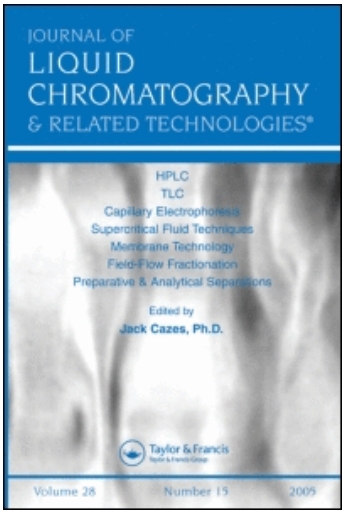

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SEPARATION OF MOLECULAR SPECIES OF PHOSPHATIDYLSERINE BY REVERSE-PHASE ION-PAIR HPLC

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ABSTRACT

Molecular species of phosphatidylserines (PS) were separated by reverse-phase ion-pair high-performance liquid chromatography (RP-IP-HPLC). HPLC mobile phases contained either tetraalkyl ammonium phosphates (TAAP) or alkyltriethyl ammonium phosphates (ATAP) in acetonitrile-methanol-water. Separations of the PS components on several different alkylbonded silica stationary phases were compared. The presence of high concentrations of TAAP (or ATAP) in mobile phases invariably enhanced the retention of the analytes on a RP column. Capacity factors (k') of the lipid components increased with increasing hydrophobicity of the ammonium counter ions. The HPLC results are indicative of solute retention via ion-pairing processes. Logarithmic k' values of analytes were linearly related to the total number of carbons in each series of the quaternary ammonium salts. However, linear relationships failed when correlated with counter-ions of different structural series. The counter-ion effects were also influenced by the structures of glycerophospholipids. A marked dependence of k' values of PS on the mobile phase acidity was observed.

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INTRODUCTION

Structures of glycerophospholipids (GPL) generally consist of nonpolar tail groups of fatty acid chains and polar head groups of phosphoric acid derivatives. There are three structural types of GPL naturally occurring in cell membranes: (1) GPL with neutral charges, (2) negatively-charged GPL, and (3) a combination of (1) and (2) with a net negative charge in the molecule. As a prototype of the polar lipids of the last structural type, phosphatidylserines (PS) are widespread but minor cell constituents found in both animals and plants. The compounds have a negative charge at the head group in addition to the pair of neutralized charges located at the serine moiety. The presence of R^1 and R^2 groups (Fig. 1) in PS is responsible for the existence of molecular species. In recent years, there are many publications (1-9) in the literature dealing with reverse-phase high-performance liquid chromatgraphy (RP-HPLC) of neutral GPL. However, little work has been done on the HPLC separation of negatively-charged GPL without chemical derivatization (10,11). Derivatives of the PS complex have been resolved by thin layer chromatography (12,13) but the parent compounds have only been resolved by RP-HPLC in a mobile phase containing choline chloride and potassium phosphate in a solution of acetonitrile-methanol-acetic acid (4). The intact molecular species of PS have not been resolved previously by RP-ion-pair-HPLC.

Analysis of GPL in soybean oil provides useful information on the stability and processing of the oil. PS and some glycerol phospholipids are among the least abundant GPL found in soybeans lecithins (14). Our continuing interest in studying the deterioration of soybean GPL during storage required the development of feasible analytical procedures for the speciation of molecular species of soybeans GPL including the minor polar lipids. Initially, attempted separations of PS by conventional RP-HPLC without any mobile phase additive failed to resolve the components. This paper reports the successful resolution of the

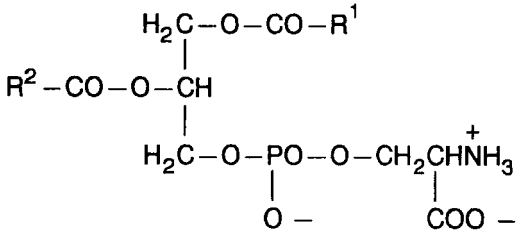

Fig. 1. Structure of phosophatidyl serine (PS. R^1 and R^2 represent alkyl or alkenyl groups of fatty acids.

PS complex by RP-ion-pair-HPLC and their retention behavior observed under various HPLC conditions.

EXPERIMENTAL

Materials:

Phosphatidylserine (dry powder or in chloroform solution) was purchased from Avanti Polar Lipids, Inc. (Pelham, Alabama), Matreya, Inc. (Pleasant Gap, Pensylvania), Calbiochem Corporation (San Diego, California), or from Sigma Chemical Co. (Saint Louis, Missouri). Other glycerophospholipids (GPL) were obtained from Avanti Polar Lipids. Tetramethyl ammonium phosphate (TMAP), tetraethyl ammonium phosphate (TEAP), tetrapropyl ammonium phosphate (TPAP) were prepared from the corresponding hydroxides [Aldrich Chemical Co. (Milwaukee, Wisconsin)] by titration with phosphoric acid until reaching a desired pH value. Tetrabutyl ammonium phosphate (TBAP), pentyltriethyl ammonium phosphate (PTAP), hexytriethyl ammonium phosphate (HTAP), heptyltriethyl ammonium phosphate (HPTAP), and octyltriethyl ammonium phosphate (OTAP) were purchased from Regis Chemical Company (Morton Grove, Illinois). HPLC solvents acetonitrile and methanol were obtained from J. T. Baker, Inc. (Phillipsburg, New Jersey). Ultrapure

HPLC-grade water was obtained by filtering distilled water through a Millipore (Bedford, MA) Milli Q water purifier system. Other inorganic chemicals were obtained from Fisher Chemicals (Fair Lawn, New Jersey).

Methods:

In all HPLC analyses, a Spectra Physics (San Jose, California) Model SP8700 liquid chromatograph interfaced with a multiple wavelength UV detector (LDC Spectromonitor D) was used. Mobile phases comprised various concentrations (1.25 mM-5.00 mM) of different types of ion-pairing reagents, acetonitrile, methanol, and water. All mobile phases were prepared daily before analyses. Aliquots of freshly prepared samples (5-10 μl of 5 mg/mL solutions) were injected via a Rheodyne (Cotati, California) Model 7125 injector (100 ul loop) onto a reverse-phase HPLC column. In some experiments, relatively old samples that had been standing in the freezer for 5-10 days were also used. All column packings were of octadecylsilica prepacked in stainless steel columns as supplied by commercial sources: (1) Waters (Milford, Massachussette) NovaPak C18, 300 mm x 3.9 mm I.D., 4 μm, (2) Beckman (San Ramon, California) Ultrasphere ODS, 150 x 4.6 mm I.D., 5 μm, (3) Alltech (Deerfield, Illinois) Econosphere C18, 100 x 4.6 mm I.D., 3 μm, and (4) E.M. Separation (Gibbstown, N.J.) Supersphere 100 RP-18e, 250 x 4 mm I.D, 3 μm. In typical HPLC analyses, 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.

PS molecular species were isolated by collecting individual HPLC peak components and were then converted to fatty acid methyl esters using a published methanol/hydrochloric acid procedure (15). Fatty acid analyses were performed with a Varian Model 3400 gas chromatograph fitted with a 0.25 mm x 30 m fused silica capillary column coated with 0.2 μm SP 2330 (Supelco Inc.,

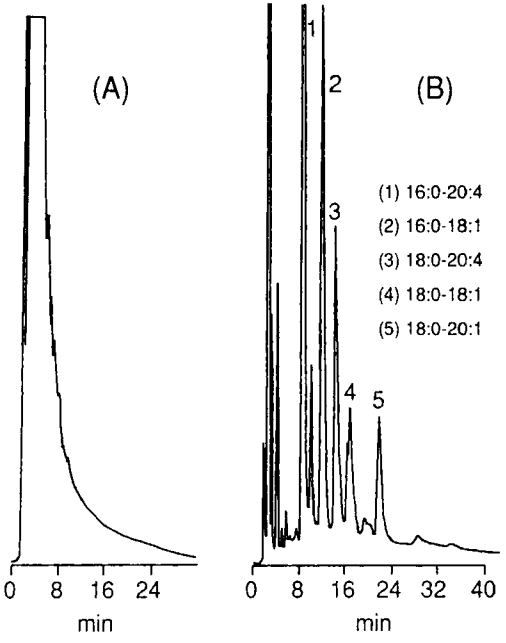

Fig. 2. HPLC separations of PS components with a mobile phase of acetonitrile-methanol-water (70:22:8) containing (A) no salt, (B) 5 mM TEAP, pH 7. Column, NovaPak C18. UV 208 nm. Fatty acids: 16:0=palmitic, 20:4=arachidonic, 18:1=oleic acid, 18:0=stearic, 20:1=eicosenoic.

Bellefonte, PA). A flame ionization detector built in the chromatograph was used throughout the analyses. A column temperature program was run from 200 to 220°C at 10°C/min after an initial hold of 15 min.

RESULTS AND DISCUSSION

Strikingly similar to the elution behavior of phosphatidyl inositols (PI) (10) and phosphatidic acid (PA) (11), RP-HPLC of PS in the absence of mobile phase additives yielded ill-defined and

unresolved peaks with little retention of the analytes on the column used. This is a common phenomenon attributable to minimum hydrophobic interactions between the highly polar negatively-charged head group of GPL with the octadecylsilica stationary phase. However, addition of a cationic ion-pairing reagent to the mobile phase significantly improved the chromatographic behavior of PS components (Fig. 2). This modification of mobile phase systems also resulted in considerable enhancement in the retention of the negatively-charged analytes on the RP-HPLC column. To further understand the nature of mobile phase salt effects, two series of quaternary ammonium salts were chosen to serve as ion-pairing counter-ions: (1) alkyltriethyl ammonium phosphates (ATAP) and (2) tetraalkyl ammonium phosphates (TAAP). The first type includes ethyltriethyl ammonium phosphate (ETAP), pentyltriethyl ammonium phosphate (PTAP), hexyltriethyl ammonium phosphate (HTAP), heptyltriethyl ammonium phosphate (HPTAP), and octyltriethyl ammonium phosphate (OTAP). The second type includes homologues of symmetrically substituted alkyl ammonium compounds: tetramethyl ammonium phosphate (TMAP), tetraethyl ammonium phosphate (TEAP), tetrapropyl ammonium phosphate (TPAP), and tetrabutyl ammonium phosphate (TBAP).

Retention data for five PS components are summarized in Table I and II to show the effect of the type of respective ATAP- and TAAP-counter-ions on the capacity factors (k') of the molecular species. In general, the k' values of the analyte components increased with an increase in the size of the quaternary ammonium compounds in the mobile phases. This counter-ion size effect was found to be much greater in the ATAP series (Table I) than in the TAAP series (Table II) as demonstrated by the greater magnitude of slope (b) values observed in ATAP. Results of linear correlation between logarithmic k' values of PS molecular species and the total number (N) of carbon atoms in ATAP (or TAAP) are given in Table I, Table II and Fig. 3. Each straight line in the correlation plots can be expressed by the following equation:

$$\ln k' = a + bN$$

Table I. Effect of alkyltriethyl ammonium (ATAP) counter ions on k′ of phosphatidylserine components and linear relation between Ln k′ and the total number of carbon atoms in ATAP (Ln k′ = a + bN)*

Component	k′* Mobile phase counter ion				
	ETAP	PTAP	HTAP	HPTAP	OTAP
1.	3.82	6.05	7.13	8.63	9.50
2.	5.26	6.42	10.0	12.8	14.3
3.	5.99	10.4	12.3	14.5	17.8
4.	6.82	12.4	14.5	18.2	22.3
5.	9.03	16.1	19.6	25.0	27.8

	Ln k′= a + bN	
	a	b
1	0.12	0.155
2	0.32	0.166
3	0.35	0.183
4	0.37	0.200
5	0.63	0.196

* RP-HPLC column: NovaPak-C18. Mobile phase conditions: acetonitrile-methanol-water (70:22:8) containing 5 mM of an alkyltriethyl ammonium salt.
Detector UV 208 nm. Flow rate 1 ml/min.
ETAP=ethyltriethyl ammonium phosphate.
PTAP=pentyltriethyl ammonium phosphate.
HTAP=hexyltriethyl ammonium phosphate.
HPTAP=heptyltriethyl ammonium phosphate.
OTAP=octyltriethyl ammonium phosphate.
See text on Ln k′= a + bN

Table II. Effect of tetraalkyl ammonium phosphate (TAAP) counter ions on k' of phosphatidylserine components and linear relation between ln k' and the total number of carbon atoms in TAAP (Ln k'= a + bN)*

Component	k'* Mobile phase counter ion				Ln k'= a + bN	
	TMAP	TEAP	TPAP	TBAP	a	b
1.	1.69	2.25	2.99	4.02	0.24	0.070
2.	2.37	3.25	4.31	5.87	0.57	0.074
3.	2.89	3.88	5.21	7.01	0.76	0.075
4.	3.39	4.75	6.11	7.88	0.93	0.073
5.	4.26	6.00	8.25	10.9	1.12	0.076

* RP-HPLC column: NovaPak-C18. Mobile phase conditions: acetonitrile-methanol-water (70:22:8) containing 5 mM of a tetraalkyl ammonium salt.
Detector UV 208 nm. Flow rate 1 ml/min.
TMAP=tetramethyl ammonium phosphate.
TEAP=tetraethyl ammonium phosphate.
TPAP=tetrapropyl ammonium phosphate.
TBAP=tetrabutyl ammonium phosphate.
See text on Ln k'= a + bN

where k', b and N are defined as above; a represents the k' value at N=0. Under specified HPLC conditions, these linear relationships can be used for the prediction of retention times of PS analytes when a new homologous member of the quaternary ammonium compounds in the same structural series is considered for incorporation into the mobile phase. This facilitates quantification of PS on a RP column of particular specifications within optimal analysis times.

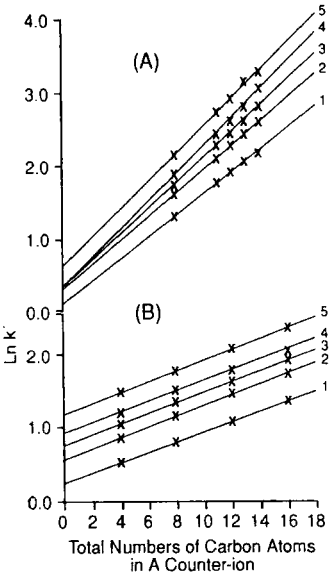

Fig. 3. Linear correlation between Ln k' of PS components and the total number of carbon atoms in (A) ATAP, pH 6.5 (B) TAAP, pH 7.5. Mobile Phase, acetonitrile-methanol-water (70:22:8) containing 5 mM of quaternary ammoniiiium phosphates. Column, NovaPak C18. See Fig. 2 for component identification.

In comparison with an earlier HPLC study (10) of PI, linear relationships established between logarithmic k' values of PI molecular species and the total number of carbon atoms generally resembled those of PS. Each of the k' values of PI obtained with TBAP in the mobile phase coincided with the appropriate correlation line at its abscissa corresponding to a total number of carbon atoms of sixteen. Thus, based on the retention data from RP-HPLC of PI, it was possible to draw straight lines from Ln k'-N plots, regardless of structural types of the ion-pairing reagents used. However, the situation in HPLC of PS was quite different. Attempts to correlate k'values of PS obtained with

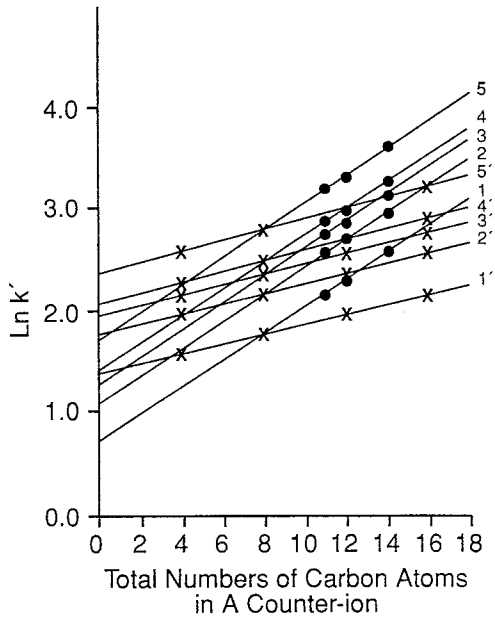

Fig. 4. Linear correlation between Ln k' of PS components and the total number of carbon atoms at ATAP (dot) and in TAAP (Cross). Mobile Phase, acetonitrile-methanol-water (70:22:8) containing 5 mM of quaternary ammonium phosphates, pH 6.5. Column, Ultrasphere ODS. See Fig. 2 for component identification.

mobile phases containing counter-ions derived from different structural series with their total number of carbon atoms were unsuccessful. For example, each of the k' values of PS obtained with TBAP failed to fall on its correlation line at an abscissa corresponding to a total number of carbon atoms of sixteen. In other words, the k' values of PS can only be linearly related with the total number of carbon atoms of a quaternary ammonium compound in the same series as illustrated in Fig. 4. The correlation curves obtained with NovaPak C18 column (Fig. 3) are similar to those obtained with Beckman ODS column (Fig. 4). The versatility

Table III. Effect of the concentration of quaternary ammonium phosphates on k' of phosphatidylserines components

Component	k'* Mobile phase counter ion concentration 0.00	1.25	2.50	5.00 (mM)
HTAP				
1.	0.00	2.75	4.38	7.13
2.	0.00	4.25	5.63	10.0
3.	0.00	5.00	7.25	12.3
4.	0.00	6.52	8.38	14.5
5.	0.00	7.81	11.3	19.6
TBAP				
1.	0.00	2.13	2.87	4.02
2.	0.00	3.53	4.03	5.87
3.	0.00	4.05	4.54	7.01
4.	0.00	5.25	5.63	7.88
5.	0.00	6.00	6.75	10.9

* RP-HPLC column: NovaPak C18. Mobile phase conditions: acetonitrile-methanol-water (70:22:8) containing variable concentrations of HTAP or TBAP. Abbreviations and other conditions same as in Tables I and II.

and analytical applicability of the correlation method are of practical significance.

Table III shows the HPLC results obtained with different concentrations of quaternary ammonium counter-ions. From the table, the general trends of counter-ion concentration effects on the K' values of PS components are clearly demonstrated. The

Table IV. Effect of mobile phase pH on k′ of phosphatidylserine components.

Mobile phase pH	k′ Component 1	2	3	4	5
TMAP					
7.50	1.69	2.37	2.89	3.39	4.26
7.00	5.63	8.38	10.3	12.3	16.4
6.15	10.3	15.5	19.65	22.8	32.0
4.32	16.3	24.8	30.3	32.3	47.8

*HPLC conditions: acetonitrile-methanol-water (70:22:8) containing 5 mM of quaternary ammonium salt, stationary phases: NovaPak C18.
Abbreviations and other chromatographic variables as in Tables I and II.

polar lipid analytes were more strongly retained on the reverse-phase column when higher concentrations of the ammonium counter ions were used in the mobile phases. The observations are like those found in previous studies on PI (10) and on aromatic carboxylic acids (16), but are opposite to those of high-molecular-weight cationic compounds (17,18) and neutrally-charged GPL (19) It was of interest to note that the k′ values of PS were invariably more responsive to the change in the concentration of the unsymmetrically substituted ammonium counter ions (ATAP) than that of the symmetrically substituted ammonium counter-ions (TAAP). Such differential influence of structures of quaternary ammonium counter ions in association with their concentrations on k′ values of PS molecular species are parallel to those derived from size effects described above. It is not clear why the unsymmetrical ion-pair reagents in the ATAP series participated

in stronger hydrophobic interactions with the analyte solutes than those in the symmetrical counter-ions in the TAAP series.

As depicted in Fig. 1, the PS structure contains an amino acid moiety along with a negative charge in the phosphoryl moiety. Due to the presence of these acidic functionalities in the molecule, the retention characteristics of PS would be expected to be sensitive to the variation in mobile phase pH. Table IV presents the retention data for PS studied under four different mobile phase pH. Obviously, there was no indication of improvement in component separation by the modification of mobile phase pHs. However, a decrease in pH caused a large increase in the k' values of the lipid components being separated. For example, when the mobile phase pH was changed from pH 7.5 to 4.32, there were corresponding drastic increases in the k' values of component 1 changing from 1.69 to 16.3, while the k' values of component 5 sharply increased from 4.26 to 47.8 in response to the above change in the mobile phase pH. Consistent with previous observations in HPLC of PA (11), the large pH-dependence of the k' values of PS molecular species is believed to arise from the reduced ionic character of analyte solutes by way of ion- suppression in the HPLC separation processes. The results of the pH study indicate that mobile phase pH is an important chromatographic variable in HPLC optimization experiments for obtaining reasonable retention parameters of PS components.

Fig. 5 shows RP-HPLC separations of PS molecular species using three octadecyl columns of different particle sizes and carbon contents. Apparently, the combination of column efficiency and the percentage of carbon loading on a given column was a critical factor to show selectivity for the analyte components. As expected, the PS solutes tended to be more strongly retained by a column with a higher percentage of carbon loading. Thus, HPLC

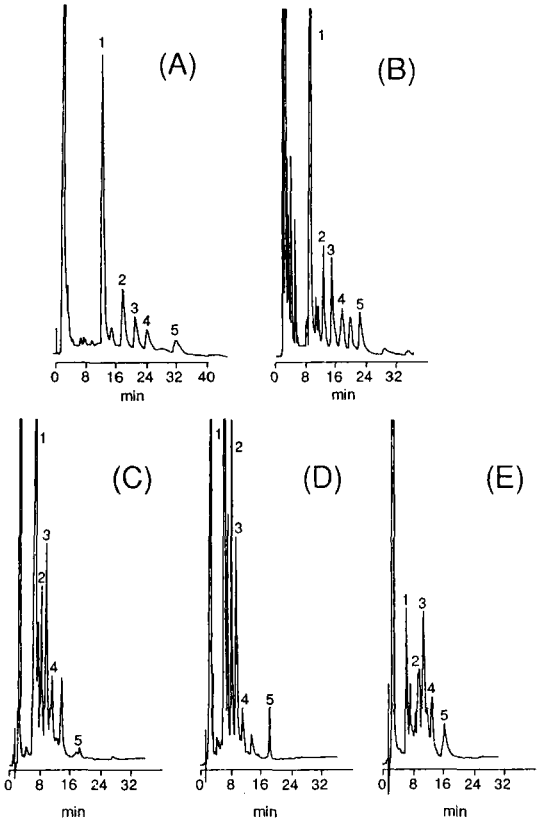

Fig. 5. HPLC separations of PS. Mobile phases, acetonitrile-methanol-water (70:22:8) containing (A) 5 mM TBAP, pH 6.5 (B) 5mM TEAP, pH 7; (C), (D) acetonitrile-methanol-water (70:25:5) containing 5 mM TBAP, pH 7.5. Column, (A) Ultrasphere ODS, (B) NovaPak C18, (C), (D) Supersphere 100 RP-18e. See Fig. 2 for peak identification.

with Ultrasphere C18 column (4.6 x 150 mm, 5 μm, 10,000 plates per meter, 12% carbon loading) produced the same number of peak components as NovaPak C18 column (3.9 x 300 mm, 4 μm, 20,000 plates per meter, 7% carbon loading). Best separations of PS molecular species were obtained with Supersphere 100 RP-18 column (4 x 250 mm, 3 μm) which has an efficiency of 25,000 plates per meter and a carbon loading of 22 %. On the other hand, few components of the polar lipid were resolved on Econosphere C18 column (4.6 x 100mm, 3 μm, 10,000 plate per meter, 10% carbon loading) despite the high efficiency of the column. Chromatographic profiles of the PS molecular species varied somewhat with the source of samples and with the length of time in solution. Chromatograms of older samples often showed a few more early-eluting peaks than freshly prepared samples presumably due to autooxidation of PS.

In summary, molecular species of PS, a minor constituent of soybean GPL, can be separated by RP ion-pair HPLC. Quantitative analyses of the compounds can be accomplished by HPLC-UV detection only if the analyses are conducted in conjunction with phosphorus analyses of the individual PS components. As the mobile phase systems employed in this study are not compatible with an evaporative light scattering (ELS) detector, it is not possible to quantitate PS by RP ion-pair HPLC-ELS detection. The HPLC results represent the first report on the separation characteristics of the PS compounds. The method can be used for practical analyses of PS derived from various sample matrices. The components can be efficiently resolved within reasonable retention times by optimizing HPLC conditions including column specifications.

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