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# Reversed-phase high performance liquid chromatography of phosphatidylcholine: a simple method for determining relative hydrophobic interaction of various molecular species

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**Abstract** A convenient method for the separation of molecular species of phosphatidylcholine (PC) by reversed-phase high performance liquid chromatography (HPLC) is described. PC species from egg, bovine brain, and porcine liver were resolved into 11–13 separate peaks on a Nucleosil-5-$C_{18}$ reversed-phase column with methanol–1 mM potassium phosphate buffer, pH 7.4, 9.5:0.5 (v/v) as the solvent. Detection was at 205 nm. PC species were primarily resolved due to specific hydrophobic interaction of their fatty acid side chains with the alkyl ligand of the stationary phase. The retention time and therefore the hydrophobic interaction of acyl chains of PC species increased logarithmically as the total number of carbon atoms in the chains increased in the homologous series. The retention times decreased nonlinearly as the number of double bonds in the fatty chains increased. Introduction of the first double bond in the side chain reduced the retention time to the greatest extent. From the chromatography data the reduction in the retention time was calculated to be equivalent to 1.8 carbon atoms compared to the fully saturated PC. Further introduction of 2, 3, 4, 5, and 6 double bonds in the same side chain reduced the retention time additionally, equivalent to 1.4, 1.2, 1.0, 1.0, and 1.0 less carbon atoms. Each molecular species of PC was assigned a "Hydrophobic Carbon Number" (HCN) based upon the total number of carbon atoms and double bonds in the side chain. PC molecules with the same HCN had the same retention time. The average HCN was calculated for various PC preparations and found to be remarkably the same for all the three different naturally occurring sources considering a wide variety of different fatty acid compositions of each type. HCN provides a simple measure of relative hydrophobicity of each PC molecule.—**Smith, M., and F. B. Jungalwala**. Reversed-phase high performance liquid chromatography of phosphatidylcholine: a simple method for determining relative hydrophobic interaction of various molecular species. *J. Lipid Res.* 1981. **22:** 697–704.

**Supplementary key words** egg phosphatidylcholine · bovine phosphatidylcholine · porcine phosphatidylcholine · hydrophobic carbon number

Previously, we have described HPLC methods for the quantitative analysis of phospholipids and glycolipids after derivatization (1, 2). We have also described HPLC analysis of phospholipids with the detection in the region of 200 nm without derivatization (3, 4). Separation of molecular species of sphingomyelins, cerebrosides, and ceramides by reversed phase HPLC has been reported (4–7). Porter, Wolf, and Nixon (8) have reported the separation of molecular species of egg phosphatidylcholine by reversed-phase HPLC. Egg phosphatidylcholine was resolved into four identifiable peaks. Here we describe greatly improved molecular species separation of egg, bovine brain, and porcine liver phosphatidylcholine, resolved into 11–13 separate peaks.

The physico-chemical interaction involved in the reversed-phase chromatographic process has been

Abbreviations: HPLC, high performance liquid chromatography; PC, phosphatidylcholine; HCN, hydrophobic carbon number; GLC–MS, gas–liquid chromatography–mass spectrometry.

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explained in light of the 'solvophobic theory' (9–11). The important feature of hydrophobic interaction is that the magnitude of the nonpolar contact area between the solute and the ligand of the stationary phase determines the degree of interaction. The actual molecular geometry involved in the binding of the solute to the ligand is unknown, due to scant knowledge of the topography of the stationary phase surface and the arrangement of the hydrocarbonaceous ligands. Nevertheless, in practical chromatographic systems it has been determined that the free energy change is minimized when the contact area between the solute and ligand and the net energy of interaction with the solvent are maximal (10). Previously, we have reported the application of this basic theory to interpret the order of elution of various molecular species of sphingomyelin in relation to molecular structure and conformation (5). Here we have extended the application of this theory to understand the complex elution order of molecular species of phosphatidylcholines. It is also shown how HPLC could be a useful and efficient tool for gaining information regarding the hydrophobic properties of biological substances.

## EXPERIMENTAL METHODS

### Materials

Egg and bovine brain phosphatidylcholine was from Supelco (Bellefonte, PA). Each sample gave a single spot on a thin-layer chromatographic plate with two different developing solvents. Porcine liver phosphatidylcholine was purchased from Serdary Research Laboratories (Ontario, Canada). This sample had a minor contamination of lyso-PC which will be shown later to be resolved from PC and did not interfere in the HPLC analysis. HPLC solvents were from Burdick and Jackson, Inc. (Muskegan, MI). Osmium tetroxide was from Stevens Metallurgical Corp. (New York, NY). All other chemicals were of reagent grade from Fischer Scientific Co. (Medford, MA).

### Instrumentation

The HPLC analysis was performed with a Waters Associates (Medford, MA) Model 6000A solvent delivery system and a Rheodyne Model 7105 syringe-loading sample injector. The chromatographic column was a stainless steel tube (4 mm × 30 cm) slurry-packed with Nucleosil-5-$C_{18}$ 5 $\mu$m particles (Machery-Nagel, Duren, Germany) in carbon tetrachloride with the aid of a Micrometrics Instrument Co. (Norcross, GA) slurry packer. A variable wave length spectromonitor model I from Laboratory Data Control (Riviera Beach, FL) was used for detection. Electron impact mass spectrometric identification of samples was with a Finnegan Model 4000 system and a Finnegan 6110 data system.

### Method

Egg, bovine brain, or porcine liver PC (0.1–2 mg) dissolved in 10–100 $\mu$l of chloroform was injected on the reversed-phase column. The HPLC solvent was methanol–1 mM potassium phosphate buffer, pH 7.4, 9.5:0.5 (v/v) pumped at a flow rate of 1 ml/min. The detection was at 205 nm. Individual molecular species of PC resolved on the column were collected and the fatty acid species associated with the peaks were identified by GLC on SP2340 and OV-1 column and also by GLC–mass spectrometry as previously described (5). Additional confirmation on the position of the double bonds in the fatty acid species was obtained by the $OsO_4$ oxidation and GLC–MS method of Dommes, Wirtz-Peitz, and Kunau (12) and Schmitz and Egge (13). The percentage composition and amount of various fatty acids associated with individual HPLC peaks was determined by the GLC analysis. The GLC–MS method was useful in determining relative amounts of the fatty acids that were unsaturated positional isomers.

In order to determine the content of disaturated fatty acid-containing PC in the commercially obtained standards, a 2-mg sample of PC was oxidized by $OsO_4$ according to Mason, Nellenbogen, and Clements (14). The disaturated PC was separated from the rest of the oxidized PC by TLC with dichloromethane–methanol–water 60:30:4 (by volume). The disaturated PC spot was scraped from the TLC plate and extracted with the same solvent. The extract was made to a volume and the fatty acid species associated with the lipid were identified by GLC as previously described (5).

## RESULTS

### Egg PC

The reversed-phase HPLC pattern of the egg PC (100 $\mu$g) on the Nucleosil $C_{18}$-5 $\mu$m column is shown in **Fig. 1.** The fatty acid composition of the individual PC peaks is listed in **Table 1.** The major fatty acids of egg PC as analyzed by GLC were 16:0 (30%), 18:0 (14%), 18:1 (30%), 18:2 (14%), 20:4 (4%), 22:5 (1.5%), and 22:6 (2%), Fig. 1, inset. The detection of PC in the HPLC analysis results primarily from the absorbance of double bonds at 205 nm (3). Thus the peak area does not represent the true quantity of PC present in the sample but rather indicates the extent



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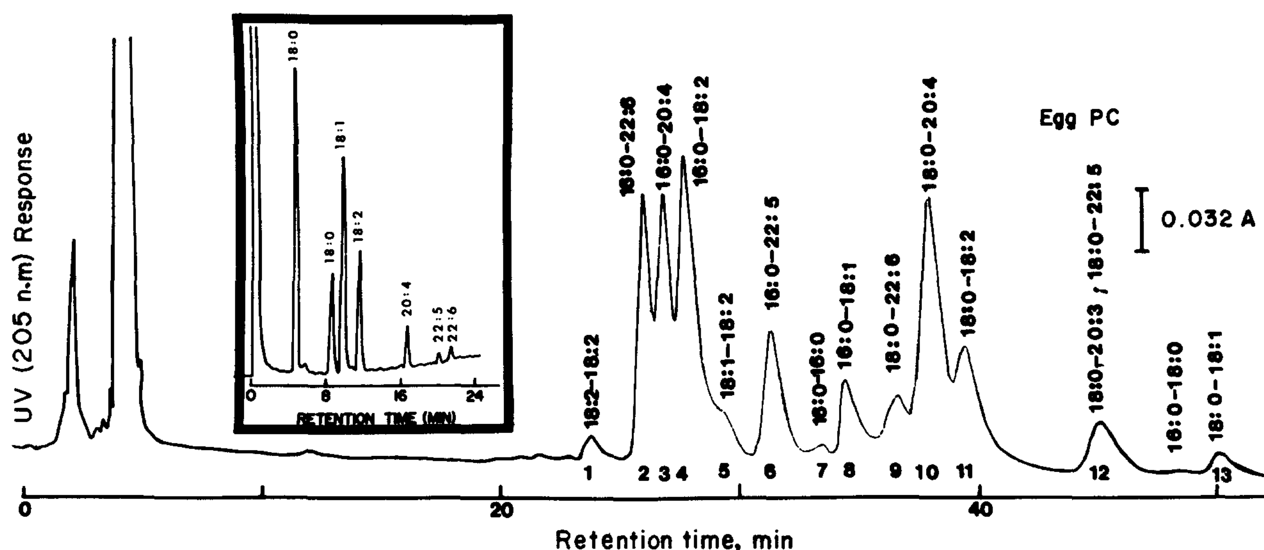


**Fig. 1.** HPLC analysis of egg phosphatidylcholine on Nucleosil-5-$C_{18}$ column. The solvent was methanol–1 mM phosphate buffer, pH 7.4, 9.5:0.5 (v/v) at a flow rate of 1 ml/min. PC, 500 μg dissolved in dichloromethane–methanol 1:1, 10 μl, was injected. The PC species eluted were collected as indicated in the figure by numbers and analyzed (see Table 1). The major fatty acid composition of the PC in an individual peak is given near the peak. Inset: GLC analysis of fatty acid methyl esters of egg total PC on SP-2340 column.

of unsaturation. Egg PC was resolved into 13 separate peaks by the reversed phase HPLC and about 20 different possible major molecular species could be identified (Table 1). Due to the indirect way of quantitation and the complex contents of various fatty acids, it is expected that some variations would result and therefore exact reconstitution cannot be achieved.

Peak 1 when analyzed by GLC and MS showed the presence of 70% of 18:2 and thus appeared to represent di-18:2-containing PC. Peak 4 was mostly 16:0–18:2-containing PC and it represented about 19% of the total PC. Peak 5 did not resolve completely from peak 4 and thus appeared to contain at least 40% 16:0–18:2-containing PC, but about 60% of this peak should represent PC with 18:1 and 18:2 fatty acids. Fraction 7 contained 95% of 16:0 and thus contained a disaturated fatty acid-containing species, which was also confirmed later by analyzing the saturated species after oxidation of egg PC and also by cochromatography on HPLC of commercially available synthetic di-16:0 PC. Peak 8 with 16:0–18:1 represented 37% of the total PC species. Peak 9 appeared to be a mixture of 18:0–22:6-, di-18:1-, and 16:0–18:1 containing PC. The latter arose from the large quantities present in the previous peak. The area between peak 12 and 13 was due mostly to 16:0–18:0-containing PC.

It was of interest to note that most of the egg PC fatty acid-containing species could be divided into two major groups, one with the 16:0 and the other with 18:0 as the saturated fatty acid. The major PC species were with 16:0–18:1, 16:0–18:2, and 18:0–18:1, 18:0–18:2. Both 16:0 and 18:0 groups also had 22:6,

TABLE 1. Percentage composition of fatty acid species of egg phosphatidylcholine fractions obtained by HPLC

| Peak No. | Fatty Acid 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:3 | 20:4 | 22:5 | 22:6 | Probable Major Molecular Species of PC | Percent Composition of PC | HCN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 8 | | 9 | 70 | 13 | | | | | 18:2–18:2;16:0–18:3;18:1–18:3 | 0.4 | 29.6,29.9 |
| 2 | 40 | | 12 | | | | | | 48 | 16:0–22:6;18:1–22:6 | 1.7 | 30.4,30.6 |
| 3 | 37 | | 18 | | | | 45 | | | 16:0–20:4;18:1–20:4 | 2.8 | 30.6,30.7 |
| 4 | 47 | | 2 | 48 | | | 3 | | | 16:0–18:2 | 18.9 | 30.8 |
| 5 | 22 | | 30 | 48 | | | | | | 16:0–18:2;18:1–18:2 | 3.2 | 30.8,31.1 |
| 6 | 43 | | 8 | 8 | | 10 | | 31 | | 16:0–22:5, 16:0–20:3, 18:1–18:2 | 2.0 | 31.5,31.6 |
| 7 | 95 | | 5 | | | | | | | 16:0–16:0 | 0.5 | 32 |
| 8 | 47 | | 53 | | | | | | | 16:0–18:1 | 37.0 | 32.2 |
| 9 | 36 | 6 | 52 | | | | | | 6 | 16:0–18:1;18:1–18:1;18:0–22:6 | 9.3 | 32.3,32.6 |
| 10 | | 35 | 27 | | | | 38 | | | 18:1–18:1;18:0–20:4 | 6.3 | 32.3,32.6 |
| 11 | | 50 | | 50 | | | | | | 18:0–18:2 | 7.3 | 32.8 |
| 12 | | 45 | | | | 25 | | 30 | | 18:0–22:5;18:0–20:3 | 0.6 | 33.6 |
| 13 | | 49 | 51 | | | | | | | 18:0–18:1 | 10.0 | 34.2 |



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20:4, 20:3, and 22:5 fatty acids and they appeared to elute from the HPLC column in a precise sequence. Disaturated and diunsaturated fatty acid-containing species were also present but they appeared to be only in small quantities.

In order to confirm the presence of disaturated species of PC, total egg PC was oxidized with osmium tetroxide and analyzed according to Mason et al. (14). The disaturated species isolated from TLC plates when analyzed by GLC showed the presence of mostly 16:0 (74%) and some 18:0 (18%) and 20:0 (8%). These results confirmed the presence of di-16:0 PC and 16:0–18:0 PC present in peak 7 and between peak 12 and 13, respectively, by HPLC.

The fatty acid methyl esters were analyzed by GLC–MS after oxidation with osmium tetroxide and trimethyl silylation. Peak 1 had mostly 18:2 with double bonds at positions 9, and 12, and small amounts of 18:1 (Δ9) and 18:3 (Δ 6, 9, 12, and 9, 12, 15).

The fatty acid methyl esters of peak 6 when similarly analyzed by GLC–MS confirmed the assignment of the fatty acids. 18:1 had a double bond at Δ9, 18:2 at Δ9, 12, and 20:3 had Δ8, 11, 14 double bonds. Peaks 4 and 11 also contained 18:2 with Δ9, 12 double bonds.

### Bovine brain PC

The HPLC pattern of the bovine PC (2 mg) is shown in **Fig. 2**. The major fatty acid species of total bovine PC as analyzed by GLC were 16:0 (30%), 18:0 (12%), 18:1 (43%), 20:1 (4%), 20:4 (2%), and 22:6 (1%) (Fig. 2 inset). Bovine PC was resolved into eleven different peaks by HPLC and about 24 different molecular species were identified.

Peaks 1 and 2 appeared to be broad peaks merged into each other. Peaks 3 and 4 contained PC with an odd chain fatty acid, 17:1. Peak 5 contained almost 65% of the total bovine PC with 16:0–18:1. This species also tailed somewhat into later eluting peaks 6 and 7. Peak 8 was a mixture of PC containing various fatty acids, including odd chain 17:0, 17:1, and 19:1.

GLC–MS indicated that the 16:1 in peak 1 was mostly with Δ9 double bond but it also contained some Δ7 double bond. Peaks 2, 3, 6, and 7 also contained PC with small but significant amounts of 18:1 with a Δ11 double bond in addition to the normally occurring Δ9. The content of disaturated species of PC in the bovine PC was analyzed (14). The disaturated species contained 82% 16:0 and 18% 18:0, indicating the presence of mostly di-16:0-containing PC.

### Porcine liver PC

The HPLC pattern of the porcine liver PC is given in **Fig. 3.** The major fatty acid species of the total porcine liver PC were 16:0 (26%), 18:0 (25%), 18:1 (23%), 18.2 (17%), and 20:4 (6%) (Fig. 3 inset). Other minor fatty acids were 16:1, 17:0, 17:1, 18:3, 20:3, 20:5, and 22:5. Porcine liver PC was resolved into 13

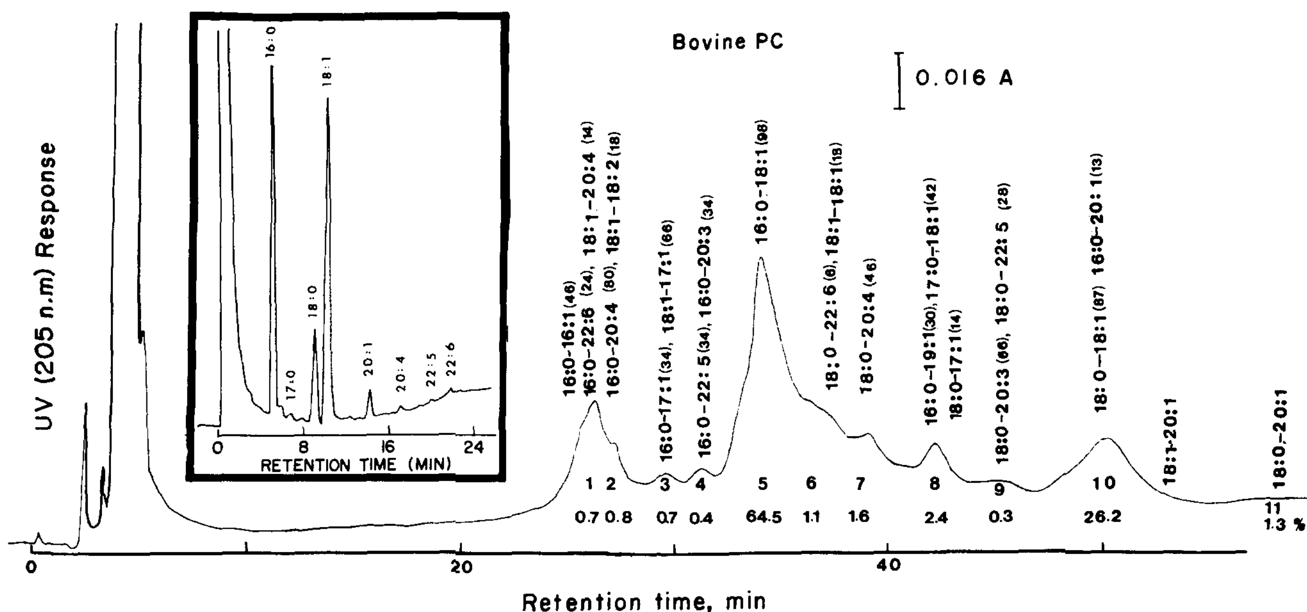


**Fig. 2.** HPLC analysis of bovine brain phosphatidylcholine, 2.0 mg on Nucleosil-5-$C_{18}$ column. The chromatographic conditions were the same as in Fig. 1. Probable major molecular species and percentage composition (in parentheses) of PC is given near each peak. Minor and overlapping molecular species are not listed. Figure below each peak number is the percent of total amount of bovine PC. Inset: GLC analysis of fatty acid methyl esters of bovine brain total PC on SP-2340 column.



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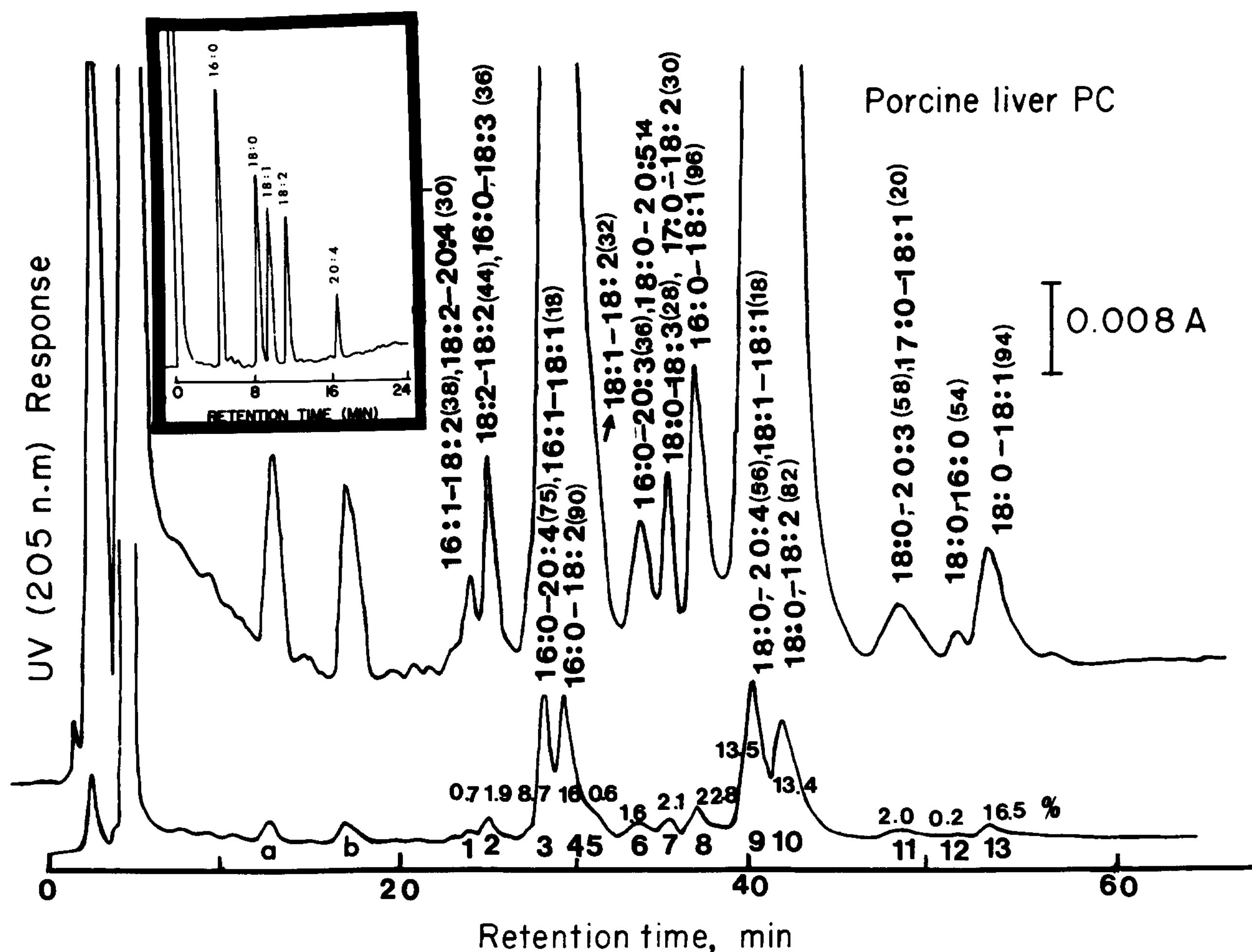


**Fig. 3.** HPLC analysis of porcine liver phosphatidylcholine, 100 $\mu$g on Nucleosil-5-$C_{18}$ column. The chromatographic conditions were the same as in Fig. 1. The lower curve was generated at 10-fold lower sensitivity than that of the upper curve. Probable major molecular species and percentage composition (in parentheses) of PC is given near each peak. Minor and overlapping molecular species are not listed. The following species were also present; in Peak 1, 16:0–20:5 (15%), 18:1–20:5 (15%); Peak 2, 18:1–18:3 (14%); Peak 3, 18:2–22:5 (7%); Peak 4, 18:1–20:4 (10%); Peak 6, 16:0–17:1 (11%), 17:0–20.4: (11%); Peak 7, 16:0–16:0 (30%); Peak 11, 18:0–17:1 (6%), 16:0–17:0 (20%); and peak 12, 18:0–18:1, 46%. Inset: GLC analysis of fatty acid methyl esters of porcine liver total PC on SP-2340 column.

different peaks by HPLC and various possible combinations of molecular species that could represent individual peaks are listed.

Peaks (a) and (b) were fatty acid oxidation products of PC and were present in porcine PC obtained from various commercial sources. These peaks were not lyso-PC since lyso-PC was eluted near the solvent front. When the same sample of PC was injected and detected at 234 nm instead of at 205 nm, the peak area of (a) and (b) was greatly increased (15, 16). This indicated that peaks (a) and (b) were the oxidation products.

Two different types of 18:3 with double bonds in Δ6, 9, 12(72%) and Δ9, 12, 15 (28%) positions were found in peaks 2 and 7 by GLC–MS of the $OsO_4$ oxidation reaction (12, 13). Peak 5 was at the tail end of peak 4 and was due to PC with 18:1–18:2. Peaks 6 and 7 contained mixtures of various fatty acids including the odd chain acids 17:0, 17:1. The exact composition of PC of these minor peaks is difficult to determine due to the presence of many different fatty acids. Peak 8 contained almost exclusively PC with 16:0–18:1 and represented about 23% of the total PC. Peak 13 was entirely due to 18:0–18:1 and represented almost 17% of the total porcine liver PC. GLC–MS showed the position of double bonds in the fatty acids as follows: Δ9 17:1; Δ9 18:1; Δ9, 12 18:2; Δ5, 8, 11, 14 20:4, and Δ8, 11, 14 20.3.

It was observed by the $OsO_4$ oxidation procedure that porcine liver PC had significant amounts of disaturated fatty acid-containing species with mainly 16:0 (66%) and 18:0 (34%). This was also confirmed



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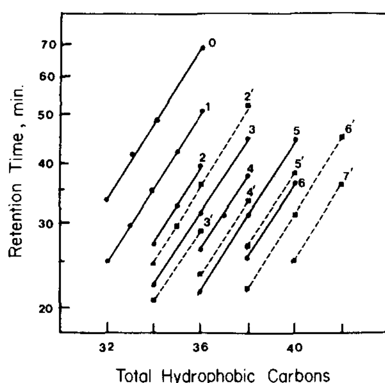


**Fig. 4.** Semilogarithmic plots of total hydrophobic carbons versus retention time in minutes of PC species on Nucleosil-5-$C_{18}$ reversed-phase column. ● —— ●, indicates PC species with either saturated fatty acids, 0; or PC with one saturated and one unsaturated fatty acid. The number of double bonds in the unsaturated fatty acid is given near the lines. ■ —— ■ indicates PC species with both unsaturated fatty acids. The numbers with primes near the lines indicate the total number of double bonds in both unsaturated fatty acids of PC species.

by HPLC analysis. The position of double bonds in the fatty acid was established by GLC–MS of the oxidation products.

## DISCUSSION

It has been generally accepted that elution patterns of lipids from a reversed-phase column are dependent upon the hydrophobic interaction between the solute and the ligand of the stationary phase. The magnitude of this interaction is dependent upon the contact area between the ligand and the solute. The contact area is determined by the molecular structure and conformation of the ligand and the solute. It has been shown previously that the extent of hydrophobic interaction between the $C_{18}$ side chain of the stationary phase and the various lipids is mainly determined by the number of carbon atoms as well as by the number and type of double bonds in the hydrophobic chains of the lipids (5, 17, 18). Thus retention times are progressively increased as the number of carbon atoms in the homologous series increased, whereas the retention times are decreased as the number of double bonds in the same fatty chains are increased.

In order to understand the hydrophobic interaction between the ligand of the stationary phase and various molecular species of phosphatidylcholine, the log of retention time was plotted against total number of hydrophobic carbon atoms in the fatty acid side chains of the PC, according to the degree of unsaturation (**Fig. 4**). As expected, for the same number of total hydrophobic carbon atoms, the retention time decreased as the number of double bonds in the molecule increased. However, the rate of decrease in the retention time with increasing number of double bonds was not the same (Fig. 4). Thus, for example, the retention time decreased by 19 min for the introduction of the first double bond in a side chain, for a total of 36 carbon atoms. The retention time decreased by an additional 11 min for the introduction of a second double bond in the same side chain. However, further introduction of 3rd, 4th, 5th, and 6th double bonds reduced the retention time only by an additional 8, 5, 4.5, and 4 min, respectively. This would indicate that introduction of the lst double bond in the molecule with the same number of carbon atoms in the chains reduced the retention time and therefore the hydrophobic interaction to the greatest extent. On introduction of more than one double bond in the molecule, the hydrophobic interaction decreased gradually.

Introduction of a second double bond in the different (2nd) side chain reduced the retention time more than a second double bond in the same side chain. Thus, for example, PC with two monounsaturated fatty acids, 18:1–18:1, was eluted earlier (retention time 36.5 min) than PC with 18:0–18:2 (retention time 40.0 min). This would indicate that the hydrophobic interaction with the stationary phase is reduced more in the former case than in the latter case, even though both types of PC have the same number of carbon atoms and same number of double bonds.

The hydrophobicity (in terms of retention time) due to carbon atoms and number of double bonds can be correlated to each other. A plot (**Fig. 5**) of the number of carbon atoms versus the number of double bonds can be derived from Fig. 4. From the plot it is noted that the effect of introduction of the first double bond in a saturated PC is equivalent to 1.8 less carbon atoms in terms of hydrophobicity. Introduction of 2nd and 3rd double bonds in the same side chain is equivalent to a total of 3.2 and 4.4 less carbon atoms. Further introduction of double bonds is equivalent to only one less carbon atom per double bond (**Table 2**). The carbon equivalence for double bonds in PC with unsaturation in both side chains is slightly different (Fig. 5). Thus for a PC with two monounsaturated fatty acids (two double bonds) the carbon equivalence was 3.7. Introduction of additional double bonds in either side chain reduced the carbon number by an additional 1.2 carbon atoms per double bond (Table 2).

Based on this carbon equivalence per number of



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double bonds in the molecule, a total 'hydrophobic carbon number', HCN, can be calculated for the side chains in each PC molecule. Thus PC molecules with different numbers of carbon atoms and double bonds but having the same HCN are eluted together in the same peak. The overlap shown in Tables 1 is due to tailing peaks. The retention time is increased proportionately as the HCN is increased. HCN provides a simple measure of relative hydrophobicity of each PC molecule. An average HCN was calculated for naturally occurring PC as follows:

$$\text{Average HCN} = \frac{\text{HCN of the peak} \times \%\ \text{of PC in the peak}}{100}$$

Thus for egg, bovine, and porcine liver PC, the average HCN was calculated to be 32.3, 32.7, and 32.2, respectively. This number is remarkably the same for all three different naturally occurring PC, considering a wide variety of different fatty acid compositions of each type. The HCN also provides valuable information for the identification of molecular species in unknown mixtures, since the order of elution of compounds on the reversed-phase column is dictated by HCN.

The HPLC method described here for the separation of molecular species of intact PC is more effective than any other procedure previously described (8, 19–22). Though critical pairs having the same HCN are not resolved on the reversed phase column, it is possible to separate these pairs by argentation HPLC. Bonded silver columns are now commercially available which separate molecular species based upon the number of double bonds in the molecules. We have recently separated egg phosphatidylcholine into six separate peaks on a silver column, based upon the degree of unsaturation in the molecular species. These species could be further resolved on the reversed-phase HPLC system described here. We have also reported the molecular species separation of benzoylated sphingomyelins and ceramides by the combined use of argentation and reversed-phase HPLC (23).

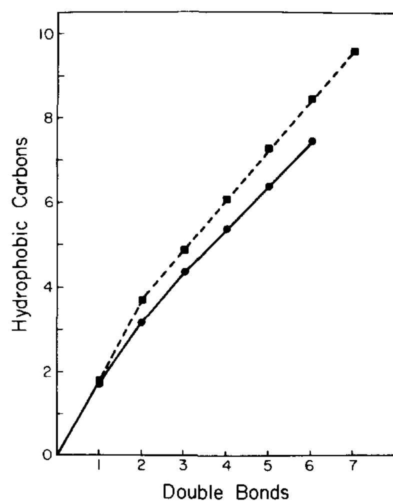


**Fig. 5.** Plots of total number of double bond versus equivalent hydrophobic carbons. Solid line with circles represents PC species with one saturated and one unsaturated fatty acid; broken line with square symbols represents PC with both unsaturated fatty acids. The plots are derived from Fig. 4 by measuring the distance (in terms of hydrophobic carbons) between the lines for saturated and the corresponding unsaturated fatty acid-containing PC.

TABLE 2. Relationship between degrees of unsaturation in phosphatidylcholine and hydrophobic interaction measured in terms of the reduced number of carbon atoms in the fatty acid side chain

| No. of Double Bonds in the Fatty Acid(s) | Equivalent Lesser Number of Carbon Atoms | |
|---|---|---|
| | A[a] | B[b] |
| 1 | 1.8 | |
| 2 | 3.2 | 3.7 |
| 3 | 4.4 | 4.9 |
| 4 | 5.4 | 6.1 |
| 5 | 6.4 | 7.3 |
| 6 | 7.4 | 8.5 |
| 7 | | 9.6 |

[a] These values represent a lesser hypothetical number of carbon atoms in the side chain when the double bonds are in the single side chain.

[b] These values represent a lesser number of carbon atoms when the double bonds are in both side chains.

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