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## Note

# General method for the analysis of phosphatidylcholines by high-performance liquid chromatography

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Individual molecular species of phosphatidylcholines (PCs) have been analyzed by a number of methods, *e.g.*, reductive ozonolysis[1], argentation thin-layer chromatography (TLC)[2], enzymatic procedures coupled with gas chromatography (GC)[3], counter-current distribution[4], Sephadex column chromatography[5] and cryo-TLC[6]. The major problems encountered with these methods include low resolution, poor reproducibility, long separation time and difficulty of quantitation.

Although reversed-phase high-performance liquid chromatography (HPLC) has been used for the separation of fatty acid methyl[7,8] and aromatic[9–11] esters, separation of individual molecular species of the higher-molecular-weight zwitterionic PCs and other phosphoglycerides has been limited[12,13]. A few reports exist on the HPLC separation of PCs from other lipid classes[14,15] and from classes of other phosphoglycerides[16–18].

We report a simple and universal method for the separation and identification of multiple molecular species of egg PCs. The method involves the preparation and HPLC of phosphatidic acid dimethyl esters (compounds 3, PAMEs), which are readily and quantitatively obtained by enzymatic hydrolysis of parent PC (compounds 1) and subsequent esterification of the phosphatidic acid molecules (compounds 2) with diazomethane (Fig. 1).

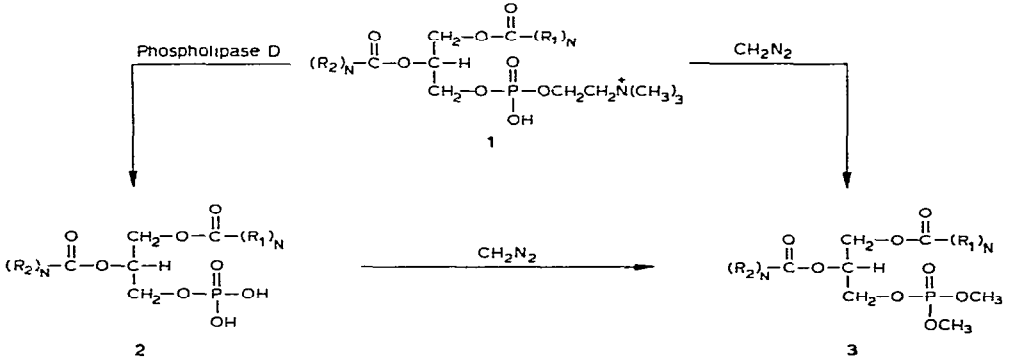


Fig. 1. Preparation of phosphatidic acid dimethyl esters from phosphatidylcholines.

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## EXPERIMENTAL

*Chemicals and derivatization procedure*

Single molecular species of PCs (standards) were obtained from Serdary Research Labs. (London, Canada) or were synthesized by adapting published methods[19,20]. Egg PC was isolated and purified from fresh hen eggs by aluminum oxide chromatography as described by Singleton *et al.*[21]. Fully hydrogenated PCs were prepared by catalytic hydrogenation of isolated natural egg PCs in chloroform–ethanol (1:1) over platinum oxide at room temperature and 50 p.s.i. The reduced egg PCs were purified by preparative TLC (chloroform–methanol–water, 65:25:4). The completeness of the hydrogenation was determined by gas–liquid chromatographic analysis of the methyl esters derived upon saponification of the corresponding parent PC molecules and esterification of the fatty acids.

PAMEs were prepared from standard and egg PCs by both direct (diazomethanolysis, Fig. 1, 1 → 3) and indirect (phospholipase D/$CH_2N_2$, Fig. 1, 1 → 2 → 3) methods according to Renkonen[22]. The identities of the PC and PAME molecules were checked and confirmed by proton magnetic resonance spectral ($^1$H NMR) analysis (Fig. 2); the homogeneities of each of these molecular classes also were confirmed by TLC.

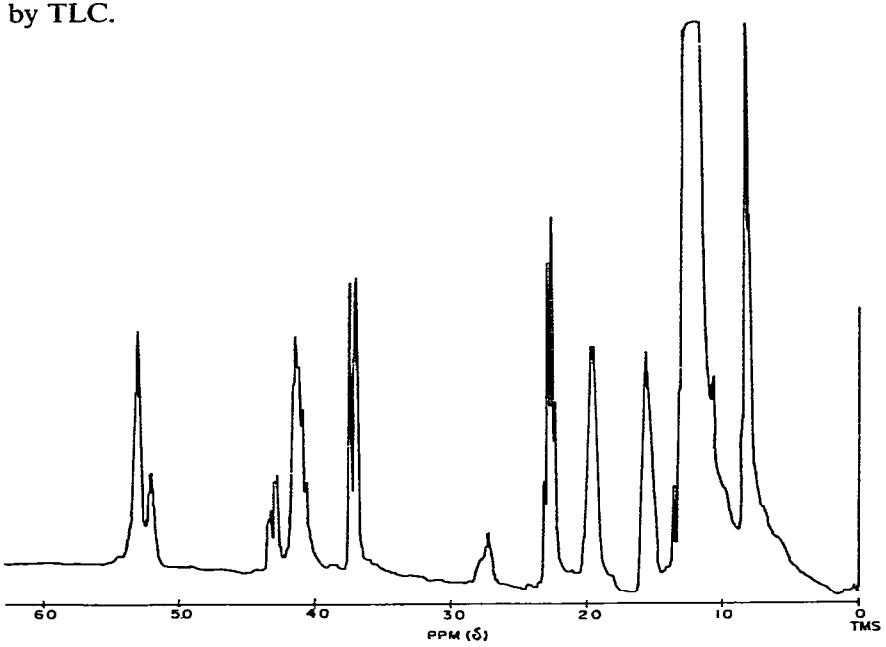


Fig. 2. 270 MHz $^1$H NMR spectrum of PAMEs derived from egg PCs. Resonances at $\delta$ ($C^2HCl_3$) 0.86 ($CH_3$), 1.30 ($CH_2$), 1.51 ($CH{=}CHCH_2CH_2$, $CH_2CH_2CO$), 2.00 ($CH{=}CHCH_2$), 2.29 ($CH_2CO$), 2.79 ($CH{=}CHCH_2CH{=}CH$), 3.69 ($POCH_3$), 3.92–4.20 ($POCH_2$, $CH_2OCO$), 5.14 (CHO), 5.28 ($CH{=}CH$) ppm

*Instrumentation and lipid analysis*

HPLC analyses were done with a Model ALC 202 liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a M6000 pumping system and a R401 differential refractometer. A Pye LCM2 flame-ionization detector (Philips Elec-

tronics Inst., Mahwah, NJ, U.S.A.) also was employed. Partisil 10 ODS and Partisil 10 ODS-2 columns (Whatman, Clifton, NJ, U.S.A.) were used separately for reversed-phase chromatography. Acetonitrile and methanol were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The HPLC conditions, including mobile phase, flow-rate, pressure and sample type are given in the legend of respective figures. The total fatty acid compositions were determined by GC of the corresponding methyl esters following saponification and methylation[23]. The GC analyses were carried out isothermally (195°C) on a column (6 ft. × 1/8 in. O.D.) of 10% SP-2330 on Chromosorb W AW (Supelco, Bellefonte, PA, U.S.A.) using a HP Model 5750 gas chromatograph (Hewlett-Packard, Paramus, NJ, U.S.A.). Quantification of chromatographic peak areas was determined using a Hewlett-Packard 3380A digital integrator. High-resolution $^1$H NMR spectra were obtained with a Bruker 270-MHz NMR spectrometer at Yale University, New Haven, CT, U.S.A.

## RESULTS AND DISCUSSION

The $^1$H NMR spectrum (270 MHz) of PAMEs derived from egg PCs is shown in Fig. 2. The doublet at $\delta$ 3.69 ($POCH_3$) and the absence of resonances at $\delta$ 3.31 and $\delta$ 3.83 [$C\mathit{H}_3)_3N^+$, $C\mathit{H}_2N^+(CH_3)_3$] diagnostic of precursor PCs proved unequivocally the identity of the PAME molecules[22]. The total fatty acid compositions of egg PCs and their corresponding PAMEs are presented in Table I. The principal fatty acids of egg PCs in order of decreasing amounts were 18:1, 16:0, 18:2 and 18:0. Since little change was found in the total fatty acid composition of PAMEs after conversion of the PC molecules to their respective PAME derivatives (Table I), the PAME molecules can be considered as authentic representatives of original individual molecular species of intact PCs. Baer and Maurukas[24] and Crone[25] have reported preparation of PAMEs directly by reaction of PCs with diazomethane (Fig. 1). Since formation of fatty acid methyl esters[26] and other side products may be expected under such reaction conditions, the indirect method, *i.e.*, enzymatic hydrolysis of parent PCs followed by methylation of the corresponding phosphatidic acids with diazomethane is preferred, especially when derivatizing highly unsaturated molecular species.

TABLE I
COMPARISON OF THE TOTAL FATTY ACID COMPOSITIONS (MOLE-%) OF EGG PC AND EGG PC-DERIVED PAMEs

| *Fatty acid* | *PC (%) ± S.D., n = 4* | *PAME (%) ± S.D., n = 4* |
|---|---|---|
| 16:0* | 30.2 ± 3.4 | 30.6 ± 2.8 |
| 16:1 | 3.5 ± 0.5 | 4.8 ± 0.5 |
| 18:0 | 10.9 ± 0.8 | 11.5 ± 1.2 |
| 18:1 | 34.8 ± 4.1 | 34.4 ± 5.0 |
| 18:2 | 18.3 ± 1.5 | 17.1 ± 2.0 |
| 20:4 | 2.3 ± 0.2 | 1.6 ± 0.5 |

* Chemical notation 16:0 designates 16 carbons and zero double bonds

A HPLC separation of PAMEs derived from egg PCs is shown in Fig. 3. The major components of the three peaks, identified by comparison of their retention times with those of known synthetic standards, were: 16:0/18:2-PAMEs (peak 1),

16:0/18:1-PAMEs (peak 2) and 18:0/18:1-PAMEs (peak 3). The observed separation was related to both the carbon chain length and the degree of unsaturation of the two fatty acyl chains in the PAME molecules. Acetonitrile–water (94:6) gave a similar separation of the three peaks except for slightly longer retention times.

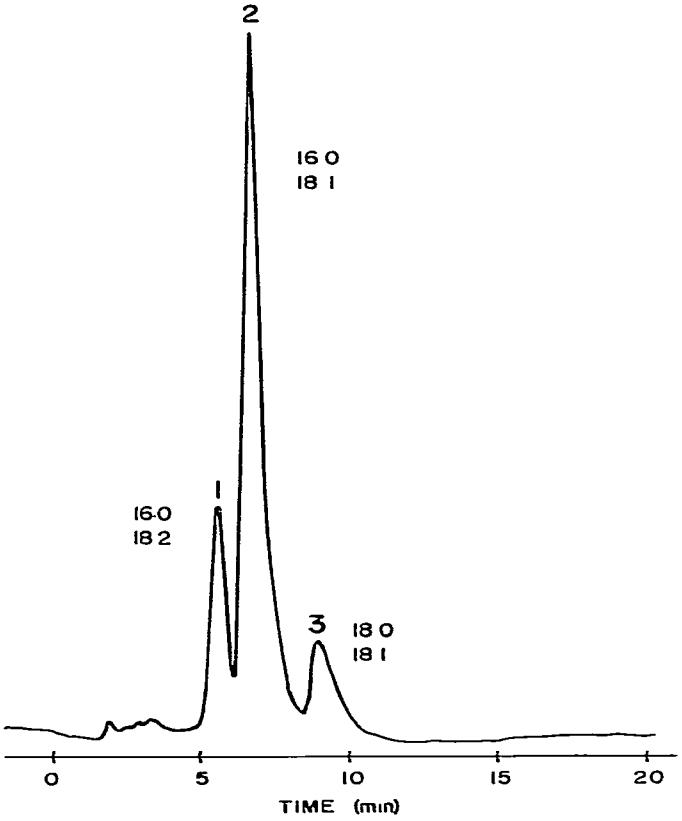


Fig 3 HPLC separation of PAMEs derived from egg PCs Column, Partisil 10 ODS, mobile phase, methanol–water (96 4) Flow-rate, 2 ml/min at 900 p s i g Detection, refractive index ×32 For composition of component peaks, see text

An identical separation of egg PAMEs was obtained using Partisil 10 ODS-2 as stationary phase and methanol as the eluent; these conditions resulted in slightly longer retention times of all three peaks. Under these chromatographic conditions, three fractions corresponding to the peaks of flame-ionization detection were collected following their separation by HPLC and pooling from ten independent runs; it was preferable to use a Partisil 10 ODS-2 column and methanol because it was easier to recover each fraction by evaporating methanol rather than methanol–water. The total fatty acid composition of each PAME fraction is compiled in Table II. Fraction 1 (F-1) consisted of PAME molecules having several combinations of paired fatty acyl chains with the major species being 16:0/18:2-PAMEs. F-2 consisted mainly of 16:0/18:1-PAMEs and 18:0/18:2-PAMEs; F-3 was comprised almost exclusively of 18:0/18:1-PAMEs with minor amounts of 16:0/20:4- and possibly 18:1/18:1-PAMEs.

TABLE II
TOTAL FATTY ACID COMPOSITIONS (MOLE-%) OF EGG PAME FRACTIONS (F) SEPARATED BY HPLC
Values are the means of two independent determinations.

| *Fatty acid* | *F-1* | *F-2* | *F-3* |
|---|---|---|---|
| 16 0 | 41.0 ± 4 1 | 37.5 ± 5 4 | 2 4 ± 0.9 |
| 16 1 | 6 4 ± 2 5 | – | – |
| 18 0 | 0 2 ± 0 4 | 6 6 ± 1 8 | 45.1 ± 2 4 |
| 18 1 | 10 2 ± 2 5 | 48 1 ± 4.5 | 50 1 ± 3 6 |
| 18 2 | 38 3 ± 4 8 | 6 3 ± 1 9 | – |
| 20 4 | 3.9 | 1 5 ± 0 4 | 2 4 ± 1.1 |

A HPLC chromatogram of PAMEs obtained from hydrogenated egg PCs is shown in Fig. 4. The same Partisil 10 ODS column that was used for non-hydrogenated PAMEs (Fig. 3) but with a different solvent system (acetonitrile–water, 95:5) was employed. The identification of peak 1 (16:0/18:0-PAMEs), peak 2 (18:0/18:0 + 16.0/20:0-PAMEs) and peak 3 (18:0/20.0-PAMEs) was established by spiking with synthetic standards and from data obtained with non-hydrogenated PAMEs (Fig. 2). Since 16:0/18:2- and 16.0/18:1-PAMEs were the major molecular species of peaks 1 and 2, respectively (Fig. 3), it is evident that the major molecular species of the corresponding hydrogenated PAMEs should be 16:0/18:0-PAMEs (Fig. 4). The separation was based on the total carbon numbers of the two esterified fatty acyl chains within the disaturated PAME molecules. Elution with acetone or acetonitrile–methanol reduced the retention times of disaturated PAMEs, resulting in poorer resolution.

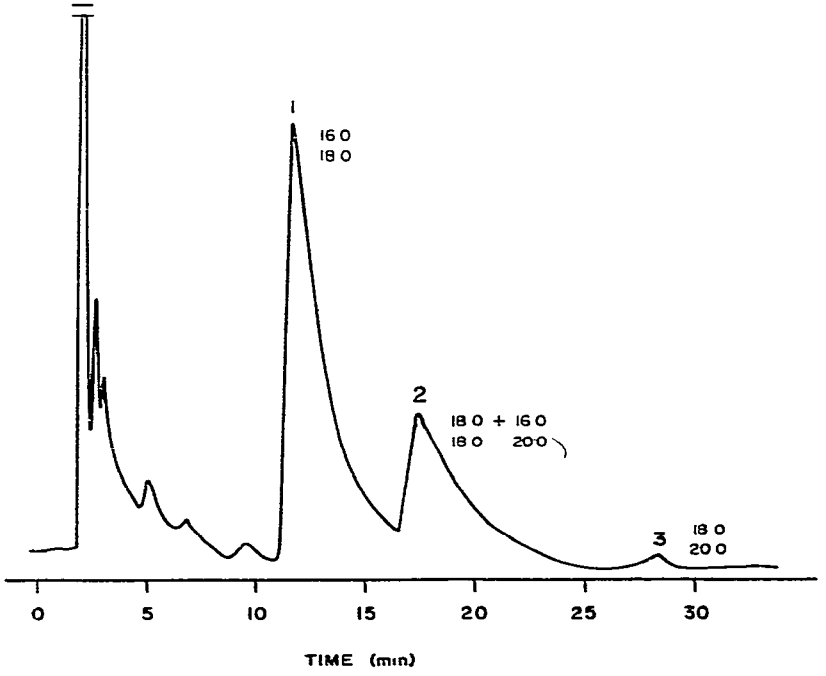


Fig 4 HPLC separation of PAMEs derived from hydrogenated egg PCs Column, Partisil 10 ODS. Mobile phase, acetonitril–water (95 5) Flow-rate, 2 ml/min at 900 p.s.i.g Detection, flame-ionization × 32. 16·0/18.0 refers to a PAME molecule (3, Fig. 1) having $R_1$ = 16.0 and $R_2$ = 18 0 at the *sn*-1 and *sn*-2 positions, respectively

In this work, PAMEs were chosen as target molecules for preliminary investigation of the development of a simple and universal method for the separation and identification of individual molecular species of naturally occurring phosphoglycerides[28] by HPLC. The main reasons for this are: molecular species such as PAMEs are more efficiently resolved than are PCs and, most likely, other classes of phosphoglycerides and phosphoglyceride-derived 1,2-diglyceride acetates[23]; phosphatidic acids also may be readily converted to other phosphate triesters[27] suitable for enhancing sensitivity; PAMEs or other phosphate triesters can be prepared from different classes of phosphoglycerides such as phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol and phosphatidylinositol, etc.; the derivatization conditions for making PAMEs from parent PCs are mild, and acyl migration which can occur during the formation of diglyceride acetates[3] is circumvented; and, the phosphorus atom would be retained in PAMEs or other phosphate triester molecules, thus permitting use of $^{32}$P-labelled phosphoglycerides for appropriate metabolic studies.

ACKNOWLEDGEMENTS

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REFERENCES

1 O. S. Privett and M L Blank, *J Amer. Oil Chem Soc*, 40 (1963) 70
2 G. A E Arvidson, *J Lipid Res*, 6 (1965) 574.
3 A Kuksis and L Marai, *Lipids*, 2 (1967) 217.
4 F. D. Collins and M A Trewhella, *Lipids*, 6 (1971) 355
5 G. A. E Arvicson, *J Chromatogr*, 103 (1975) 201.
6 R. F. Henderson and M H. Clayton, *Anal. Biochem*, 70 (1976) 440
7 C. R Scholfield, *J Amer. Oil Chem Soc*, 52 (1975) 36
8 J. D Warthen, Jr, *J Amer. Oil Chem. Soc.*, 52 (1975) 151
9 M. J. Copper and M. W Anders, *Anal. Chem*, 46 (1974) 1849
10 R F. Borch, *Anal. Chem*, 47 (1975) 2437
11 N E. Hoffman and J C Liao, *Anal. Chem*, 48 (1976) 1104
12 N A. Porter, R. A Wolf and J. R. Nixon, *Lipids*, 14 (1979) 20
13 C G. Crawford, R. D. Plattner, D. J Sessa and J. J. Rackis, *Lipids*, 15 (1980) 91
14 O. S. Privett, K. A. Dougherty, W. L. Erdahl and A. Stolyhwo, *J. Amer Oil Chem Soc*, 50 (1973) 516
15 K. Kiuchi, T. Ohta and H. Ebine, *J Chromatogr. Sci*, 13 (1975) 416.
16 F B. Jungalwala, J E Evans and R. H McCluer, *Biochem J*, 155 (1976) 55
17 K Kiuchi, T. Ohta and H. Ebine, *J Chromatogr*, 133 (1977) 226
18 W M. A. Hax and W S. M. Geurts van Kessel, *J. Chromatogr.*, 142 (1977) 735.
19 R. G Jensen and R E Pitas, *Advan Lipid Res.*, 14 (1976) 213
20 D. H Hanahan, H. Brockerhoff and E. J Barron, *J Biol Chem*, 235 (1960) 1917
21 W. S. Singleton, M. S. Gray, M. L. Brown and J. L. White, *J. Amer Oil Chem Soc*, 42 (1965) 53
22 O. Renkonen, *Biochim. Biophys. Acta*, 152 (1968) 114
23 J Y.-K. Hsieh, Ph.D. *Dissertation*, University of Rhode Island, Kingston, RI (1979)
24 E. Baer and J. Maurukas, *J. Biol Chem*, 212 (1955) 39.
25 H. D. Crone, *Biochim. Biophys. Acta*, 84 (1964) 665
26 W. O. Ord and P. C. Bamford, *Chem Ind*, (1967) 2115
27 J Y.-K Hsieh and J G Turcotte, *J. High Resolut Chromatogr Chromatogr. Commun*, (1980) 481.
28 J. G. Turcotte, S P. Srivastava, W. A Meresak, B A Rizkalla, F. Louzon and T P. Wunz, *Biochim Biophys. Acta*, 619 (1980) 604.