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High-performance liquid chromatography of phosphatidic acids and related polar lipids

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ABSTRACT

The retention behavior of phosphatidic acids (PA) and phosphatidic acid methyl esters (PM) was studied by reversed-phase ion-pair high-performance liquid chromatography (HPLC). The HPLC systems consisted of mobile phases of acetonitrile–methanol–water containing tetraalkylammonium phosphates (TAAP) and stationary phases of alkyl-bonded silica and polystyrene–divinylbenzene resins. The lipid solutes were more strongly retained when using mobile phases containing larger TAAP at higher concentrations. The results are indicative of an ion-pair retention mechanism. Molecular species of PM were readily resolved despite the complete inseparability of PA under all conditions used. Except for tetrabutylammonium phosphate, there was a linear correlation between the logarithmic capacity factors (k') of PM (or PA) and the total number of carbon atoms of TAAP. The significant concentration dependence of separation factors for certain PM components was related to the size effect of TAAP. A normal-phase HPLC method for the separation of PA from other polar lipids is described.

INTRODUCTION

Phospholipids (PL) are widely distributed in plant and animal cells. These polar lipids play an important role in regulating the movement of charged and uncharged molecules. Soybean oil contains phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) as the four major consitutents of its polar lipids. It also contains a small percentage of phosphatidylserine (PS). The change in PL composition with time has been related to the stability of soybean oil during storage [1]. The determination of PL in crude and degummed oil provides useful information on the extent of PL deterioration caused by stressed soybeans. As PA has been found to be the predominant product of PL decomposition, it was necessary to assess the variation in its molecular species distribution with the period of storage. To this end, an effective analytical method was required for the separation of PA components.

A number of techniques for the separation of molecular species of neutral PL (*i.e.*, PC and PE) are known and numerous reports on the reversed-phase high-performance liquid chromatography (HPLC) of PC and PE have been published [2–10]. Whereas the ionic charges in PC and PE are internally neutralized within the molecules, PA, PI and PS are all negatively charged compounds requiring external counter ions to form neutral species. Few studies have been conducted for the speciation of these negatively charged PL into molecular species using ion-pair methodology. Most recently, mixtures of soybean PI have been resolved by reversed-phase ion-pair HPLC [11]. Although sporadic reports on indirect determinations of PA derived from PC are available [12–14], methods for the direct determination of PA molecular species have remained obscure. This paper describes reversed-phase ion-pair HPLC techniques for the separation of molecular species of PA and its monomethyl ester, phosphatidic acid methyl ester (PM).

There are many publications [6,15–26] dealing with normal-phase HPLC separations of PL class-

es. However, the scarcity of published information on reproducible normal-phase HPLC methods for the separation of PA from other PL classes provided the impetus for this normal-phase HPLC work.

EXPERIMENTAL

Chemicals and reagents

Phospholipid standards PA, PC, PE, PI, PM and PS were obtained either from Avanti Polar Lipids (Pelham, AL, USA) or Sigma (St. Louis, MO, USA). Commercial PA and PM were used in all HPLC analyses. Both compounds were prepared from egg PC by the manufacturers. Commercial PM was prepared by methanolysis of PC in the presence of phospholipase D. Tetraalkylammonium phosphates were obtained from Regis (Morton Grove, IL, USA). HPLC-grade acetonitrile and methanol were purchased from J. T. Baker (Phillipsburg, NJ, USA). HPLC solvents tetrahydrofuran and ammonia solution were obtained from EM Science (Gibbstown, NJ, USA). Chloroform used in normal-phase HPLC was a product of Mallincrodt (Paris, KY, USA). A Waters–Millipore (Milford, MA, USA) Milli-Q water purifier was used for obtaining ultra-pure HPLC water.

High-performance liquid chromatography

In all reversed-phase HPLC experiments, a Spectra-Physics (San Jose, CA, USA) liquid chromatograph equipped with a Model SP8700 solvent delivery system was interfaced with an LDC Analytical (Riviera Beach, FL, USA) SpectroMonitor D variable-wavelength UV detector. For normal-phase HPLC work, a Varex (Rockville, MD, USA) Model ELSD II evaporative light-scattering (ELS) detector was used.

Reversed-phase HPLC mobile phases consisting of acetonitrile–methanol–water and various concentrations (0.1–5.0 m*M*) of quaternary ammonium phosphates were filtered, degassed and pumped through a column under isocratic conditions at a flow-rate of 1 ml/min. Normal-phase HPLC analyses were carried out by gradient elution with mobile phase solvents of (1) chloroform–tetrahydrofuran (THF) and (2) methanol–ammonia solution–chloroform at a flow-rate of 1 ml/min.

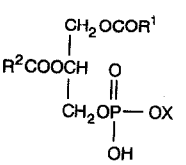
Several stationary phases were evaluated: (1) Waters NovaPak C_{18} (30 cm × 3.9 mm I.D.; 4 μm), (2) polymeric resins of macroporous polystyrene–divinylbenzene (MPD), PLRP-S-100 (25 cm × 4.6 mm I.D.; 5 μm) (Polymer Labs., Amherst, MA, USA), (3) EM Science LiChrosorb RP-18 (25 cm × 4.6 mm I.D.; 10 μm) (Alltech, Deerfield, IL, USA), (4) Brownlee Spheri-5 RP-8 (22 cm × 4 mm I.D.; 5 μm) (Applied Biosystems, Foster City, CA, USA), (5) Adsorbsphere HS C_{18} (25 cm × 4.6 mm I.D.; 5 μm (Alltech), (6) EM Science LiChrosorb Si-60 (25 cm × 4.6 mm I.D.; 10 μm) and (7) EM Science LiChrosorb Si-100 (25 cm × 4.6 mm I.D.; 5 μm).

Aliquots (5–10 μl of 1% solutions) of PL samples in chloroform were injected onto a column via a Rheodyne (Cotati, CA, USA) Model 7125 injector fitted with a 10-μl loop. Detector signals were monitored with an OminiScribe recorder (Houston Instruments, Houston, TX, USA).

RESULTS AND DISCUSSION

Reversed-phase high-performance liquid chromatography

Individual PL classes consist of molecular species that differ in the R^1 and R^2 groups of fatty acids, as shown in Fig. 1 for the strcture of PA. Notwithstanding the existence of non-polar tail groups in the fatty acid moieties, reversed-phase HPLC of PA in mobile phases containing no ion-pairing reagents produced unresolved peaks with little retention (Fig. 2A) owing to the influence of polar head groups of phosphoric acid derivatives. Much like phosphatidylinositols (PI), molecular species of PA including its monomethyl ester, PM, could be retained on a reversed-phase column only when cationic counter ions (ion-pairing reagents) were pres-



I . X = H

II . X = CH_3

Fig. 1. Structures of (I) phosphatidic acid (PA) and (II) phosphatidic acid methyl ester (PM). R^1 and R^2 are alkyl or alkenyl groups of fatty acids.

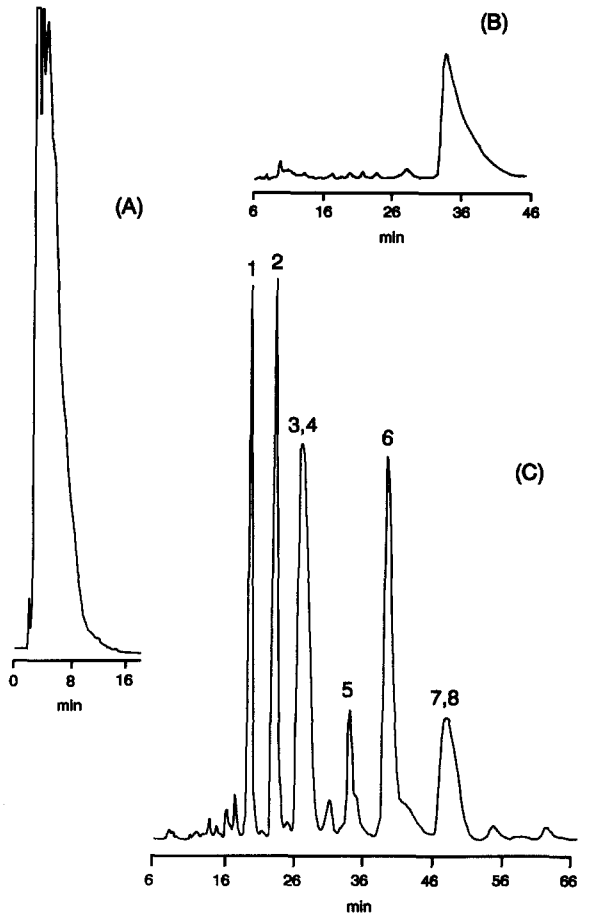


Fig. 2. Reversed-phase HPLC of (A) PM without an ion-pairing reagent, (B) PA with an ion-pairing reagent (5 m*M* PTAP) and (C) PM with an ion-pairing reagent (5 m*M* PTAP). Conditions: stationary phase, NovaPak C_{18}; mobile phase, acetonitrile–methanol–water (70:22:8); UV detection at 208 nm. Peaks: 1 = 16:0–20:4; 2 = 16:0–18:2; 3 + 4 = 16:0–18:1; 5 = 18:0–18:2; 6 = 18:1–18:1; 7 + 8 = 18.0–18:1, corresponding to R^1 and R^2 groups of fatty acids in PM (Fig. 1). Fatty acid designations: 16:0 = palmitic; 18:0 = stearic; 18:1 = oleic; 18:2 = linoleic; 20:4 = arachidonic.

ent in the mobile phases. In order to optimize the HPLC conditions and obtain various degrees of retention and separation of the compounds, a series of tetraalkylammonium phosphates (TAAP) were evaluated, including tetrabutylammonium phosphate (TBAP), pentyltriethylammonium phosphate (PTAP), hexyltriethylammonium phosphate (HTAP), heptyltriethylammonium phosphate (HTAP), octyltriethylammonium phosphate (OTAP) and dodecyltriethylammonium phosphate (DTAP). As there are two negative charges in the PA molecule, it requires two cationic ions for neutralization. In reversed-phase ion-pair HPLC of PA, an enhancement of analyte retention was invariably observed despite the inability of the HPLC systems to resolve PA molecular species (Fig. 2B). It is probable that concurrent ion-pair formation from doublely charged PA might be sterically disfavored, resulting in inadequate hydrophobic interactions in chromatographic processes. On the other hand, molecular species of phosphatidic acid methyl ester, PM, were separated on a reversed-phase column (Fig. 2C). In this instance, the singlely charged anion presumably formed an ion pair with a TAAP cation to facilitate hydrophobic interactions with the hydrophobic phase employed.

Results of the reversed-phase ion-pair HPLC of PM are summarized in Table I. The retention characteristics of PM components were markedly affected by both the size and concentration of quaternary ammonium counter ions present in the mobile phases. The capacity factors (k') of molecular species increased with an increase in the chain length of the alkyl group in an alkyltriethylammonium counter ion. There was a linear correlation between the logarithmic k' values and the total number of carbon atoms or the longest carbon chain length of alkyl groups in the TAAP counter ions (Fig. 3). The linear correlation data indicate that the total area of alkyltriethyl groups in TAAP is available for solvophobic interactions [27,28]. It should be pointed out that all but TBAP in the TAAP series are homologues of alkyltriethylammonium phosphates. The log k' values of PM in mobile phases containing TBAP were found to deviate from the correlation lines obtained with other TAAP in the series. Evidently, structural effects of TAAP on the k' values of PM were primarily controlled by the carbon chain length of the largest alkyl group of TAAP. These observations are in contrast with those in reversed-phase ion-pair HPLC of PI, where the k' values were entirely governed by the total number of carbon atoms in the tetraalkyl groups of TAAP [11]. Hence, among the investigated TAAP counter ions, HPLC of PM with TBAP (longest carbon chain length = 4) yielded the least retained components, whereas molecular species of PI were least retained in HPLC with mobile phases containing

TABLE I

EFFECTS OF THE CONCENTRATION AND THE TYPE OF QUATERNARY AMMONIUM COUNTER IONS ON THE CAPACITY FACTORS k' OF PHOSPHATIDIC ACID METHYL ESTER (PM)

HPLC conditions: stationary phase, NovaPak C_{18}; mobile phase, acetonitrile–methanol–water (70:22:8) containing a tetraalkylammonium phosphate (TAAP) at various concentrations. Commercial PM are derived from egg phosphatidylcholines. For abbreviations, see Results and Discussion. For peak identification, see Fig. 2C.

Counter ion	Concentration (mM)	Capacity factor, k'									
		Component									
		1	2	3	(α)	4	5	6	7	(α)	8
TBAP	5.0	5.60	6.68	7.48	(1.07)	8.02	10.7	12.5	15.7	(1.04)	16.4
	2.5	3.04	3.71	4.25	(1.06)	4.52	5.46	6.41	7.62	(1.04)	7.89
	1.25	2.30	2.84	3.44	(1.04)	3.58	4.39	5.33	6.34	(1.02)	6.50
HTAP	5.0	5.60	6.54	7.75	(1.00)	7.75	9.84	11.7	14.5	(1.00)	14.5
	2.5	3.31	3.98	4.86	(1.00)	4.86	6.14	7.48	8.97	(1.00)	8.97
	1.25	2.10	2.64	3.38	(1.00)	3.38	4.05	5.53	6.62	(1.00)	6.62
OTAP	5.0	9.51	11.7	13.9	(1.08)	15.0	18.5	22.4	27.0	(1.05)	28.4
	2.5	5.33	6.28	7.40	(1.07)	7.92	9.58	11.7	14.2	(1.03)	14.6
	1.25	3.13	3.90	4.91	(1.00)	4.91	5.93	7.77	9.44	(1.00)	9.44
DTAP	2.5	12.3	15.0	17.4	(1.08)	19.0	23.8	27.9	36.5	(1.07)	39.0
	1.25	7.08	8.70	10.2	(1.05)	10.7	12.7	15.8	188	(1.06)	19.9
	0.544	3.85	4.79	5.73	(1.00)	5.73	7.22	8.97	11.0	(1.00)	11.0

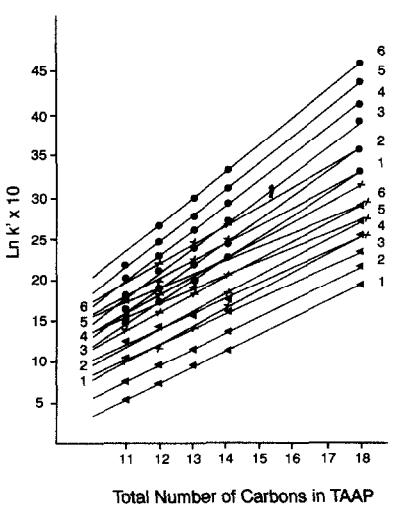


Fig. 3. Correlation of ln k' of six major molecular species of PM with the total number of carbon atoms in TAAP. Reversed-phase ion-pair HPLC conditions as in Fig. 2 except for TAAP concentrations: ● = 5 mM; × 2.5 mM; ▲ = 1.25 mM. UV detection at 208 nm.

PTAP (smallest total number of carbon atoms = 11) [11]. When TBAP was used as the counter ion, the retention of PI corresponded to a total of sixteen carbon atoms on correlation plots [11].

Separations of PM components 3, 4, 7 and 8 were particularily susceptible to variations in the structure of TAAP used in the mobile phases (Table I). The ammonium counter ions having a larger total number of carbon atoms, as in TBAP (C_{16}), OTAP (C_{14}) and DTAP (C_{18}), tended to offer better selectivity [higher values of separation factors (α)] for the lipid components of interest. Such structural effects appeared to diminish (lower α values) as the counter ion concentrations decreased (Table I). Component peaks 3 and 4 and 7 and 8 may be attributed to reverse positonal isomers. The k' values of PM analytes increased with increasing counter ion concentration. In general, the observed concentration effects of TAAP on the retention behavior of PM are similar to those found previously in HPLC of PI [11] and aminobenzoic acids [29], but are different

TABLE II

REVERSED-PHASE ION-PAIR HPLC OF PHOSPHATIDIC ACID ON OCTADECYLSILICA STATIONARY PHASES

Mobile phase: acetonitrile–methanol–water (70:22:8). The commercial phosphatidic acids are derived from egg phosphatidylcholine.

Stationary phase	TAAP concentration (m*M*)	Capacity factor, *k'*				
		Quaternary ammonium counter ion				
		TBAP	PTAP	HTAP	HPTAP	OTAP
LiChrosorb RP-18	5	4.03	8.17	8.54	9.36	9.78
	2.5	2.17	4.90	6.05	7.21	8.67
	1.25	2.08	2.83	3.60	4.63	5.75
NovaPak C_{18}	5	4.78	8.27	8.70	9.77	10.1
	2.5	2.34	5.10	6.68	7.89	9.87
	1.25	2.14	2.89	3.74	4.93	6.23

to those of aprotic ionic compounds [30,31]. The cationic counter ion effects (with respect to both the concentration and size of TAAP) discussed so far are indicative of an ion-pair retention mechanism by which solvophobic interactions of PM solutes with stationary phases are believed to proceed during HPLC separation processes.

Table II summarizes HPLC data for PA to show the influence of TAAP on the capacity factors, *k'*. The general trends of counter-ion effects on the retention of PA are similar to those noted in the HPLC of PM, even though there was no indication of component resolution of the former in all experiments performed. Correlation of ln *k'* values with the total number of carbons or the number of the carbons on the largest alkyl group in TAAP, excluding TBAP, yielded linear plots. It is unclear how TAAP participated in ion-pair formation with the double negative charges of PA molecules. Nevertheless, an ion-pair mechanism appeared to be

TABLE III

REVERSED-PHASE ION-PAIR HPLC OF PHOSPHATIDIC ACID METHYL ESTER (PM) UNDER VARIOUS CONDITIONS

Mobile phase: (A) and (B) acetonitrile–methanol–water (65.8:13.7:20.5); (C) and (D) acetonitrile–methanol–water (70:22:8). The source of PM is same as in Table I. For peak identification, see Fig. 2C.

Stationary phase	TAAP	Concentration (m*M*)	Capacity factor, *k'*									
			Component									
			1	2	3	(α)	4	5	6	7	(α)	8
(A) Spheri-RP-C_8 (5 μm)	DTAP	3.11	15.6	17.8	19.6	(1.14)	22.4	26.6	29.4	33.4	(1.13)	37.6
		0.78	4.61	5.20	5.82	(1.00)	5.82	7.19	8.24	9.68	(1.11)	10.7
(B) PLRP-S (5 μm)	DTAP	0.78	14.0	14.0	17.8	(1.08)	19.3	26.4	26.4	37.1	(1.00)	37.1
		0.195	6.41	6.41	7.89	(1.04)	8.19	11.9	16.5	11.9	(1.00)	16.5
(C) LiChrosorb RP-18 (10 μm)	HTAP	5.0	5.05	6.11	7.29	(1.00)	7.29	8.86	10.4	12.9	(1.00)	12.9
		2.5	3.21	4.00	4.93	(1.00)	4.93	5.86	7.14	8.57	(1.00)	8.57
(D) Adsorbsphere HS C_{18} (5 μm)	TBAP	5.0	13.4	15.9	18.1	(1.09)	19.7	23.7	27.6	32.3	(1.07)	34.4
		1.25	7.22	8.63	9.96	(1.06)	10.6	12.9	15.3	17.9	(1.06)	19.0
	PTAP	1.25	6.04	7.30	8.48	(1.00)	8.48	11.0	12.5	15.4	(1.00)	15.4

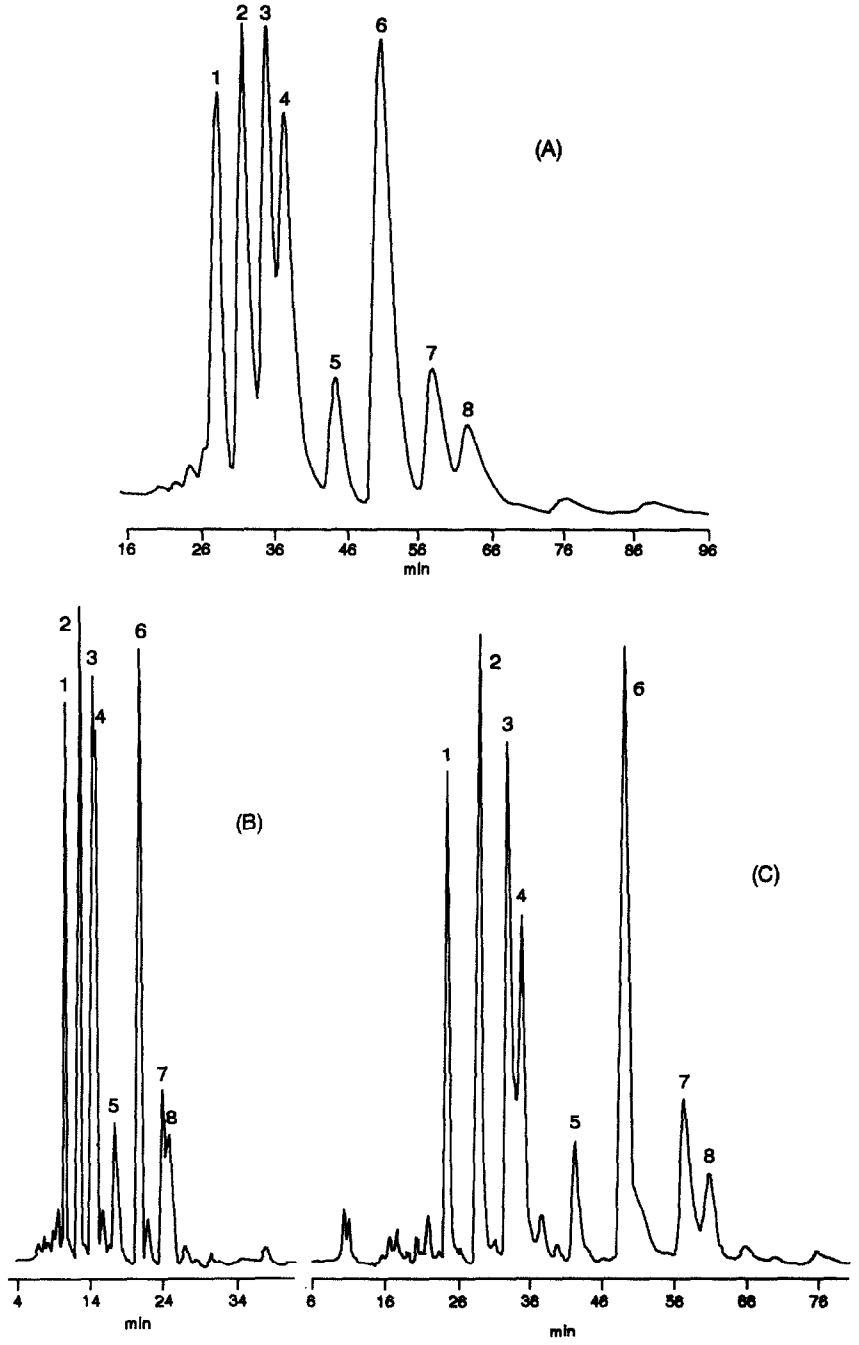


Fig. 4. Reversed-phase ion-pair HPLC separations of PM on various alkyl-bonded silica phases. Conditons: stationary phases, (A) octylsilica, Spheri-5 RP-8, (B) NovaPak C_{18}, (C) Adsorbsphere HS C18; mobile phases, (A) acetonitrile–methanol–water (65.8:13.7:20.5) containing 1.56 m*M* DTAP, (B) and (C) acetonitrile–methanol–water (70:22:8) containing 1.25 m*M* TBAP. UV detection at 208 nm.

TABLE IV

EFFECTS OF PHOSPHORIC ACID AND AMMONIA SOLUTION ON CAPACITY FACTORS, k', OF PM AND PA ON AN MPD PHASE IN REVERSED-PHASE ION-PAIR HPLC

HPLC conditions: stationary phase, MPD; mobile phase, acetonitrile–methanol–water (70:18:12) containing 5 m*M* dodecyltriethylammonium phosphate (DTAP). The sources of PM and PA are the same as in Tables I and II, respectively. For peak identification, see Fig. 2C.

Compound	Concentration (m*M*)	Capacity factor, k'								
		Component								
		1	2	3	4	5	6	7	8	PA
H_3PO_4	0.00	6.26	6.26	7.74	7.74	10.1	10.1	12.9	12.9	4.48
	5.00	8.33	8.33	9.37	9.37	12.9	12.9	16.8	16.8	9.96
	20.0	9.07	9.07	10.3	10.3	14.6	14.6	19.7	19.7	23.7
Ammonia solution	0.00	6.26	6.26	7.74	7.74	10.1	10.1	12.9	12.9	4.48
	15.0	4.93	4.93	6.56	6.56	9.22	9.22	11.3	11.3	3.74
	30.0	3.89	3.89	5.22	5.22	7.59	7.59	10.1	10.1	2.70

operative during chromatographic processes as reflected in the typical counter ion effects (Table II). As briefly stated earlier, the formation of ion pairs with both negative charges might be sterically unfavorable, leaving one unpaired acidic function that could inhibit hydrophobic interactions. Esterification of PA to its monoester PM removed one free charge and facilitated the separation of molecular species by ion-pairing retention processes. Hence, neutralization of charges in PA is an obvious prerequisite for the successful HPLC separation of its molecular species.

Table III shows the HPLC results for PM on various stationary phases. As expected, the capacity factors were higher on octadecylsilica phases than on stationary phases derived from macroporous polystyrene–divinylbenzene, MPD. In the former instances it was necessary to use lower members of TAAP in mobile phases for bringing peaks within reasonable retention times. However, the retention on octylsilica was lower (partly owing to smaller column dimensions) than on MPD, as demonstrated in the HPLC data in which the corresponding k' values of PM are compared under identical mobile phase conditions at a DTAP concentration of 0.78 m*M* (Table III). Of the silica-based stationary phases, Adsorbsphere HS C_{18} showed the greatest selectivity for PM components 3, 4, 7 and 8. This is attributed to its high carbon loading (20%), which favors hydrophobic interactions. With higher members of TAAP (total carbon number >16) in the series as counter ions, HPLC separations of component pairs 3–4 and 7–8 occurred with enhanced selectivity (higher α values). These results, coupled with the concentration dependence of the α values, are in agreement with earlier findings in analogous experiments where a higher efficiency column (NovaPak C_{18}, 4 μm) was used (Table I).

Fig. 4 compares separations of PM components on various alkyl-bonded silica phases. Although Spheri-5 RP-8, an octylsilica column, was made of relatively low carbon loading (9%) packings, significant enhancement (higher α values) in component separation (component pairs 3–4 and 7–8) was observed in experiments where DTAP was added to the mobile phases. Thus, with DTAP as counter ions, HPLC of PM led to reasonable separations of components 3, 4, 7 and 8, which remained unresolved or partially resolved in most instances.

In the light of the acidic properties of the title compounds and the stability of MPD over a wide pH range, we studied the retention behavior of PA and PM in acidic and basic media on this stationary phase. Table IV shows the effect of phosphoric acid and ammonia solution on the k' values in the reversed-phase ion-pair HPLC of the acidic polar lip-

ids. Generally, an increase in k' values of the analytes was demonstrated in HPLC with mobile phases containing higher concentrations of phosphoric acid. Conversely, an increase in the concentration of ammonia solution was accompanied by a small decrease in the k' values of the compounds analyzed. The pH dependence of both PA and PM in protic mobile phases is rationalized in terms of ion-suppression phenomena, frequently observed in the HPLC of ionic compounds [32,33]. As there was no evidence of participation of aromatic moieties of MPD in chromatographic interactions with analyte solutes, modifications of the mobile phase pH gave little improvement in column selectivity for the separation of PM molecular species. In comparison with results from the HPLC of PM, the concentration of phosphoric acid in the mobile phases had a greater influence on the retention of PA because of the doubly charged acidic characteristics of the parent compound.

Normal-phase high-performance liquid chromatography

Normal-phase HPLC separation of PA from other PL found in soybean oil has not been thoroughly investigated on account of its low natural abundance and some reported uncertainty associated with the HPLC behavior of the compound [6]. As PA is the major PL detected in degummed soybean oil, it was pertinent to include in this work the results of a normal-phase HPLC study. Table V presents separation data for PA and four other PL classes derived from soybeans. After initial attempts to reproduce a published procedure [21] failed to resolve the PL components with reported retention times (the published HPLC data [21] might have been obtained before equilibration of the HPLC system was reached), the mobile phase systems were modified as shown for systems A–F in Table V. Normally, baseline separations of PA from other PL were achieved with good reproduc-

TABLE V

NORMAL-PHASE HPLC SEPARATION OF MIXTURES OF SOYBEAN PHOSPHOLIPID CLASSES

HPLC conditions: gradient programs for changes in mobile phase solvents 1 and 2 with times (min) were run in one of the two modes 1→2→2→1 and 1→2→1 (arrows represent stepwise duration times) with duration times shown as (i) 20–20–20, (ii) 30–15–10, (iii) 40–20, (iv) 30–10–5 and (v) 35–10–5 min. For detailed description of gradient elution, see legend for Fig. 5. Mobile phases: (A) solvent 1 = $CHCl_3$, solvent 2 = CH_3OH–NH_3–$CHCL_3$ (92:7:1); (B) solvent 1 = $CHCl_3$–THF (4:1), solvent 2 = CH_3OH–NH_3–$CHCl_3$ (92:7:1); (C) solvent 1 = $CHCL_3$–THF (1:1), solvent 2 = CH_3OH–NH_3–$CHCL_3$ (92:7:1); (D) solvent 1 = $CHCl_3$–THF (1:1), solvent 2 = CH_3OH–NH_3CHCL_3 (92:4:4); (E) solvent 1 = $CHCl_3$–THF (1:1), solvent 2 = CH_3OH–$(C_2H_5)_3N$–$CHCl_3$ (92:4:4).

Stationary phase	Gradient elution	Capacity factor, k'								
		Phospholipid class								
		PE	(α)	PI	(α)	PS	(α)	PA	(α)	PC
LiChrosorb Si-60 (10 μm)	(A) (i) 20–20–20	8.52	(1.22)	10.4	(1.00)	10.4	(1.14)	11.9	(1.17)	13.9
	(ii) 30–15–10	10.2	(1.23)	12.5	(1.05)	13.1	(1.15)	15.0	(1.09)	16.3
	(iii) 40–20	13.0	(1.20)	15.6	(1.03)	16.1	(1.12)	18.0	(1.00)	18.0
	(B) (ii) 30–15–10	11.2	(1.15)	12.9	(1.11)	14.3	(1.09)	15.6	(1.15)	17.9
LiChrosorb Si-100 (5 μm)	(C) (i) 20–20–20	8.24	(1.10)	9.10	(1.02)	9.29	(1.18)	11.0	(1.18)	13.0
	(ii) 30–15–10	10.4	(1.13)	11.8	(1.03)	12.1	(1.17)	14.2	(1.16)	16.5
	(D) (i) 20–20–20	8.90	(1.04)	9.30	(1.06)	9.86	(1.18)	11.6	(1.20)	13.9
	(ii) 30–15–10	10.4	(1.07)	11.1	(1.06)	11.8	(1.19)	13.9	(1.19)	16.5
	(iv) 30–10–5	10.9	(1.05)	11.4	(1.06)	12.1	(1.21)	14.6	(1.16)	16.9
	(E) (ii) 30–15–10	9.10	(1.25)	11.4	(1.00)	11.4	(1.04)	11.9	(1.35)	16.1
	(iv) 30–10–5	8.81	(1.20)	10.6	(1.00)	10.6	(1.14)	12.1	(1.35)	16.3
	(v) 35–10–5	8.29	(1.48)	12.3	(1.00)	12.3	(1.07)	13.1	(1.35)	17.7

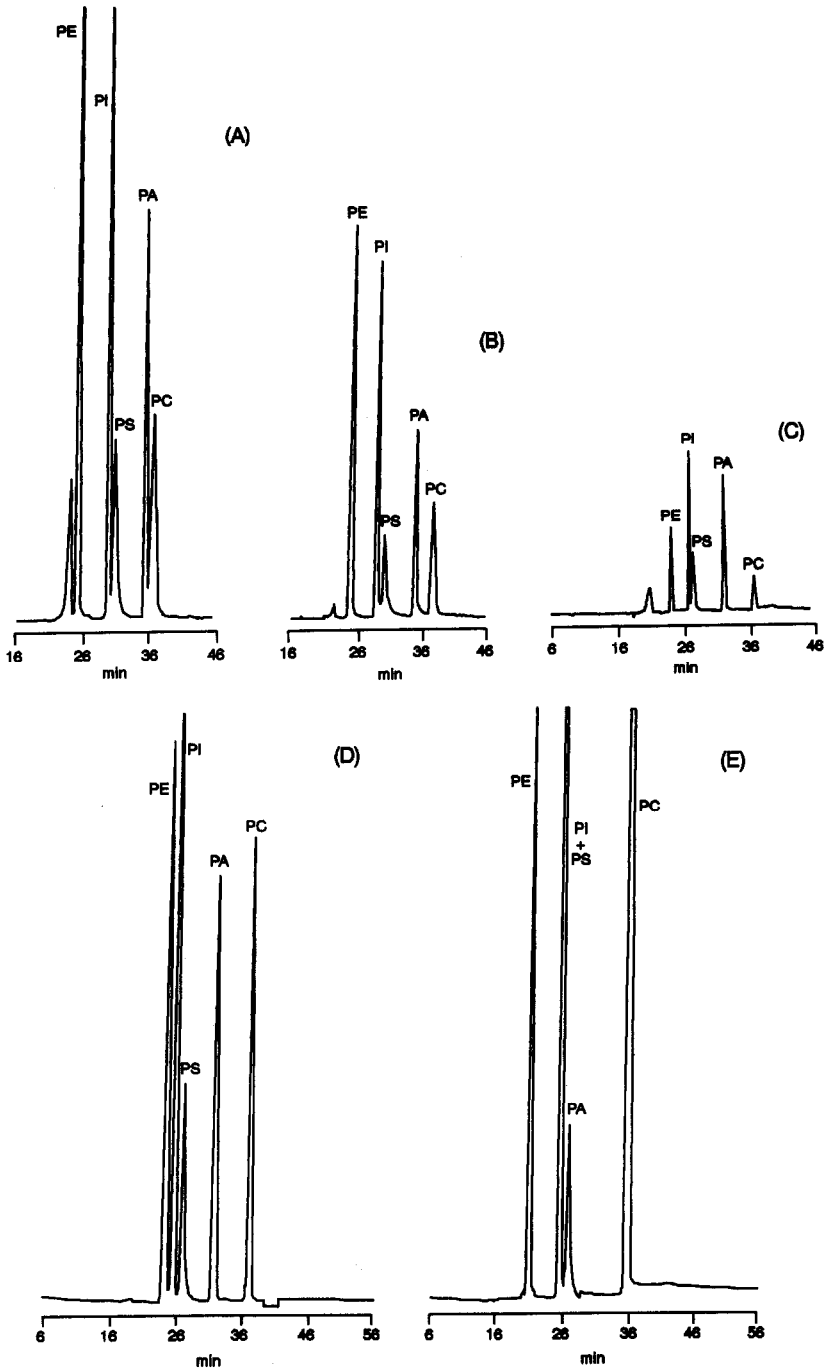


Fig. 5. Normal-phase HPLC separations of PL classes under gradient elution starting from solvent 1 to solvent 2 in 30 min, staying at solvent 2 for 15 min and then returning to solvent 1 in 10 min (solvent cycle 1→2→2→1, time period 30–15–10 min). HPLC conditions for (A), (B), (C), (D) and (E) as in Table V. ELS detector temperature for (A), (B), (C) and (D) 150°C and for (E) 160°C.

ibility and high efficiency. Normal-phase HPLC with mobile phases of different polarity and solvent strength yielded chromatographic profiles largely determined by the polarity of PL solutes. Under the conditions employed, the polar lipids exhibited polarity characteristics in the increasing order PE < PI < PS < PA < PC.

The data in Table V indicate that the mode of elution had a profound effect on the separation of negatively charged PL (PI, PS and PA). For a baseline separation of all five components, PE, PI, PS, PA and PC, the elution mode of choice was a gradient program of 1→2→2→1 (arrows represent stepwise duration times) in which solvent 1 [chloroform tetrahydrofuran (THF)] was pumped through a cycle via solvent 2 (methanol–ammonia solution–chloroform) with corresponding periods of 30, 15 and 10 min for the duration times (30 min from solvent 1 to solvent 2, 15 min hold at solvent 2, then 10 min from solvent 2 to solvent 1). Representative normal-phase HPLC chromatograms showing separations of PA from other PL under various conditions are given in Fig. 5. The order of elution, PE < PI < PS < PC, is the same as that obtained with Christie's systems [23,24]. Sphingomyelin would elute after PC in the present system. The extent of separation between PI and PS, which have close k' values, appeared to be more sensitive to the change in the mode of gradient elution than other adjacent pairs. Regardless of the stationary phases used, incorporation of THF [34] in mobile phases led to better dispersed chromatographic peaks of PA and PC [(A) (ii) *vs.* (B) (ii) and (C) *vs.* (D), Table V]. Further, separations of these two components were notably augmented by reducing the basicity of mobile phases, as indicated in experiments (D) *vs.* (E) and (E) *vs.* (F) in Table V. The chromatographic outcome can be explained in the context of decreasing polarity of PA (less ionized) in mobile phases containing decreased amounts of ammonia solution or in mobile phases whose ammonia contents are replaced with triethylamine of reduced basicity.

CONCLUSIONS

This reversed-phase ion-pair HPLC method represents the first direct approach to tackling the analytical problems associated with the separation of complex mixtures of molecular species derived from PA compounds. The ion-pairing technique provides a mean to delineate the chromatographic characteristics of polar lipids in general and other charged PL in particular. The correlations data can be used for the prediction of unknown k' values of PA and PM analyzed under given mobile phase conditions. For achieving separations under specific HPLC conditions, the method is versatile and can be applied to various analytical systems by optimizing reversed-phase HPLC variables such as stationary phase specfications, mobile phase compositions and types and concentrations of ion-pair reagents. The normal-phase HPLC–ELS detection method permits the simultaneous separation and quantification of PL classes in various samples of plant origin (*e.g.*, soybeans). The method is reliable and useful for the routine analysis of PL samples containing high levels of PA.

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REFERENCES

1 T. L. Mounts and A. M. Nash, *J. Am. Oil. Chem. Soc.*, 67 (1990) 757.
2 N. A. Porter, R. A. Wolf and J. R. Nixon, *Lipids*, 14 (1979) 20.
3 F. B. Jungalwala, V. Hayssen, J. M. Pasquini and R. H. McCluer, *J. Lipid Res.*, 20 (1979) 579.
4 B. J. Comton and W. C. Purdy, *J. Liq. Chromatogr.*, 3 (1980) 1183.
5 M. Smith and F. B. Jungalwala, *J. Lipid Res.*, 22 (1981) 697.
6 G. M. Patton, J. M. Fasulo and S. J. Robins, *J. Lipid Res.*, 23 (1982) 190.
7 B. J. Compton and W. C. Purdy, *Anal. Chim. Acta*, 141 (1982) 405.
8 A. Cantafora, A. Di Biase, D. Alvaro, M. Angelico, M. Martin and A. F. Attili, *Clin. Chim. Acta*, 134 (1983) 281.
9 W. W. Christie and M. L. Hunter, *J. Chromatogr.*, 325 (1985) 473.
10 N. Sotirhos, C. Thorngren and B. Herslof, *J. Chromatogr.*, 331 (1985) 313.
11 S. L. Abidi, T. L. Mounts and K. A. Rennick, *J. Liq. Chromatogr.*, 14 (1991) 573.
12 J. G. Turcotte, J. Y. Hsieh and D. K. Welch, *Lipids*, 16 (1981) 761, and references cited therein.
13 J. Y. Hsieh, D. K. Welch and J. G. Turcotte, *J. Chromatogr.*, 208 (1981) 398.
14 Y. Nakagawa and K. Waku, *J. Chromatogr.*, 381 (1986) 225.
15 L. A. Dethloff, L. B. Gilmore and G. E. R. Hook, *J. Chromatogr.*, 382 (1986) 79.

16 P. Juaneda and G. Rocquelin, *Lipids*, 21 (1986) 239.
17 A. G. Andrews, *J. Chromatogr.*, 336 (1984) 139.
18 H. P. Nissen and H. W. Kreysel, *J. Chromatogr.*, 276 (1983) 29.
19 M. Seewald and H. M. Eichinger, *J. Chromatogr.*, 469 (1989) 271.
20 R. W. Gross and B. E. Sobel, *J. Chromatogr.*, 197 (1980) 79.
21 A. Stolyhwo, M. Martin and G. Guiochon, *J. Liq. Chromatogr.*, 10 (1987) 1237.
22 J. L. Perrin and A. Prevot, *Rev. Fr. Corps Gras*, 11 (1986) 437.
23 W. W. Christie, *J. Lipid Res.*, 26 (1985) 507.
24 W. W. Christie, *J. Chromatogr.*, 361 (1986) 396.
25 P. Juaneda, G. Rocquelin and P. O. Astorg, *Lipids*, 25 (1990) 756.
26 W. M. A. Hax and W. S. M. Geurts van Kessel, *J. Chromatogr.*, 142 (1977) 735.
27 H. Colin and G. Guiochon, *J. Chromatogr.*, 141 (1977) 289.
28 M. C. Hennion, C. Picard and M. Caude, *J. Chromatogr.*, 166 (1978) 21.
29 S. L. Abidi, *J. Liq. Chromatogr.*, 12 (1989) 595.
30 S. L. Abidi, *J. Chromatogr.*, 362 (1986) 33.
31 S. L. Abidi, *J. Chromatogr.*, 255 (1983) 101.
32 Z. Iskandarani and D. J. Pietrzyk, *Anal. Chem.*, 53 (1981) 489.
33 P. C. White and A. M. Harbin, *Analyst (London)*, 114 (1989) 877.
34 S. L. Abidi, *J. Chromatogr.*, 288 (1984) 277.