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High-performance liquid chromatographic separation of molecular species of neutral phospholipids

S. L. Abidi* and T. L. Mounts

Food Quality and Safety Research, National Center for Agricultural Utilization Research, Agricultural Research Service, US Department of Agriculture, 1815 N. University Street, Peoria, IL 61604 (USA)

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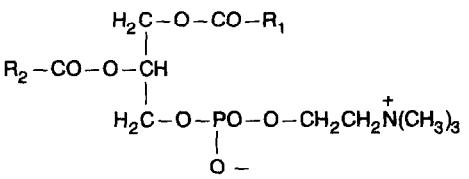
ABSTRACT

Molecular species of neutral phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were resolved by reversed-phase high-performance liquid chromatography (HPLC) using mobile phases of acetonitrile–methanol–water containing tetraalkylammonium phosphates (TAAPs). Competitive interactions of TAAPs and analyte solutes with a reversed-phase HPLC column resulted in reduced retention of PC or PE with concomitant increase in detection sensitivity. The chromatographic data for PC and PE were distinctly different from those for negatively charged phospholipids where ion-pair retention mechanisms prevailed. While PC (or PE) components eluted at longer retention times with a larger size of TAAP, an increase in the TAAP concentration invariably caused a decrease in phospholipid retention times. Optimization of HPLC conditions by using high concentrations (25–100 m*M*) of tetramethylammonium phosphate in acetonitrile–methanol–water (70:22:8) facilitated elution of components with improved peak symmetry. HPLC separations of neutral phospholipids derived from animal sources were more complex than those from soybeans.

INTRODUCTION

During the course of another study on the analysis of phospholipids (PLs) in crude and degummed soybean oil, it was necessary to monitor the change in molecular species distributions of individual PL classes in the oil samples stored for various periods of time [1,2]. The four major PLs found in soybeans are phosphatidic acids (PAs), phosphatidylcholines (PCs), phosphatidylethanolamines (PEs) and phosphatidylinositols (PIs). Of these, PAs and PIs are negatively charged compounds, whereas PCs and PEs are internally neutralized molecules (Fig. 1). Although many chromatographic methods for analysis of PLs are known in the literature [3–10], reliable analytical methods for the quantification of the two ionic type of intact PL molecules have not been clearly defined. Only recently have high-performance liquid chromatographic (HPLC) methods been reported specifically for the separation of negatively charged PAs and PIs [11,12]. Subcomponents of the two classes of polar lipids can be separated only by reversed-phase ion-pair HPLC. Using the ion-pairing technique, HPLC of the phos-



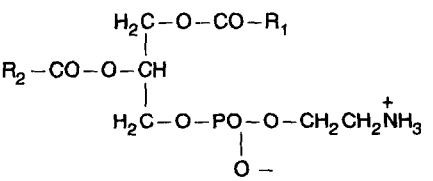


Fig. 1. Structures of phosphatidylcholine and phosphatidylethanolamine. R_1 and R_2 represent alkyl or alkenyl groups of fatty acids.

phatides leads to well-resolved molecular species with little structural disruption (especially at the glyceroyl–phosphoryl junction). On the other hand, conventional general methods [13–17] of PL analysis require conversion of PIs to derivatives of diglycerides by the removal of the phosphoryl moiety and PAs to dimethyl esters [18] prior to separation by HPLC.

In an earlier attempt to separate molecular species of PCs and PEs isolated from crude and degummed soybean oil according to a published procedure [19], it was difficult to obtain a good chromatogram for the later-eluting components due to severe peak broadening and tailing. These peaks of high hydrobicity often emerged from a HPLC column at unreasonably long retention times. In view of the success of separating negatively charged PAs and PIs by reversed-phase ion-pair HPLC, the same chromatographic methodology was applied to the analysis of samples of PCs and PEs to examine the effect of commercial ion-pairing reagents on the separation of their molecular species. Preliminary results showed that PCs and PEs in the presence of a tetraalkylammonium phosphate were less retained and had better shaped peaks than in the absence of the ammonium salt in the mobile phase. More importantly, a significant improvement in detector response was observed. Such distinct differences in the chromatographic behavior between the neutral and charged PLs are indicative of diverse retention mechanisms involved in the HPLC separation processes. The retention of neutral PLs via ion interactions with mobile phase electrolytes has not been thoroughly investigated previously. This paper reports the results of a comprehensive study of HPLC separation of molecular species of PCs and PEs under various mobile phase electrolyte conditions to demonstrate the analytical utility of the methods in practical application.

EXPERIMENTAL

Materials

PCs and PEs were purchased either from Avanti Polar Lipids (Pelham, AL, USA) or Sigma (St. Louis, MO, USA). Other PLs were obtained from Avanti. Tetramethylammonium phosphate (TMAP) and tetraethylammonium phosphate (TEAP) were prepared by treating corresponding tetraalkylammonium hydroxide (Aldrich, Milwaukee, WI, USA) with phosphoric acid (Fisher, Fair Lawn, NJ, USA) until pH 7. Tetrabutylammonium phosphate (TBAP), pentyltriethylammonium phosphate (PTAP), heptyltriethylammonium phosphate (HPTAP) and sodium pentanesulfonate (SPS) were purchased from Regis (Morton Grove, IL, USA). Tetramethylammonium sulfate (TMAS) was the high-purity product of Aldrich. HPLC-grade acetonitrile and methanol were obtained from J. T. Baker, (Phillipsburg, NJ, USA). Ultrapure water for HPLC was obtained by filtering distilled water through a Millipore (Bedford, MA) Milli-Q water purifier.

High performance liquid chromatography

All HPLC experiments were performed with a Spectra-Physics (San Jose, CA, USA) Model SP8700 liquid chromatograph interfaced to a variable-wavelength UV detector (SpectroMonitor D:LDC, Riviera Beach, FL, USA). HPLC column effluents were monitored at 208 nm. Mobile phases were prepared by adding measured amounts of tetraalkylammonium phosphates to various proportions of acetonitrile–methanol–water. These solutions were filtered, degassed and pumped through an analytical column at a flow-rate of 1–2 ml/min. Aliquots (5–10 μl) of analytical samples (10–20 mg/ml) were injected via a Rheodyne (Cotati, CA, USA) Model 7125 injector (10-μl loop) onto a reversed-phase HPLC column. All samples were freshly prepared prior to analysis. Several reversed-phase columns were used: (i) NovaPak C_{18}, 300 × 3.9 mm I.D., 4 μm (Waters Assoc., Milford, MA, USA), (ii) polymeric resins of macroporous polystyrene–divinylbenzene (MPD), PLRP-S-100, 250 × 4.6 mm I.D., 5 μm (Polymer Labs., Amherst, MA, USA), (iii) Brownlee Speri-5 RP-8, 220 × 4 mm I.D., 5 μm, (Applied Biosystems, Foster City, CA, USA), (iv) Waters Resolve C_{18}, 150 × 3.9 mm I.D., 5 μm, and (v) Alltech (Deerfield, IL, USA) Adsorbosphere HS C_{18}, 250 × 4.6 mm I.D., 5 μm.

HPLC peaks were collected and isolated molecular species were converted to fatty acid methyl esters by a known HCl–methanol procedure [20]. Fatty acid composition was determined using a Varian Model 3400 gas chromatograph equipped with a 0.25 mm × 30 m fused-silica capillary column coated with 0.2 μm SP 2330 (Supelco, Bellefonte, PA,

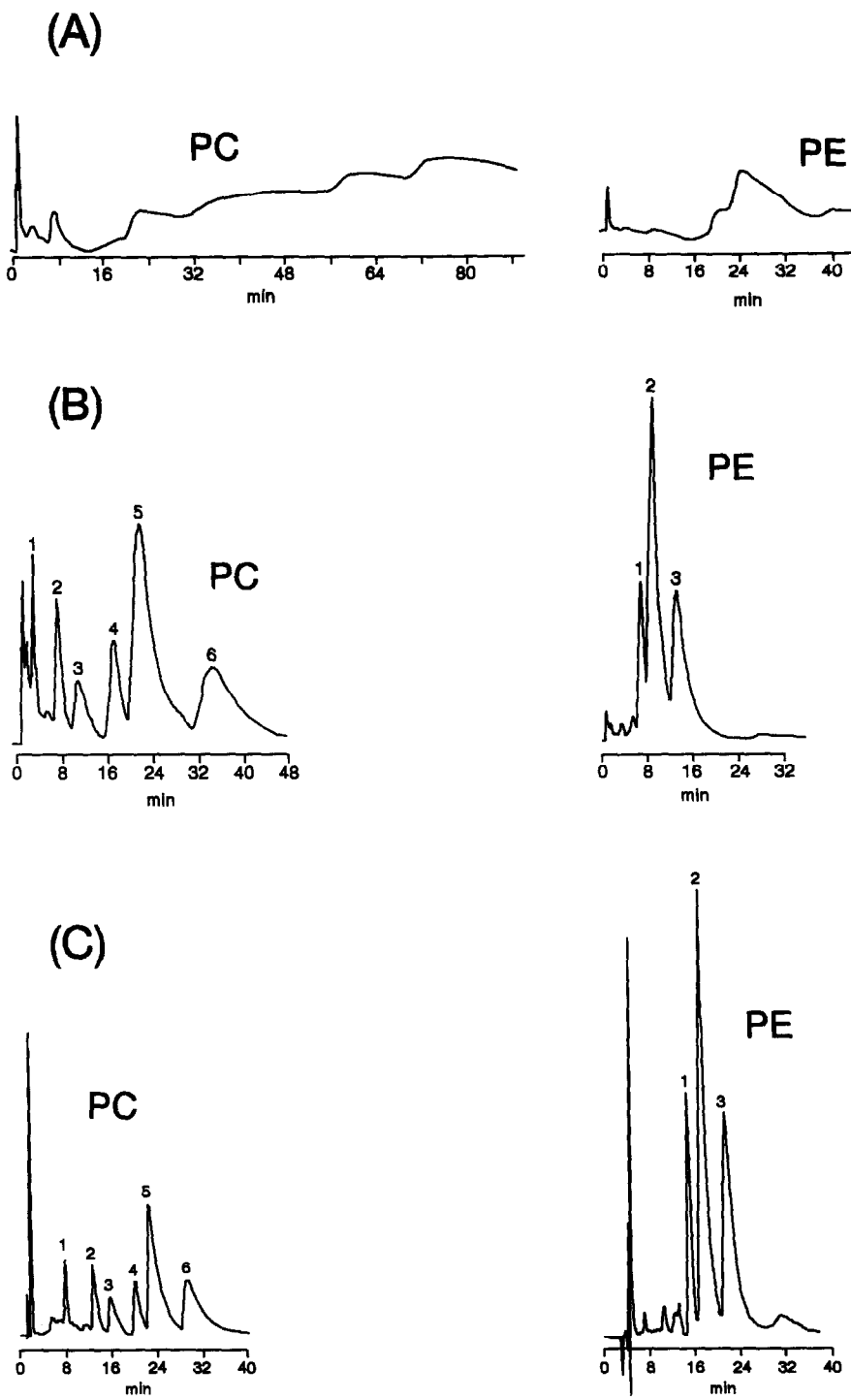


Fig. 2. HPLC separation of molecular species of soybean PCs and PEs in the absence of mobile phase salts (A) and in the presence of mobile phase salts (B, C). Columns: (A, B) Waters Resolve C_{18}, 150 × 3.9 mm, flow-rate, 2 ml/min; (C) Brownlee Spheri-5 RP-8, 220 × 4 mm, flow-rate, 2 ml/min for PCs and 1 ml/min for PEs. Mobile phases: acetonitrile–methanol–water (70:22:8) containing (A) no salt, (B) 5 m*M* HPTAP (C) 5 m*M* PTAP. UV detection at 208 nm. For peak identification, see footnote to Table I.

USA). The chromatograph was interfaced with a flame ionization detector. Helium was the carrier gas. A column temperature program was run from 200 to 220°C at 10°C/min after an initial hold of 15 min. Peak area integration was computed automatically by the data processor in the Model 3400 instrument.

RESULTS AND DISCUSSION

In an exploratory study on the HPLC separation of a pair of closely related PCs the fatty acid chains of which are interchanged at the β- and γ-carbons of the glycerol moiety, unusually long retention times of analytes having broad unsymmetrical peaks were observed. In recent reversed-phase ion-pair HPLC studies [11,12] of negatively-charged PLs, modification of mobile phases with quaternary ammonium salts produced dramatic effects on the chromatographic separation of their molecular species. A similar series of tetraalkylammonium phosphates (TAAPs) as used in those studies were employed to explore their impact on the chromatographic behavior of neutral phospholipids, PCs and PEs. The six TAAPs chosen in this study, TMAP, TEAP, TBAP, PTAP, HPTAP and TMAS, have alkyl chains considerably shorter than those used in HPLC of PIs [11] and PAs [12].

HPLC chromatograms obtained under various conditions are presented in Fig. 2 to illustrate the beneficial effect of mobile phase TAAPs on the peak characteristics of PCs and PEs. It is evident that adding TAAPs to mobile phases led to significant shortening of retention times of the lipid components with concurrent increase in peak heights. Such an improvement in peak shapes was more prominent for PCs than for PEs because of the greater degree of hydrophobicity inherent in PCs, the trimethylamino analogues of PEs (Fig. 1). Without mobile phase electrolytes in the HPLC systems, the neutral PL solutes were strongly adsorbed on a reversed-phase hydrocarbonaceous column. In other words, the amounts of injected analytes failed to elute quantitatively from the columns. The adsorption phenomenon was thwarted by incorporation of TAAPs into the mobile phases. HPLC on longer-alkyl-chain bonded silica (Resolve C_{18}) tended to give broader analyte peaks than with a shorter-alkyl-chain bonded silica (Brownlee C_8) column (Fig. 2B *vs.* C).

TABLE I

HPLC SEPARATION OF MOLECULAR SPECIES OF SOYBEAN PCs AND PEs ON A RESOLVE C_{18} COLUMN

Flow-rate 2 ml/min. Mobile phase solvents were acetonitrile–methanol–water (70:22:8). HPTAP = Heptyltriethyl ammonium phosphate.

HPTAP concentration (m*M*)	Capacity factor, k'					
	Component[a]					
	1	2	3	4	5	6
PCs						
5.00	2.11	6.78	11.0	18.1	23.4	37.9
2.50	2.56	7.89	12.8	22.6	29.7	49.2
1.25	3.22	9.00	17.2	29.2	38.1	63.0
0.625	3.89	12.3	21.2	35.0	45.2	76.8
0.00	Too broad to be measurable					
PEs						
5.00	6.56	8.78	13.2			
2.50	7.00	9.67	16.8			
1.25	10.6	13.7	23.0			
0.625	13.2	17.2	28.3			
0.00	23.4	28.3	45.2			

[a] Component identification: PCs: 1 = 18:3–18:3; 2 = 18:2–18:3; 3 = 18:2–18:2; 4 = 18:1–18:2; 5 = 16:0–18:2; 6 = 16:0–18:1; PEs: 1 = 18:2–18:3; 2 = 18:2–18:2; 3 = 16:0–18:2.

TABLE II

HPLC SEPARATION OF MOLECULAR SPECIES OF SOYBEAN PCs ON A BROWNLEE SPHERI-5 RP-8 COLUMN

Flow-rate 2 ml/min. Mobile phase solvents were acetonitrile–methanol–water (70:22:8). PTAP = Pentyltriethyl ammonium phosphate; SPS = sodium pentyl sulfonate. For component identification, see footnote to Table I.

Electrolyte concentration (m*M*)	Capacity factor, *k'*					
	Component					
	1	2	3	4	5	6
PTAP						
5.00	7.00	12.2	15.0	19.4	21.8	28.6
2.50	7.80	13.0	16.2	20.6	23.7	29.8
1.25	10.2	17.4	21.8	28.6	32.2	42.6
0.00	13.0	22.6	29.0	38.2	44.2	59.0
SPS						
5.00	9.80	17.4	21.8	32.2	35.8	46.6
2.50	10.6	19.4	25.0	37.4	42.6	56.2

Table I shows the concentration effect of HPTAP on the capacity factors (*k'*) of PC and PE. For both the PL classes, the *k'* values decreased with increasing HPTAP concentrations. Under the same conditions, molecular species of PCs eluted at longer retention times than PEs. Consequently, in the ab-

TABLE III

HPLC SEPARATION OF MOLECULAR SPECIES OF SOYBEAN PCs AND PEs ON A BROWNLEE SPHERI-5 RP-8 COLUMN UNDER VARIOUS MOBILE PHASE CONDITIONS

Flow-rate 1–2 ml/min. Mobile phase were solvents A–solvent B (92:8), where solvent A = acetonitrile–methanol (70:22), solvent B = 5 m*M* electrolyte in water. PTAP = Pentyltriethylammonium phosphate; TEAP = tetraethylammonium phosphate; TMAP = tetramethylammonium phosphate; TMAS = tetramethylammonium sulfate. For component identification, see footnote to Table I.

Electrolyte in solvent B	Capacity factor, *k'*					
	Component					
	1	2	3	4	5	6
PCs (2 ml/min)						
K_2HPO_4	7.00	12.2	15.4	23.0	27.0	37.0
NH_4PO_4	12.6	22.6	27.8	39.4	45.4	59.8
PTAP	9.85	16.2	19.8	26.6	29.8	37.8
TEAP	9.63	15.1	18.4	24.8	28.4	36.0
TMAP	6.19	9.38	11.4	15.0	17.8	22.9
TMAS	6.57	11.2	13.8	17.8	21.4	27.8
No salt	11.0	20.2	27.8	44.2	55.8	71.8
PE (1 ml/min)						
K_2HPO_4	1.33	1.74	2.23			
NH_4PO_4	4.20	5.21	6.98			
PTAP	3.92	4.36	5.00			
TEAP	3.24	3.61	4.58			
TMAP	2.41	2.79	3.40			
TMAS	6.40	8.63	10.2			
No salt	Too broad to be measurable					

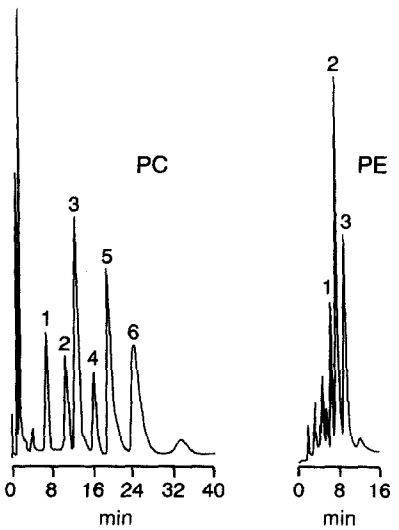


Fig. 3. HPLC separation of molecular species of soybean PC and PE on a Brownlee Spheri-5 RP-8 column. Mobile phase: [(acetonitrile–methanol) (70:22)]–(5 m*M* TMAP in water) (92:8); flow-rate, 2 ml/min for PCs and 1 ml/min for PEs. UV detection at 208 nm. For peak identification, see footnote to Table I.

sence of HPTAP in the mobile phase, the PC components were too broad to be quantifiable. Regardless of the column length, chromatographic peak characteristics were affected by the alkyl-chain length of the bonded-silica phases used. Thus, the peak separations obtained in HPLC with a Resolve C_{18} (150 × 3.9 mm) column were less compact (Table I) than those found in experiments with a Brownlee C_8 (220 × 4 mm) column (Table II). It was of interest to note that similar inverse relationships existed between the concentration effects of electrolytes PTAP and SPS on the k' values of PCs (Table II). Traditionally, both PTAP and SPS have been used as representative ion-pairing counter ions for respective anions and cations in ion-pair HPLC.

Table III shows the effect of various mobile phase electrolytes on the retention behavior of PCs and PEs on octylsilica (Brownlee). Generally solute retention was found to be stronger in HPLC with ammonium phosphate than with potassium phosphate. In comparison with the ammonium salt, the potassium salt could be more effective in alleviating analyte adsorption by way of ion suppression. With TAAPs in the mobile phases, the higher member of the electrolyte in the series gave rise to the higher k' values of the component analytes (PTAP > TEAP > TMAP). The HPLC data in Table III confirm earlier findings (Fig. 2, Tables I and II) that the presence of an electrolyte in mobile phases was es-

TABLE IV

HPLC SEPARATION OF MOLECULAR SPECIES OF SOYBEAN PCs AND PEs ON A MACROPOROUS POLYSTYRENE–DIVINYLBENZENE COLUMN

Flow-rate 1 ml/min. Mobile phase solvents were (i) acetonitrile–methanol–water (70:10:20), (ii) acetonitrile–methanol–water (70:15:15) and (iii) acetonitrile–methanol–water (70:22:8). PTAP = Pentyltriethyl ammonium phosphate. For component identification, see footnote to Table I.

Mobile phase + PTAP concentration (m*M*)	Capacity factor, k'					
	Component					
	1	2	3	4	5	6
PC						
(i) + 5.00	2.85	3.44	6.26	7.59	9.81	12.2
2.50	4.00	3.74	6.56	7.89	10.1	13.1
0.00	3.15	4.04	6.85	8.19	10.6	13.2
(ii) + 0.00	2.41	2.85	4.63	5.81	8.30	9.07
(iii) + 0.00	1.07	1.37	1.96	2.56	3.15	3.74
PEs						
(i) + 5.00	7.44	9.96	12.6			
2.50	7.59	10.4	13.4			
0.00	7.89	10.9	14.1			
(ii) + 0.00	5.67	7.40	9.52			

sential for obtaining useful chromatographic results. When the mobile phase contained no salt, component peaks had either retention times which were too long or peak shapes which were too broad to be of any analytical value. The use of sulfate in lieu of phosphate (TMAS *vs.* TMAP) in the HPLC solvent systems gave more strongly retained PL solutes, presumably due to stronger interactions of the phosphate ions with the octylsilica phase modified by the added electrolyte in the mobile phase. Some examples of chromatograms run with TMAP are shown in Fig. 3.

Separation of rat liver PC on a macroporous polystyrene–divinylbenzene (MPD) column using mobile phases without added salts has been described [21]. Our HPLC results of neutral lipids on the polymeric resins of the same MPD phase are shown in Table IV. Although the observed trends of TAAP concentration effects were similar to those found in HPLC on silica-based columns, the k' values of PCs and PEs were less sensitive to the change in the concentration of PTAP. Adsorption of the neutral PL analytes on MPD seemed to be much less likely to occur than on bonded-silica phases. In view of the low retentivity of the MPD column toward polar lipids, mobile phases normally required a higher water content than other phases to further separate the analyte peaks. The influence of mobile phase solvent compositions on the retention characteristics of PC and PE is also shown in Table IV. As usually observed in reversed-phase HPLC, higher k' values were obtained when the mobile phases contained higher percentages of water.

Table V presents HPLC data obtained with an octadecylsilica column (NovaPak C_{18}, 4 μm) which has a longer column length, but smaller particle size relative to other columns discussed. There were few differences in the number of resolved molecular species with the variation in the particle size or efficiency of the column. This observation is unlike the situation in reversed-phase ion-pair HPLC of negatively charged PAs and PIs where component reso-

TABLE V

HPLC SEPARATION OF MOLECULAR SPECIES OF SOYBEAN PCs AND PEs ON A NOVAPAK C_{18} COLUMN

Flow-rate 2 ml/min. For component identification, see footnote to Table I.

Electrolyte concentration (m*M*)	Capacity factor, k'					
	Component					
	1	2	3	4	5	6
PCs						
PTAP (acetonitrile–methanol–water) (74:23:3)						
10.0	3.44	10.1	16.8	25.7	31.9	52.3
5.00	4.33	11.4	19.4	29.7	36.3	58.6
2.50	5.67	17.7	29.2	48.8	61.7	104
Solvent B[a]						
TMAP, 5[b]	4.33	15.0	24.8	47.9	66.1	105
TMAP, 10	3.89	10.6	15.4	25.7	34.1	53.2
TMAP, 50	1.67	7.00	11.4	19.4	27.4	45.2
TMAP, 100	1.67	6.56	11.0	19.0	27.3	45.2
PEs						
PTAP (acetonitrile–methanol–water) (74:23:3)						
10.0	3.05	6.56	11.4			
5.00	4.78	8.33	12.8			
2.50	10.6	13.2	27.4			

[a] Mobile phases were solvent A–solvent B (95:5), where solvent A = acetonitrile–methanol (70:22), solvent B = various concentrations of TMAP in water.

[b] Mobile phase was solvent A–solvent B (92:8) PTAP = pentyltriethyl ammonium phosphate; TMAP = tetramethyl ammonium phosphate.

lution was notably affected by a change in column efficiency [11,12]. Since the NovaPak column is quite retentive for PCs, it is recommended to use a smaller percentage of water in the mobile phase. Examination of the data in Table V revealed similar patterns of inverse relationships for both PCs and PEs in the concentration dependence of their k' values. To obtain narrow peaks, mobile phases containing the lowest member of TAAPs (TMAP in solvent B, Table V) in the quaternary ammonium series were employed. When k' values obtained from mobile phases containing 50 mM TMAP were compared with those from mobile phases containing 100 mM of the same salt, few differences were noticeable between the corresponding retention data for PCs. The results suggest that the degree of ion interactions between PL solutes and TMAP in the HPLC processes reached a maximum at a concentration of $\leqslant$ 50 mM TMAP.

HPLC separations of molecular species of PCs and PEs from plant and egg sources are shown in Fig. 4. All of the HPLC experiments were carried out under optimized conditions. Separations of molecular species on octylsilica (Fig. 4A) are generally similar to those on octadecylsilica (Fig. 4B), but the degree of component resolution appears to be somewhat more efficient in HPLC with the octadecylsilica column. In Fig. 4B, peak profiles for the negatively charged PAs and PIs are also given with those for the neutral PLs to demonstrate the general applicability of the mobile phase quaternary ammonium electrolytes in HPLC separations of subcomponents of polar lipids. HPLC separations of molecular species of bovine brain PEs, bovine heart PCs, and platelet-activating factor are shown in Fig. 5. In comparison to plant PEs and PCs, the ether-linked species in the two bovine samples tended to have longer retention times. In general, the distribution patterns of molecular species derived from animal sources are more complex than those from plant sources.

For reversed-phase HPLC of ionic compounds (anions and cations), solute partition mechanisms have been postulated based on ion-pair, ion-exchange, and ion-interaction retionales [22]. Hence, it was logical to study the effect of mobile phase electrolytes on the chromatographic behavior of PL ions. While negatively charged PI and PA molecular species have been reported to undergo HPLC separation via ion-pairing processes [11,12], retention of neutral PL (PCs and PEs) components in the presence of counter ions appeared to follow mechanistically different pathways as each of the analytes showed less tendency to be retained by a reversed-phase HPLC column. The latter observations are reminiscent of previous studies on quanternary ammonium compounds [23] and quinoidal imminium compounds [24]. Although there are ionic charges present in both PCs and PEs, they are neutralized by intra-molecular pairing of the charges. This is

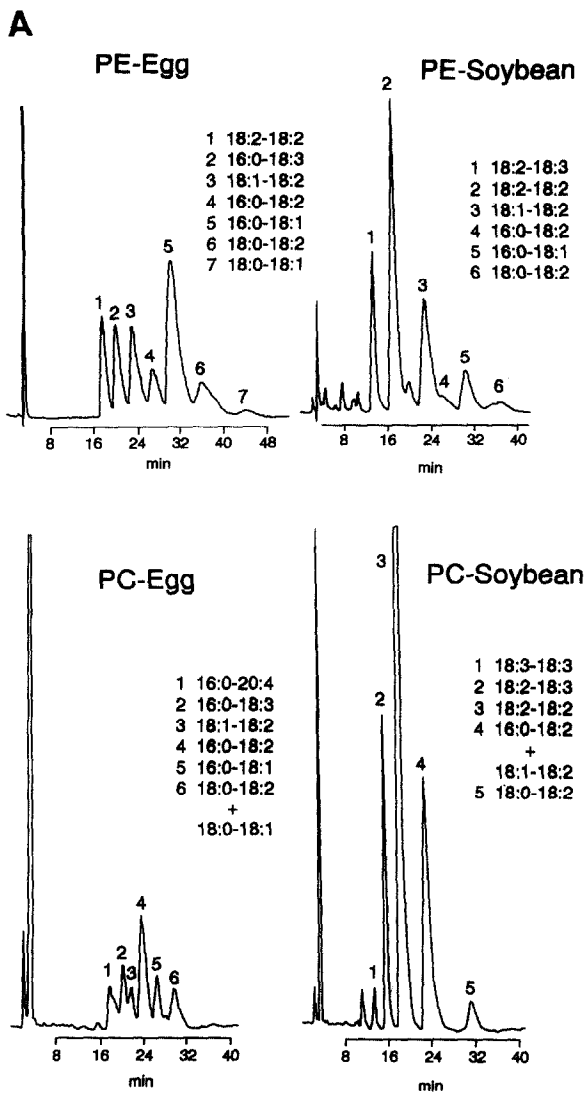


Fig. 4.

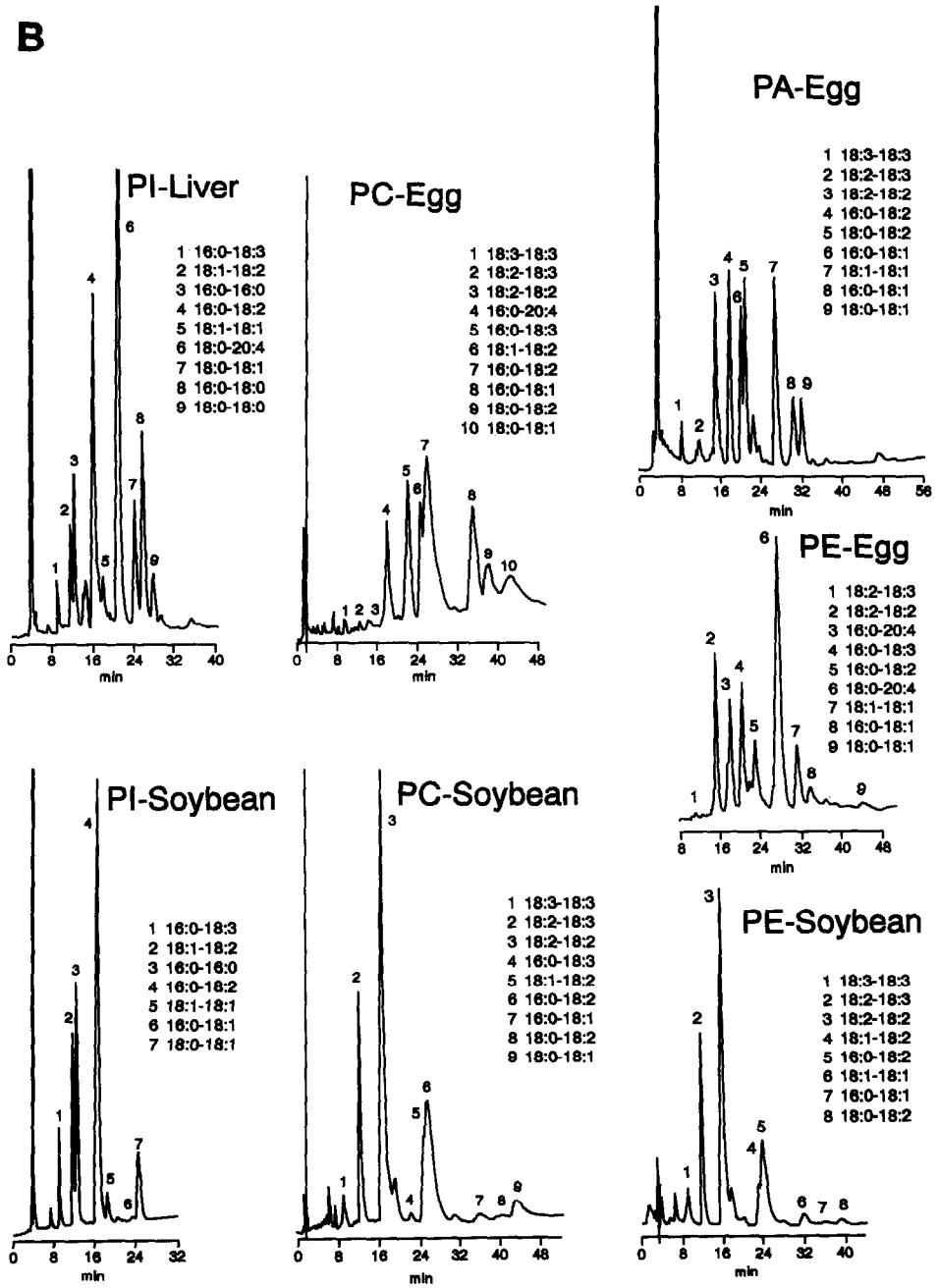


Fig. 4. (A) HPLC of animal *vs.* soybean PCs and PEs on a Brownlee Spheri-5 RP-8 column. Mobile phase: [(acetonitrile–methanol) (70:22)]–(50 m*M* TMAP in water) (92:8); flow-rate, 1 ml/min. UV detection at 208 nm. (B) HPLC of animal *vs.* soybean PAs, PCs, PEs and PIs on an Alltech Adsorbsphere HS C_{18} column. Mobile phase: acetonitrile–methanol–water (70:28:2) containing 25 m*M* TMAP; flow-rate, 2 ml/min for PCs and 1 ml/min for others. UV detection at 208 nm.

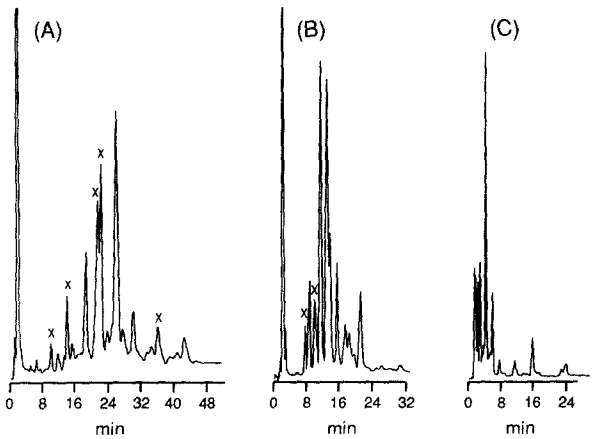


Fig. 5. HPLC of bovine heart PCs (A), bovine brain PEs (B), and platelet-activating factor (C) on a Waters NovaPak C_{18} column. Mobile phase conditions same as Fig. 4B. Flow-rate, 1 ml/min. UV detection at 208 nm. Peak components marked with × are identical with those in soybean oil. All other peak components are ether-linked species.

evidenced by the absence of ion-pairing retention characteristics observed in HPLC of negatively charged PLs (PAs and PIs). Hence, HPLC separations of molecular species of neutral PLs apparently proceeded via an ion-exchange or an ion-interaction retention mechanism.

In conclusion, the methods developed in this study are useful for direct analysis of molecular species of PCs and PEs present in various sample matrices at low levels. The method is also useful for the analysis of samples containing ether-linked aliphatic moieties at the *sn*-1 position of PEs and PCs. This is the first report documenting the mechanistically different chromatographic pathways by which separations of molecular species of neutral and charged PLs are believed to proceed. Deployment of the separation methodology in conjunction with other detection systems (mass spectrometry and nuclear magnetic resonance spectrometry) may lead to viable alternatives for characterization of molecular species. Although the methods are simplified by eliminating the need for chemical derivatization, the procedure requires peak collection and phosphorus determination for analyte quantitation. Using this technique, quantitative analysis of the neutral PLs by HPLC–evaporative light scattering detection is unsuitable because of the incompatibility of the detection systems with the mobile phase electrolytes. The limitation of HPLC-UV detection may be overcome by interfacing the HPLC system with a flame photometric detector.

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