molecule as illustrated in method 19 (Table 2), distinct separations of GPL classes with decent peak characteristics were obtained without the use of a mobile phase buffer. Another example of achieving separations with straight solvents was reported by M.V. Bell et al., [53]. They literally removed the polar head group of PS by molecular destruction and successfully separated PS molecular species as the DNB derivatives even though no electrolyte was present in the mobile phase (method 32).

Excluding the PS carboxyl group, the remainder of PS has the same basic molecular structure as PE (Fig. 1). Thereby, it is of interest to compare the reversed-phase separation behavior of DAN-PS with that of DAN-PE. Upon dansylation, the ionic structure of PE is changed from neutrally-charged to negatively-charged species, while the negative charged PS acquires an additional negative charge. Consequently, the change in ionic characteristics of PE and PS has dramatic influence on the chromatographic outcome of the polar lipids in mobile phases with added electrolytes. Reversed-phase HPLC separations of the dansylated GPL duo has recently been investigated [63,66]. In these studies, it was found that better ion-pair HPLC separations were obtained with DAN-PE molecular species than with the underivatized analyte species. Conversely, reversed-phase ion-pair HPLC of the parent PS seemed to give superior component resolution than DAN-PS. In HPLC of the latter compounds (DAN-PS) that possess double negative charges as in PA, mobile phases of high ionic strength with high counter-ion concentrations are required to effect reasonable separations [67].

6. Other separation techniques

Molecular species of PS have been separated and identified by direct probe positive or negative ion fast atom bombardment MS using a surface precipitation technique [68,69]. The negative ion MS method is particularly useful to distinguish isomers and minor species [69]. Most recently, two groups of investigators have taken new approaches [31,70] for the separation of GPLs. They succeeded in the simultaneous separation and quantitation of PS along with other GPLs in lecithin samples by high-performance capillary electrophoresis or micellar electrokinetic capillary chromatography (MEKC). The MEKC method allowed rapid analyses of PS with high peak capacity and required minimal solvent and reagent consumption.

List of abbreviations

CDP	Cytidine diphosphate
DAN	5-Dimethylamino-naphthalene-sulfonyl
DAG	Diacylglycerol
DPG	Diphosphatidylglycerol
DNB	3,5-Dinitrobenzoyl
ELS	Evaporative light scattering
FI	Flame ionization
FL	Fluorescence
GPL	Glycerophospholipid
IID	Isoindole
LPC	Lyso-PC
LPE	Lyso-PE
MEKC	Micellar electrokinetic capillary chromatography
MS	Mass spectrometry
MPA	Methylphosphonic acid
ODS	Octadecylsilica
OPA	*o*-Phthalatealdehyde
PA	Phosphatidic acid

PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
PRD	1-Pyrenyl dodecanoyl
QAAP	Quaternary alkyl ammonium phosphate
RI	Refractive index
SP	Sphingomyelin
TEAP	Tetraethyl ammonium phosphate
TNBS	Trinitrobenzenesulfonic acid
TNP	Trinitrophenyl

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