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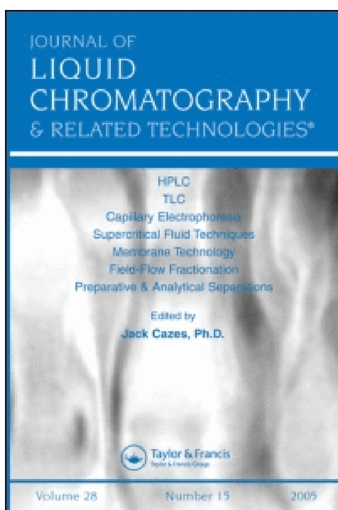

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REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PHOSPHATIDYLINOSITOLS

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ABSTRACT

Reversed-phase ion-pair high-performance liquid chromatographic (HPLC) separations of molecular species of phosphatidylinositols (PI) were studied. Mobile phases of acetonitrile-methanol-water containing various tetraalkylammonium phosphates (TAAP) were used for optimization. Stationary phases of macroporous polystyrene divinylbenzene (MPD), octylsilica, and octadecylsilica exhibited varying degrees of retentivity toward PI solutes. Without exception, capacity factors (k') of PI on either alkylsilica or MPD increased with the alkyl chain length and the concentration of the quaternary ammonium counter ions evaluated. The results can be interpreted in terms of an ion-pair retention mechanism. Logarithmic k' values were linearly related to the total number of carbons in TAAP. Perbenzoylation of PI yielded derivatives readily resolvable without the use of any mobile phase additive. Incorporation of TAAP to a mobile phase facilitated component separations of early-eluting perbenzoates. In HPLC with

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MPD, hydrophobic interactions of the perbenzoate analytes with the stationary phase appeared to arise from the nonpolar tail groups of PI molecules. Potential applicability of the reversed-phase ion-pair HPLC method for analysis of molecular species of PI in soybean oil is demonstrated.

INTRODUCTION

Phospholipids (PL) are glycerides of fatty acids and phosphoric acid derivatives. The hydrocarbonaceous fatty acid chains and the phosphorus-containing moieties constitute the respective nonpolar tail group and polar head group of the PL structures. The acidic function of the phosphatidyl unit is neutralized by amino groups present in some PL, but remains anionically charged in others. Phosphotidylinositol (PI) is an example of the latter class of PL. Chromatographic analysis of the compounds with mixed polarity often requires laborious efforts in optimizing chromatographic conditions. Although there are numerous publications in the literature (1-9) on reversed-phase high-performance liquid chromatography (HPLC) of intact molecules of PL, most of the studies were concerned with the separation of neutral PL and reversed-phase ion-pair HPLC separation of molecular species of anionic PL has not been reported.

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In connection with another study on the stability of soybean oil during storage, it was necessary to monitor the PL content in crude and degummed soybean oil at various stages of storage. With little difficulty, reversed-phase HPLC analysis of neutral PL in methanol-acetonitrile-water systems provided useful information about olecular species distributions in each class of PL, phosphotidylethanolamine (PE) and phosphatidylcholine (PC), found in soybean oil. However, the molecular species of anionic PL (phosphatidic acid and PI) were not separated by conventional reversed-phase HPLC. In this case, only a single peak coeluting with the solvent front or unresolved ill-defined peaks were observed. Subsequent HPLC experiments with ion-pairing techniques led to excellent separations of molecular species of PI. The results are reported in this paper.

EXPERIMENTAL

Materials:

Soybean phosphatidylinositol was purchased from Avanti Polar Lipids, Inc. (Pelham, Alabama). Tetrabutylammonium phosphate (TBAP), pentyltriethyl ammonium phosphate (PTAP), hexytriethylammonium phosphate (HTAP), heptyltriethylammonium phosphate (HPTAP), octyltriethylammonium phosphate (OTAP) and pyridine were purchased from Regis Chemical Company (Morton Grove, Illinois). Benzoyl chloride was obtained from Aldrich Chemicals (Milwaukee, Wisconsin). Perbenzoates were prepared by treating PI with benzoyl chloride in the presence of pyridine at 60°. After removal of solvent, the reaction mixture was dissolved in chloroform, washed with water and dried over anhydrous sodium sulfate to give the derivatives suitable for HPLC analysis. HPLC solvents, acetonitrile and methanol were obtained from J. T. Baker, Inc. (Phillipsburg, New Jersey). Ultrapure HPLC grade water was obtained by purification through a Millipore Milli Q water purifier system. Other inorganic reagents were products of J.T. Baker, Inc.

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Methods.

A Spectra-Physics (San Jose, California) Model SP8700 liquid chromatograph equipped with a multiple wavelength UV detector [LDC (Riviera Beach, Florida) spectroMonitor D] was used in all HPLC work. In the initial phase of this work, a Varex (Rockville, Maryland) evaporative light scattering (ELS) detector Model ELSD II was also used. Mobile phases containing different concentrations of an ion-pairing reagent were prepared by mixing a solution of acetonitile-methanol-water with an exact amount of the quaternary ammonium salt. Analytical samples (5-10 ul of 10 mg/ml solutions) were injected via a rheodyne (Cotati, California) Model 7125 injector (10 or 100 ul loop) onto a reversed-phase HPLC column. Five different stationary phases were used:(1) Waters (Milford, Massachusetts) NovaPak C18, 3.9 x 300 mm, 4 um, (2) polymeric resins of macroporous polystyrene divinylbenzene (MPD),

PLRP-S-100, 4.6 x 250 mm, 5 um (Polymer Laboratories, Amherst, MA), (3) EM Science (Gibbstown, New Jersey) Lichrosorb RP-C18, 4.6 x 250 mm, 10 um, (4) Brownlee (Applied Biosystems, Foster City,California) Spheri-5 RP-8, 4 x 220 mm, 5 um, and (5) Alltech (Deerfield, Illinois) Adsorbsphere C18, 4.6 x 250 mm, 5 um. Mobile phases were filtered, degassed, and pumped through the column at a flow rate of 1 ml/min unless specified otherwise. Capacity factors (k') were determined from the equation $k' = t/t_o - 1$, where t and t_o are the retention times of an analyte and an unretained solute, respectively.

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RESULTS AND DISCUSSION

As mentioned, HPLC separation of molecular species of neutral phospholipids PE and PC can be readily accomplished with a number of reversed phase stationary phases using appropriate mobile phases of water and organic solvents. However, in these simple HPLC solvent systems, components of phosphatidylinositol (PI) are neither separable nor retained by the column because of the presence of a negative charge and five hydroxy groups in the molecule (structure I, Fig. 1). In light of our previous successful experience with HPLC of anionic carboxylates derived from aminobenzoic acids (10), a series of tetraalkyl ammonium phosphates (TAAP) were chosen to study the retention behavior of the title polar lipids. The quaternary ammonium compounds include tetrabutyl ammonium phosphate (TBAP), pentyltriethyl ammonium phosphate (PTAP), hexyltriethyl ammonium phosphate (HTAP), heptyltriethyl ammonium phosphate (HPTAP), and octytriethyl ammonium phosphate (OTAP). Each of these ammonium salts serves as a counter ion to the anion situated at the head group of the lipid and contributes some degree of hydrophobicity to the PI analytes of interest.

At the onset of this work, reversed-phase HPLC of PI was carried out under the same mobile phase conditions [acetonitrile-methanol-water (70:15:15)] as those used previously for HPLC of PC

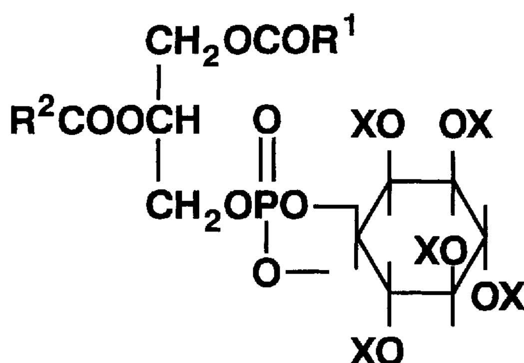

Figure 1. Structures of phosphatidyinositol. I, parent compound; II, perbenzoate. R^1 and R^2 = alkyl or alkenyl groups of fatty acids.

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(8). The results were unsatisfactory. Fig. 2 compares HPLC separations of PI in the absence and in the presence of a tetraalkylammonium salt in the specified mobile phase. It was clearly demonstrated that addition of TAAP to an aqueous mobile phase containing organic modifiers not only benefited the separation but also improved the chromatographic peak characteristics. Furthermore, far better defined peaks were obtained when the ammonium salt was present in larger quantities (see B _vs_ C, Fig. 2). Fig. 3 shows chromatograms obtained with two other stationary phases which are different from that shown in Fig. 2. Different solvent systems were used for bringing the chromatographic peaks within reasonable retention times. Evidently, the types and particle sizes (4 um, 5um, and 10 um) of column packings had notable effects on the separation of components. The number of components being separated increased with increasing efficiency

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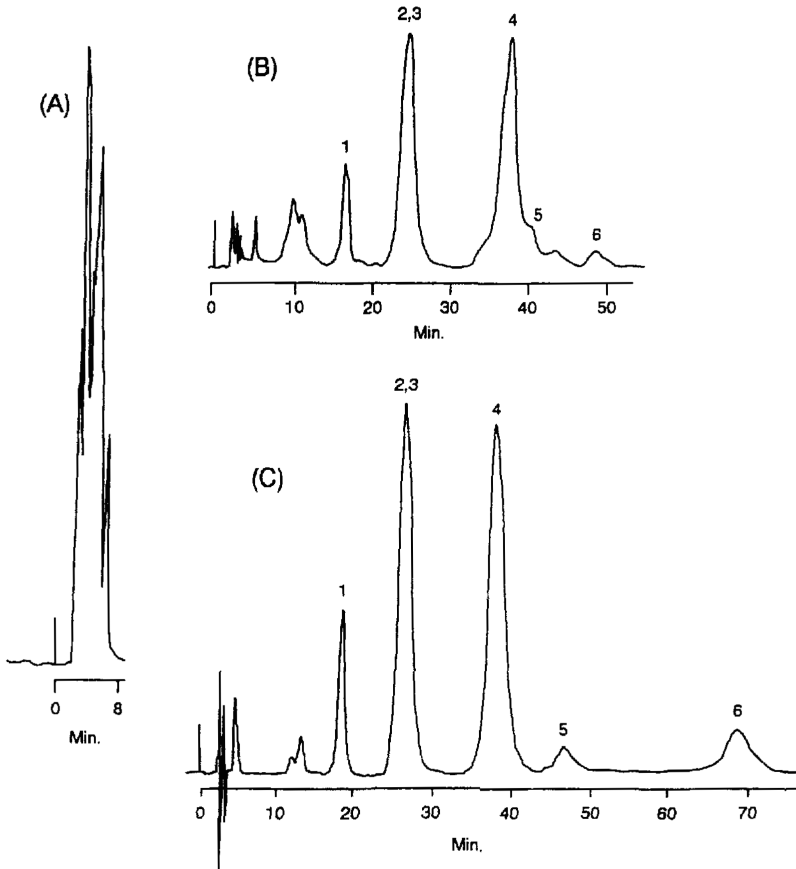
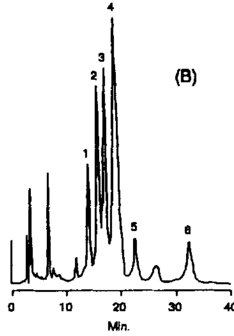


Figure 2. HPLC separation of molecular species of Phosphatidylinositol with a mobile phase of acetonitrile-methanol-water (70:15:15) containing (A) no salt, (B) 10% 5mM HPTAP, and (C) 100% 5mM PTAP.Column, Lichrosorb RP C18, 10 um. UV detector, 208 nm. See Table I for peak identification.



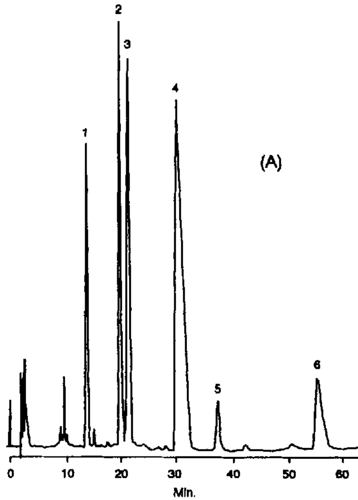


Figure 3. HPLC separation of molecular species of phosphatidylinositol on (A) NovaPak C18, 4 um, and (B) polystyrene divinylbenzene, PLRP-S-100 (MPD), 5 um. Mobile phase conditions: (A) acetonitrile-methanol-water (70:22:8) containing 5mM TBAP, (B) acetonitrile-methanol-water (70:5:25) containing 5mM HPTAP. UV detector, 208 nm.

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Table I. Effect of the size of quaternary ammonium counter ions on k' of phosphatidylinositols.

Component	k'* Mobile phase counter ion				
	PTAP	HTAP	HPTAP	OTAP	TBAP
1.	1.56	1.73	1.94	2.22	2.78
2.	2.67	2.94	3.26	3.67	4.44
3.	3.00	3.29	3.61	4.00	4.89
4.	4.67	5.16	5.65	6.22	7.56
5.	5.78	6.33	6.96	7.67	9.33
6.	8.72	9.63	10.7	11.8	14.4

* Stationary phase: NovaPak C18. Mobile phase conditions: acetonitrile-methanol-water (70:22:8) containing 5 mM of a quaternary ammonium salt. PTAP=pentyltriethylammonium phosphate. HTAP=hexyltriethylammonium phosphate. HPTAP=heptyltriethylammonium phosphate. OTAP= octyl triethylammonium phosphate. TBAP=tetrabutylammonium phosphate. Detector UV 208 nm. Flow rate 1 ml/min. Component identification: (1) 16:0-18:3, (2) 18:1-18:2, (3) 16:0-16:0, (4) 16:0-18:2, (5) 16:0-18:1, and (4) 18:0-18:0 corresponding to the R^1 and R^2 groups of fatty acid chains in PI (Fig. 1).

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of the column (smaller particle size of packings). With a macroporous polystyrene divinylbenzene (MPD) resin column, PI components were not as well resolved as with alkylsilica despite some unusual selectivity of the MPD phase for resolving components 2 and 3. As expected, hydrophobic interactions between predominantly aromatic moieties of the MPD phase and hydrophobic fatty acid moieties of PI molecules were less favored than those between alkylsilica and the nonpolar tail groups of the polar lipid.

Table II. Effect of the concentration of quaternary ammonium phosphates on k' of phosphatidylinositols

Component	k'* Mobile phase counter ion concentration (mM)			
	0.00	1.25	2.50	5.00
(A) PTAP				
1.	0.00	0.11	0.67	1.56
2.	0.00	0.44	1.22	2.67
3.	0.00	0.56	1.44	3.00
4.	0.00	1.22	2.33	4.67
5.	0.00	1.56	2.89	5.78
6.	0.00	2.61	4.67	8.72
(B) TBAP				
1.	0.00	0.44	1.00	2.78
2.	0.00	0.89	1.67	4.44
3.	0.00	1.00	1.89	4.89
4.	0.00	1.78	3.11	7.56
5.	0.00	2.22	3.89	9.33
6.	0.00	3.78	6.11	14.4

* Stationary phase: NovaPak C18. Mobile phase conditions: acetonitrile-methanol-water (70:22:8) containing variable concentrations of (A) PTAP or (B) TBAP. Abbreviations and other conditions same as in Table I.

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In reversed-phase ion-pair HPLC of PI, two fold effects of quaternary ammonium counter ions on capacity factors, k', of molecular species were observed depending upon (1) the nature of the quaternary ammonium reagent or the chain length (or the size) of alkyl groups, and (2) the concentration of the ammonium salt. The results are summarized in Table I and Table II. Examination of the retention data in Table I revealed that HPLC with larger alkyl groups of a quaternary ammonium phosphate in the mobile phase produced molecular species of higher retention characteristics. The capacity factors (k') of individual molecular species on a NovaPak C-18 column increased as the chain length of the counter ions increased from carbon-5 pentyl to carbon-8 octyl of the quaternary ammonium ions in the series. The total number of carbons in the tetraalkyl groups of the ammonium salt appeared to be the determining factor for solute retention, since the highest k' values of PI components were obtained with mobile phases containing the bulkiest ammonium compound in the series, TBAP, as the counter ion (Table I).

As shown in Table II, the concentration dependence of capacity factors, k', was evaluated using three different concentrations 1.25 mM, 2.50 mM, and 5.00 mM of TAAP. HPLC with higher concentrations of quaternary ammonium ions TBAP, PTAP, HTAP, HPTAP, or OTAP in mobile phases led to more strongly retained (higher k' values) analytes on an alkylsilica stationary phase (NovaPak C-18 was used in this case). The results are parallel with those found in a previous study on aminobenzoic acids(10). In contrast, a reversal in concentration effects (11,12) was observed when reversed-phase HPLC of PI was carried out under different mobile phase conditions in which triethylamine was used as the additive to accommodate the use of an evaporative light scattering (ELS) detector. However, separations of molecular species in the experiments employing triethylamine, shown in Fig. 4, were incomplete and inferior to those obtained with mobile phases containing quaternary ammonium compounds (Table II). The

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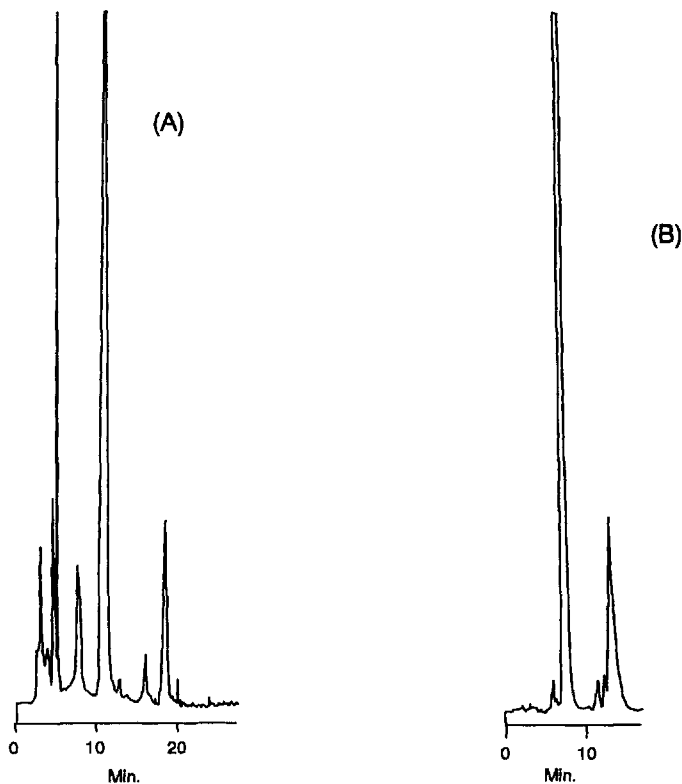

Figure 4. HPLC separation of PI on (A) Adsorbsphere (C18 mobile phase:(acetonitrile/20mM Et_3N)-(water/20 mM Et_3N) (80:20); (B) MPD, mobile phase: (acetonitrile-methanol-water) (70:15:15)-(water/20 mM Et_3N) (80:20). ELS detector, 150°C.

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latter mobile phases were not suitable for ELS detection because of nonvolatility of TAAP.

Correlation of capacity factors, k', of major PI components with the size of quaternary ammonium counter ions used in mobile phases established linear relationships between logarithmic k' values and the total number of carbons of the tetraalkyl groups on the ammonium ions (Fig. 5). In agreement with previous

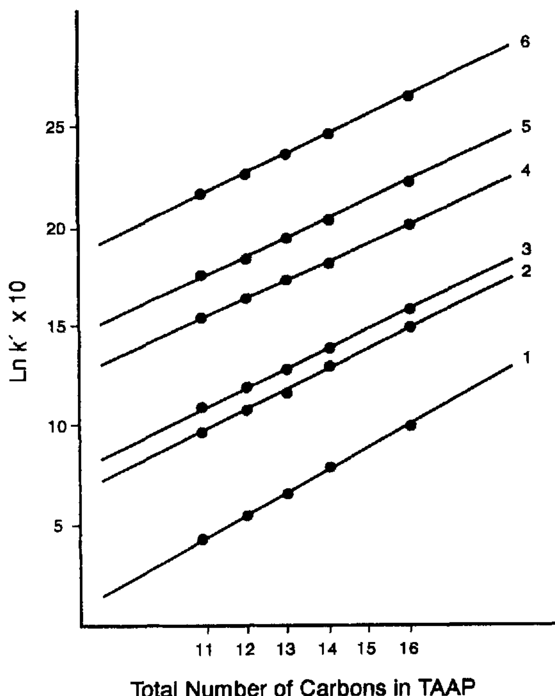

Figure 5. Linear correlation between logarithmic capacity factors, k', and the total number of carbons of tetraalkylammonium phosphates.

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observations (13, 14), the total area of the tetraalkyl groups of TAAP is available for solvophobic interactions. The correlation data allow for prediction of k' values of molecular species analyzed by reversed-phase ion-pair HPLC with mobile phases containing other members of the TAAP series. The positive slope values of the plots (Fig. 5) coupled with results from Table Iand II are indicative of an ion-pair mechanism by which HPLC separation of PI solutes are believed to proceed during the chromatographic process. On the other hand, reversed-phase HPLC of the lipid analytes in the presence of triethylamine yielded

Table III. Reversed-phase ion-pair HPLC of phosphatidylinositols under various chromatographic conditions*.

Counter ion	k' Component 1	2	3	4	5	6
(A)						
(i)						
PTAP	1.89	2.11	2.27	2.56	3.07	4.41
HPTAP	1.96	2.30	2.41	2.85	3.37	4.78
(ii)						
PTAP	5.43	8.29	8.29	12.6	15.6	23.4
HPTAP	6.86	10.4	10.4	15.9	20.0	30.2
(B)						
(i)						
HPTAP (2.5 mM)	3.30	3.89	4.19	4.78	5.74	8.78
HPTAP (5 mM)	4.33	5.00	5.37	6.11	7.44	11.2
(ii)						
HPTAP (1.25 mM)	1.60	1.71	1.79	2.39	2.72	4.11
HPTAP (2.5 mM)	2.59	3.20	3.41	4.50	5.01	7.73
(C)						
PTAP	1.71	2.43	2.43	3.86	4.57	6.64
TBAP	2.21	3.36	3.57	5.16	6.21	9.14

* HPLC conditions: (A), acetonitrile-methanol-water (70:15:15) containing 5 mM of quaternary ammonium salt, stationary phases (i) MPD (ii) Lichrosorb C-18; (B) acetonitrile-methanol-water (70:5:25) with stationary phases (i) MPD, and (ii) octylsilica (Spheri-5 RP8); (C) acetonitrile- methanol-water (70:22:8) containing 5 mM of the salts with a Lichrosorb C-18 column.
MPD=macroporous polystyrene divinylbenzene
PTAP=pentyltriethylammonium phosphate.
HPTAP=heptyltriethylammonium phosphate. Abbreviations and other conditions same as in Table I.

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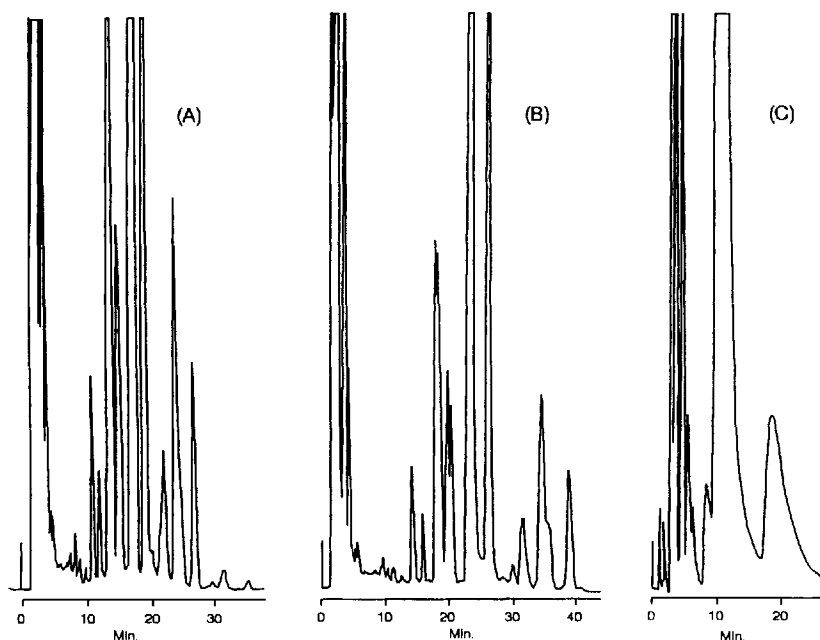

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Figure 6. HPLC separataion of perbenzoates of PI on (A) NovaPak C18, mobile phase: methanol; (B) NovaPak C18, mobile phase:(methanol)-[(acetonitrile methanol-water (70:22:8) containing 5mM PTAP] (90:10; (C) MPD, mobile phase: methanol. UV detector, 208 nm.

results incompatible with ion-pair retention rationalization. Nevertheless, the presence of triethylamine in the mobile phases significantly improved peak characteristics and peak resolution presumably due to minimization of analyte adsorption on alkylsilica by virtue of triethylamine-silinol interactions.

Inspection of HPLC data in Table III showed that mobile phase solvent compositions had insignificant effects on the separation of PI molecular species. Depending on the stationary phases used, an increase in water content of a mobile phase resulted in longer retention times of the analytes with little improvement in component resolution. The retention characteristics of PI observed

in separate HPLC experiments with MPD, Lichrosorb C-18 and octylsilica (OS) stationary phases under various mobile phase conditions (Table III) further reflect ion-pair separation processes. Of the stationary phases evaluated, the Lichrosorb C-18 column (10 um) was the least satisfactory phase for the separation of component 2 and 3, though adding TBAP to the mobile phase led to some degree of separation of these two components (experiment C in Table III).

The reversed-phase HPLC results reported here suggest that molecular species of PI can be resolved only when mobile phases contain cationic counter ions capable of forming ion-pairs with the anionic analytes in the HPLC systems. Perbenzoylation of PI markedly reduced the polarity of the compound to such an extent that its molecular species were efficiently separated by reversed-phase HPLC on octadecylsilica (ODS) in the absence of any counter ion in the mobile phases (Fig. 6A). However, incorporation of a quaternary ammonium salt in a mobile phase seemed to enhance the separation of some early eluting components (Fig. 6B). Interestingly, with HPLC on MPD, the perbenzoate derivatives (structure II, Fig. 1) of PI were poorly resolved, if not at all (Fig. 6C). In view of the aromatic structures of polymeric resins, MPD would be expected to participate in pi-pi interactions with the perbenzoates and therefore would be more retentive than what was actually observed. The apparent lack of pi-pi interactions is presumably due to prevailing hydrophobic interactions of the nonpolar tail groups of PI solutes with the stationary phases.

In conclusion, molecular species of PI can be separated by reversed-phase ion-pair HPLC with UV detection. As in common practice, it is necessary to isolate each component from the PI mixture for characterization of fatty acids and for the determination of compositions of molecular species. Since mobile phases used in ion-pair HPLC are not amenable with the evaporative light scattering detector (mass detector), the possibility of simultaneous separation and quantitation of PI by reversed-phase ion-pair HPLC with mass detection is precluded.

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