Quantitative analysis of phosphatidylcholine molecular species using HPLC and light scattering detection

Jos F. H. M. Brouwers,[1] Barend M. Gadella, Lambert M. G. van Golde, and Aloysius G. M. Tielens

Laboratory of Veterinary Biochemistry and Institute of Biomembranes, Utrecht University, P.O. Box 80.176, 3508 TD Utrecht, The Netherlands

Abstract A number of HPLC chromatographic procedures can be used to separate intact molecular species of phosphatidylcholine (PC), but on-line quantification has remained problematic due to insensitivity of UV-detection for saturated species. Here, a new method is presented, separating all major PC molecular species from a variety of biological samples in intact form using a single, short and isocratic run. Species were separated on two RP18 reverse-phase columns in series and all species displayed an exponential relation between retention time and the percentage of acetonitrile or triethylamine in the mobile phase, allowing optimization of the mobile phase on a theoretical base, rather than on time-consuming test-runs. The use of triethylamine as a volatile additive instead of choline chloride allowed the use of light scattering detection. On a molar base, the response of the detector was invariant between species and allowed quantification of as little as 50 pmoles. The method was tested using phosphatidylcholines with widely different molecular species patterns, such a PC from rat liver, porcine pulmonary surfactant, bovine heart, boar sperm cells, and the parasite *Schistosoma mansoni*. As only volatile components are present in the solvents, individual molecular species can easily be recovered in pure form from the column effluent, enabling their further analysis (e.g., scintillation counting).**—Brouwers, J. F. H. M., B. M. Gadella, L. M. G. van Golde, and A. G. M. Tielens.** Quantitative analysis of phosphatidylcholine molecular species using HPLC and light scattering detection. *J. Lipid Res.* 1998. **39:** 344–353.

Supplementary key words ether lipids • phospholipids • plasmalogens • lipid peroxidation • metabolism

The physical properties and functions of biological membranes are to a large extent mediated by their lipid composition. Not only lipid classes (such as phosphatidylcholine (PC), cholesterol, phosphatidylethanolamine (PE), etc.) but also the molecular species compositions of the various phospholipids have to be determined in the characterization of lipid membranes. The number of unsaturated carbon–carbon bonds in the aliphatic groups at the *sn*-1 and *sn*-2 position of the phospholipid is a key factor in determining phase-transition temperature and lateral diffusion velocity of membranes, thus playing important roles in events such as endo- and exocytosis, sorting of lipids, or membrane fusion. More recently PC (but also PE) has been recognized as an important precursor in signal transduction pathways (1), as the action of phospholipase C and D on this phospholipid releases the mediators diacylglycerol (DAG) and phosphatidic acid, respectively. In addition, phospholipase A_2 can release (polyunsaturated) fatty acids such as arachidonic acid from the *sn*-2 position, which can be further metabolized to biologically active molecules such as leukotrienes or prostaglandins. The exact relationship between these roles of PC and its molecular composition in biological membranes has yet to be determined, and a reliable and sensitive technique to determine molecular species of this phospholipid would be a valuable tool in lipid biochemistry.

The chromatographic separation of phospholipid molecular species requires a high resolving power, as there are only subtle distinctions between many species, such as the presence of one extra double bond or two extra carbon atoms. Reverse-phase high performance liquid chromatography (RP-HPLC) on octadecyl derivatized silica (commonly refered to as ODS or RP-18) has proven to be capable of such selectivity. The oldest and most straight-forward method in the determination of the fatty-acyl chain composition of phospholipids involves the hydrolysis of all ester bonds and

Abbreviations: ELSD, evaporative light scattering detection; RP-HPLC, reverse-phase high performance liquid chromatography; PC, phosphatidylcholine (referring collectively to all diradyl choline glycerophospholipid species); PtdCho, diacyl phosphatidylcholine; AlkCho, alkylacyl choline glycerophospholipid; PlasCho, plasmenylcholine species (alkenylacyl choline glycerophospholipids); TLC, thin-layer chromatography; DAG, diacylglycerol.

[1]To whom correspondence should be addressed.

subsequent analysis of released fatty acids as methyl esters or phenacyl esters, using GC or HPLC, respectively. However, information about the intramolecular position of a fatty acid will be lost, as well as information about the occurrence of particular combinations of fatty acids at the *sn*-1 and *sn*-2 position. To preserve this information, phospholipids can be converted to DAG and subsequently to derivatized homologs, facilitating detection by UV absorption measurements. The conversion to DAG eliminates the polar headgroup that causes tailing due to aspecific interaction with column material. However, the experimental procedures of phospholipase treatment and chemical derivatization are potential sources for the introduction of artefacts, and the biological activity of the phospholipids is lost. Therefore the separation of intact phospholipid molecular species has become more and more important. Currently, elution procedures based on the method developed by Patton, Fasulo, and Robins (2) are most frequently used to separate intact molecular species of PC. The interaction between headgroup and stationary phase is eliminated by the addition of quaternary amine salts, which compete with the headgroup for the interaction sites on the reverse phase column. Using only solvents that have no appreciable absorbance between 200 nm and 210 nm, UV detection can be used to monitor elution of components from the column, relying on the presence of double bonds in the aliphatic moieties of phospholipid. In a recent report, response factors for a wide variety of PC species have been published, allowing quantitative detection of these species, but saturated species remain undetected (3). Quantitative detection of both saturated and unsaturated species can be achieved by post-column formation of fluorescent micelles at 50°C, requiring additional equipment for the pulse free addition of the fluorescent probe and heating of the lipid–probe mixture (4).

Here we present a new method for quantitative analysis of intact PC molecular species using RP-HPLC on a RP18 column and a light scattering detector. These detectors nebulize the column effluent to an aerosol which is subsequently evaporated, leaving the solute components as fine droplets. These droplets are illuminated by a laser and the amount of scattered light is measured. The new method here described is capable of detecting (sub-)nanomolar quantities of PC regardless of the type of linkage or degree of unsaturation of the aliphatic chains at the *sn*-1 and *sn*-2 position. Furthermore, the method is able to separate molecular species that are commonly present in biological samples in a short time, using easily obtainable solvents and standard equipment. A flow splitter enables the individual molecular species to be obtained in pure and intact form as only volatile solvents are used.

MATERIALS AND METHODS

Preparation of samples

Phosphatidylcholines from different origins were isolated as follows. Lungs of specified pathogen-free pigs obtained from the Central Veterinary Institute, Lelystad, The Netherlands, were lavaged 5 times with 15-ml portions of physiologic saline per kg body weight. Surfactant was isolated from this lavage according to the method of Curstedt et al. (5). *Schistosoma mansoni* parasites were isolated from ether anesthetized hamsters, 45–48 days after infection, by perfusion of the heart at 37°C with Tris-buffered saline (TBS, 50 mm, pH 7.4). Schistosomes were repeatedly washed with ice-cold TBS and homogenized in TBS (approx. 20% v/v) (6). Homogenates (20% w/v) in ice-cold TBS were prepared from livers isolated from male Wistar rats, and from bovine heart. Sperm cells were isolated and purified from fertile Dutch Landrace boars at the Collaborative Artificial Insemination Centre, Bunnik, The Netherlands, as described before (7, 8). Lipids were extracted from all samples according to the method of Bligh and Dyer (9). From these extracts, PC was isolated using HPLC as described below. Reference compounds (individual molecular species) were obtained from Avanti Polar Lipids, Alabaster, AL.

High performance liquid chromatography

The HPLC system consisted of an LKB low pressure mixer, a model 2248 pump purchased from Pharmacia, Upsala, Sweden, a Rheodyne injector, and a Varex MKIII light scattering detector obtained from Alltech, Deerfield, IL. To isolate PC for species separation, lipid classes were separated on HPLC, using a 4-μm Lichrosphere normal-phase silica column purchased from Merck, Darmstadt, Germany as the stationary phase. Elution was performed at a flow rate of 1 ml·min^{-1} by a gradient of hexane–isopropanol–dichloromethane 40:48:12 to hexane–isopropanol–dichloromethane–water 40:42:8:8 in 15 min followed by additional elution with the latter solvent for 15 min (essentially according to Letter (10)). PC and other components were collected manually from the column effluent using a flow splitter from Alltech, Deerfield, IL. The eluate was evaporated to dryness under a stream of nitrogen and redissolved in chloroform–methanol 1:1. Resolution of molecular species was performed on two 5-μm endcapped Lichrosphere 100-RP18 columns in series, obtained from Merck, Darmstadt, Germany. Isocratic elution was applied with a solvent composed of acetonitrile, methanol, and triethylamine in varying ratios at a flow rate of 1 ml·min^{-1}. For the analysis of hydrogenated species (see below), only a single column was used. All

solvents used were from Labscan, Dublin, Ireland, were of HPLC grade, and had a residue after evaporation of less than 0.001% (w/v) or were distilled before use.

Detector setup and calibration

The light scattering detector used compressed air as the nebulizing gas. The detector was optimized by triplicate injection of 1 nmol of dilauryl phosphatidylcholine ((12:0–12:0)PtdCho). The mean height of the three peaks was divided by the height of the noise in the baseline. This signal to noise ratio was calculated for the combination of four different gasflows (1.6, 1.8, 2.0, and 2.2 $1 \cdot min^{-1}$) with six drift tube temperatures (70 to 120°C in 10°C steps). For the preparation of calibration curves, standard solutions of component numbers 6, 8, 13, 15, 22, 24, 26, and 31 (see **Table 1**) were prepared. The phosphorus content of these solutions were determined using the method of Rouser, Siakotos, and Fleischer (11). Mixtures of several species were subsequently used in the construction of calibration curves. Peaks were integrated using the EZChrom Chromatography Data System (Scientific Software, San Ramon, CA). This software was also used to calculate molar amounts of PC species from the constructed (nonlinear) calibration curve.

Identification of species

Peaks eluting from column were collected using a flow splitter between column and detector and were collected manually. The fatty acid contents of collected peaks were determined after hydrolysis in 0.3 m potassium hydroxide in methanol and subsequent derivatiza-

TABLE 1. Composition of PC molecular species

No.	Species	Liver	Surfactant	Heart	Sperm	*S. mansoni*
				mol%		
1	20:4–20:4	tr	–	tr	–	tr
2	18:2–22:6	1.3 ± 0.3	–	–	–	–
3	18:2–20:4	0.7 ± 0.2	–	1.4	0.9	2.2
4	18:2–18:2	2.7 ± 0.3	–	–	tr	tr
5	18:1–22:6	tr	–	tr	tr	0.8
6	16:0–22:6	10.7 ± 0.7	1.0	3.3	2.2	4.1
7	18:1–20:4	3.1 ± 0.4	sp. 8	sp. 8	tr	2.0
8	16:0–20:4	15.1 ± 0.1	0.8	5.6	sp. 9	6.5
9	16:0–22:5	–	–	–	19.5	–
10	18:1–18:2	5.1 ± 1.6	tr	sp. 12	tr	tr
11	16:0–16:1	tr	12.8	sp. 12	–	–
12	(16:0–22:6)PlasCho	sp. 13	–	6.9	33.2	–
13	16:0–18:2	18.0 ± 1.8	20.0	11.2	2.0	7.0
14	16:0–20:3	0.8 ± 1.3	–	sp. 13	–	0.5
15	18:0–22:6	5.5 ± 0.5	–	2.1	1.7	2.3
16	(16:0–22:6)AlkCho	–	–	5.1	8.6	–
17	(16:0–16:1)PlasCho	–	–	sp. 16	–	–
18	(16:0–18:2)PlasCho	–	–	10.7	–	–
19	(16:0–20:3)PlasCho	–	–	4.1	–	–
20	16:0–22:4	–	–	–	–	2.5
21	(16:0–22:5)PlasCho	–	–	–	22.4	–
22	18:0–20:4	11.8 ± 0.3	0.8	2.3	tr	4.2
23	18:1–18:1	2.0 ± 0.1	1.3	3.3	–	2.8
24	16:0–18:1	9.5 ± 0.5	17.8	20.0	1.0	17.2[a]
25	18:0–18:2	11.0 ± 0.1	–	3.3	–	sp. 26
26	16:0–16:0	sp. 25	42.1	sp. 25	1.0	14.5
27	(16:0–Unknown)PC	–	–	–	–	3.3
28	(16:0–18:1)PlasCho	–	–	12.9	–	–
29	(16:0–16:0)PlasCho	–	–	1.8	–	–
30	18:0–20:3	1.2 ± 0.1	–	–	–	–
31	(16:0–16:0)AlkCho	–	–	tr	tr	3.7
32	16:0–20:1	–	–	–	–	14.9
33	18:0–18:1	1.4 ± 0.3	0.8	3.1	2.9	6.0
34	16:0–18:0	tr	1.7	–	–	4.3
35	20:1–20:1	–	–	–	–	tr
36	18:0–18:0	0.8 ± 0.3	tr	–	–	0.5

All species were PtdCho unless indicated otherwise. Rat liver PC was injected in triplicate, resulting in the given mean value ± SD. When components coeluted, the total amount is given with the major component. Minor species in the same peak are indicated with sp. followed by the number of the major component; tr, trace amounts detected (<0.5%); –, none detected.

[a]Also contained (16:0–20:2) PtdCho.

tion of fatty acids to their phenacyl derivatives (12). Phenacyl esters were separated with RP-HPLC and detected at 242 nm as described previously (6). Confirmation of fatty acyl composition and determination of the type of linkage of the aliphatic groups at the *sn*-1 position of the glycerol backbone was achieved by reduction of all C=C bonds using Adams' catalyst and hydrogen as described previously (13). Retention times of hydrogenated species were compared with PtdCho and alkylacyl choline glycerophospholipid (AlkCho) standards purchased from Avanti Polar Lipids, Alabaster, AL. Determination of the positional location of fatty-acyl groups was achieved by phospholipase A_2 digestion of PC (13).

RESULTS

Reduction of head group interaction

Chromatograms showed extensive tailing of peaks due to aspecific interaction between column material and phospholipid headgroup when only methanol and acetonitrile were used to elute molecular species from PC. As an example, the elution of PC isolated from boar sperm cells is shown in **Fig. 1A**. Amine salts are known to be able to prevent this unwanted interaction by competing with the headgroup moieties for binding places on the silica (2) but the presence of a non-volatile salt in the mobile phase would exclude the use of an ELSD. Therefore, we searched for a volatile additive that would be able to prevent aspecific interaction. Addition of 1% (v/v) (or more) triethylamine to the eluent proved to be very effective in achieving this goal (Fig. 1B).

Retention times

The separation of related compounds can be enhanced by using the optimal composition of the solvent. To find these optimal conditions, in which the separation of species in maximal, we modified the characteristics of the mobile phase by changing the percentage of the individual components in the solvent. By making relatively small changes, exponential relationships between the composition of the mobile phase and the retention times of given PC species were established (**Fig. 2**). Increasing amounts of triethylamine or decreasing amounts of acetonitrile resulted in decreasing retention times of molecular species (Figs. 2A and B, respectively). The responses to the changes in mobile phase were not identical for all molecular species, so that changes in composition of mobile phase also influenced separation of components.

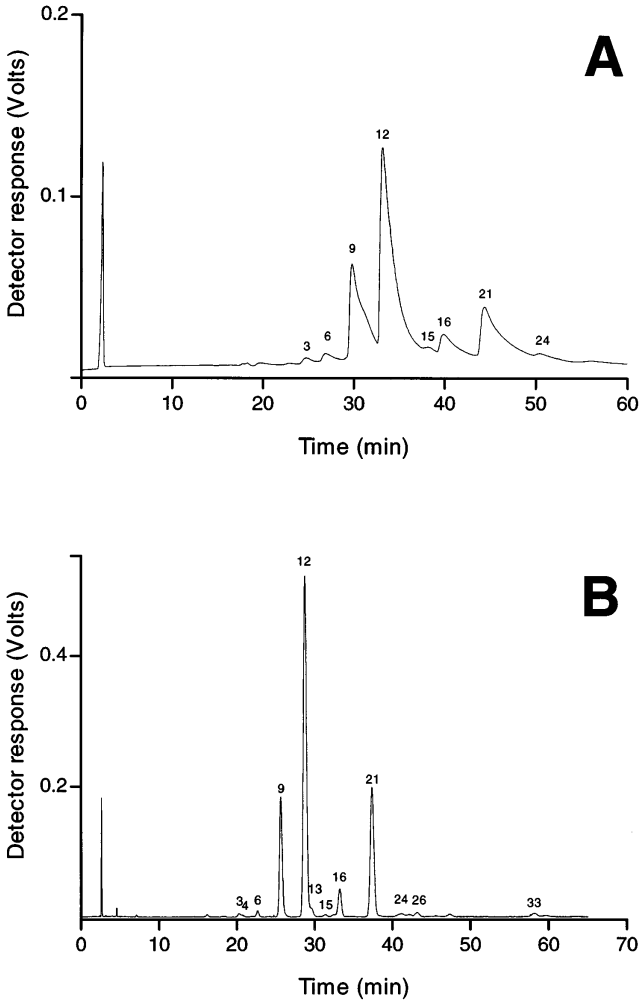

Fig. 1. Reduction of the aspecific interaction of phosphatidylcholine molecular species with the stationary phase of two Lichrosphere 100 RP18 columns in series by the addition of 1% (v/v) of triethylamine to the mobile phase consisting of methanol–acetonitrile 3:2 (v/v). Elution was performed at a flow rate of 1 ml·min^{-1}. Shown is the separation of PC molecular species derived from boar sperm cells as measured by light scattering detection. Peak numbers refer to the components listed in Table 1. Figure A: without triethylamine, B: with triethylamine.

Quantification

Due to the nature of the applied detection technique, at low quantities the response of the ELSD is non-linear with the amount of PC eluting from the column (14). Therefore, calibration curves are required in order to quantitate the separated components. Before calibrating the detector for a number of molecular species, the gas-flow rate and drift-tube temperature of the ELSD were optimized. The signal to noise ratio was determined for 24 combinations of drift-tube temperature and gas-flow as described in the Materials

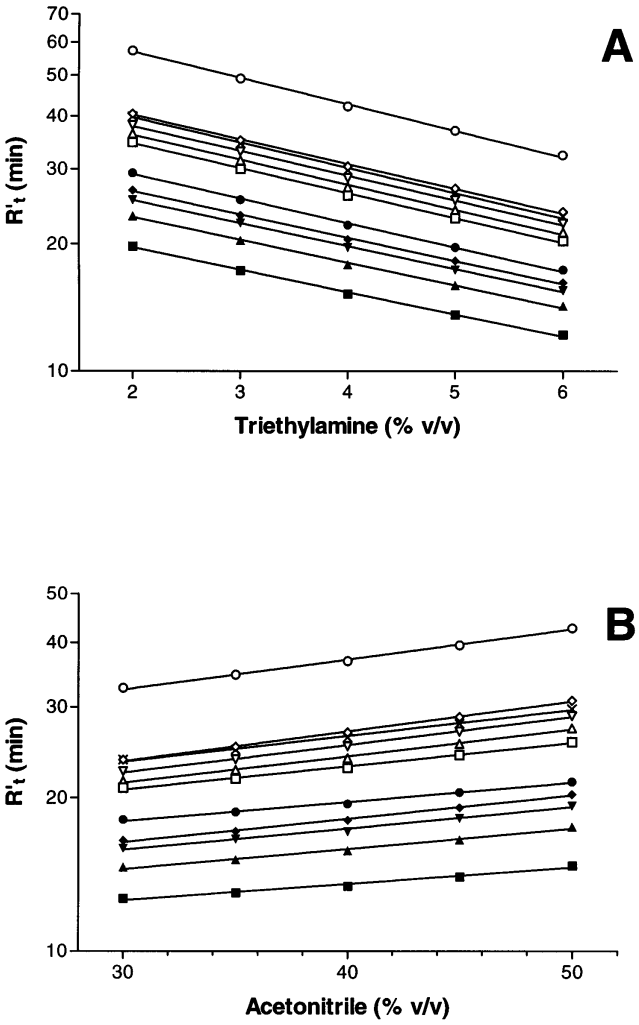

Fig. 2. Exponential relationship between mobile phase composition and the retention times of a number of reference PC species. Known mixtures of PtdCho molecular species were separated on two Lichrospere RP18 columns in series at a flow rate of 1 ml·min^{-1}. The eluting solvent consisted of 40% (v/v) acetonitrile and varying amounts of triethylamine (2%–6%) in methanol (58%–54%) (A), or of 5% (v/v) triethylamine and a variable amount of acetonitrile (30%–50%) in methanol (65%–45%) (B).■: (16:0–22:6)PtdCho; ▲: (16:0–20:4)PtdCho;▼:(16:0–16:1)PtdCho; ◆:(16:0–18:2)PtdCho; ●: (18:0–22:6)PtdCho; □: (18:0–20:4)PtdCho; △:(18:1–18:1)PtdCho;▽: (16:0–18:1)PtdCho; ×: (18:0–18:2) PtdCho; ◇: (16:0–16:0)PtdCho; ○: (18:0–18:1)PtdCho. R′t: relative retention time.

and Methods section, using triplicate injections of 1.0 nmol of (12:0–12:0)PtdCho. Optimal detector configuration was considered the one with the highest signal to noise ratio, which was achieved at 100°C and 1.80 l·min^{-1} (**Fig. 3**). Calibration curves were constructed for eight molecular species (see Materials and Methods section), saturated as well as polyunsaturated and ranging in molecular weight from 719 to 833. Although these PC species differ considerably in their molecular weight and degree of saturation, their calibration curves were almost identical. In fact, all calibration curves were within 6% deviation from the calibration curve shown for (16:0–16:0)PtdCho in **Fig. 4**, making this method also suitable for quantification and characterization of samples with unknown PC composition.

The maximum amount of sample applicable was limited by the loading capacity of the column rather than the detection range of the ELSD. When more than 150 nmol of PC was loaded, the chromatogram suffered from peak broadening and shifting retention times.

Composition of biological samples

A number of biological samples were analyzed to validate the method described **(Fig. 5)**. Samples analyzed and discussed below were either of well-described composition (rat liver and pulmonary surfactant), contained high amounts of saturated species (pulmonary surfactant) or ether-linked fatty alcohols (boar sperm and bovine heart), or were of unknown composition (schistosome PC). To assess the amount of ether lipids, a portion of each sample was hydrogenated. The reduction of C=C bonds allowed discrimination of phospholipids with an equal number of carbon atoms in the aliphatic groups on the type of radyl linkage. Due to the more hydrophobic ether linkage, ether linked species eluted after their PtdCho homologs **(Fig. 6)**.

Rat liver. When PC isolated from rat liver was injected, it resolved into 24 peaks in less than 60 min of total running time (Fig. 5A). Confirming published data (15–17) on the molecular species composition of liver and hepatocytes from rat, the major components are (16:0–20:4)PtdCho, (16:0–18:2)PtdCho, and (18:0–20:4)PtdCho. The PC molecular species composition determined here is given in **Table 1**. After hydrogenation, only saturated diacylPtdCho species were detected (Fig. 6A) with the exception of a small amount of AlkCho with equivalent carbon number 38, probably derived from the small amount of 1-O-hexadec-1′-enyl-2-O-docosahexaenoyl-*sn*-glycero-3-phosphocholine ((16:0–22:6)PlasCho).

Porcine pulmonary surfactant. The separation of PC species from lung surfactant was performed with an increased amount of acetonitrile in the mobile phase to aid in the separation of (16:0–16:1)PtdCho and (16:0–18:2)PtdCho. To prevent prolonged duration needed for analysis, the amount of triethylamine was also increased, causing all species to elute earlier from the column. In porcine lung surfactant, only four species were present in appreciable amount. The high amount of (16:0–16:0) PtdCho in the sample was clearly apparent in the chromatogram (Fig. 5B) which corresponded to literature val-

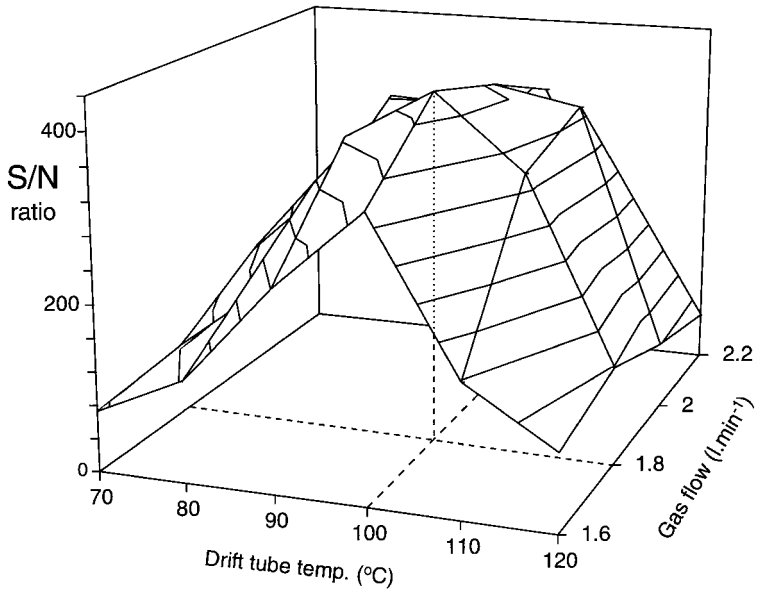

Fig. 3. Signal to noise (S/N) ratio of the response of a Varex MKIII light scattering detector to the injection of 1 nmol of (12:0–12:0)PtdCho at various drift-tube temperatures and gas flow rates. The phospholipid was injected in triplicate on two 5-μm Lichrosphere 100-RP18 columns in series and eluted using 2% triethylamine, 40% acetonitrile, and 58% methanol (v/v) at a flow rate of 1 ml·min^{-1}. The S/N ratio was calculated by dividing the height of the peak by the height of the noise in the baseline. The optimal detector configuration was found at a drift tube temperature of 100°C and a gas flow of 1.80 l·min^{-1}.

ues (4, 18). Ether-linked species were not detected after hydrogenation of the sample (data not shown).

Bovine heart and boar sperm. Heart tissue and spermatozoa are well known for the presence of high amounts of ether phospholipids. In the analysis of boar sperm PC, four major peaks were observed of which three corresponded to ether-linked species, which is in agreement with earlier work (Fig. 1B, Table 1, ref. 19). In bovine heart, mainly PtdCho and PlasCho species were detected, with PlasCho species being retained longer on the column than the corresponding PtdCho species (Fig. 5C). The amount of PlasCho and PtdCho species found compared well with data published earlier (20, 21). When the aliphatic moieties of bovine heart PC were hydrogenated, the high amount of ether phospholipids was even more notable. This is shown in Fig. 6B, were peaks marked with an asterisk represent ether-linked species of bovine heart.

Schistosoma mansoni. Analysis of PC isolated from adult schistosomes revealed unique molecular PC species that were not found in any of the other samples. The high amount of (16:0–20:1)PtdCho (peak 32, Fig. 5D) was especially notable. Although the PC species composition of *S. mansoni* has not been described before, it is known that PC from these organisms contains a significant amount of 20:1, which is synthesized via

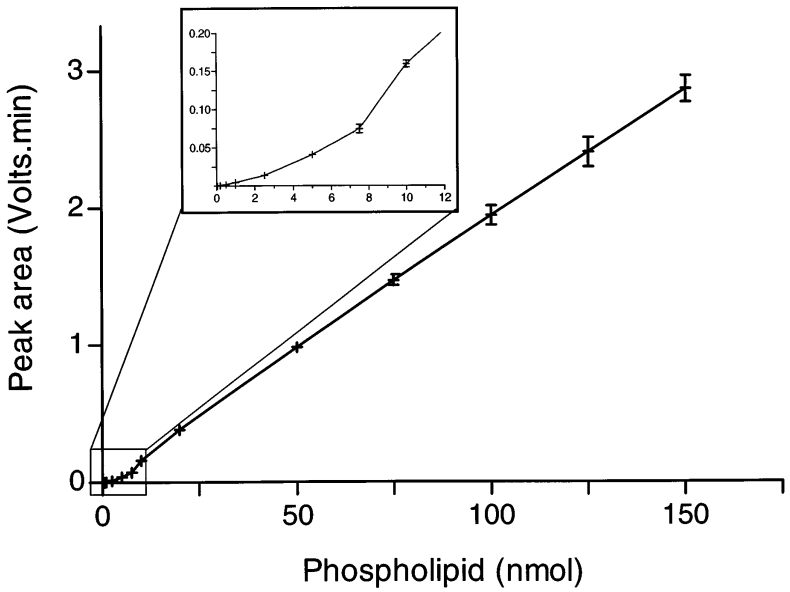

Fig. 4. Mass-response curve for (16:0-16:0)PtdCho using a Varex MKIII evaporative light scattering detector operated at a drift-tube temperature of 100°C and a gas flow of 1.8 l·min^{-1}. A standard solution of which the phosphorus contents was determined using the method of Rouser et al. (11) was used for the construction of calibration curves. Mean values ± SD are given (n=3). Where not shown, standard deviation was too small to allow graphical representation (both figure and inset).

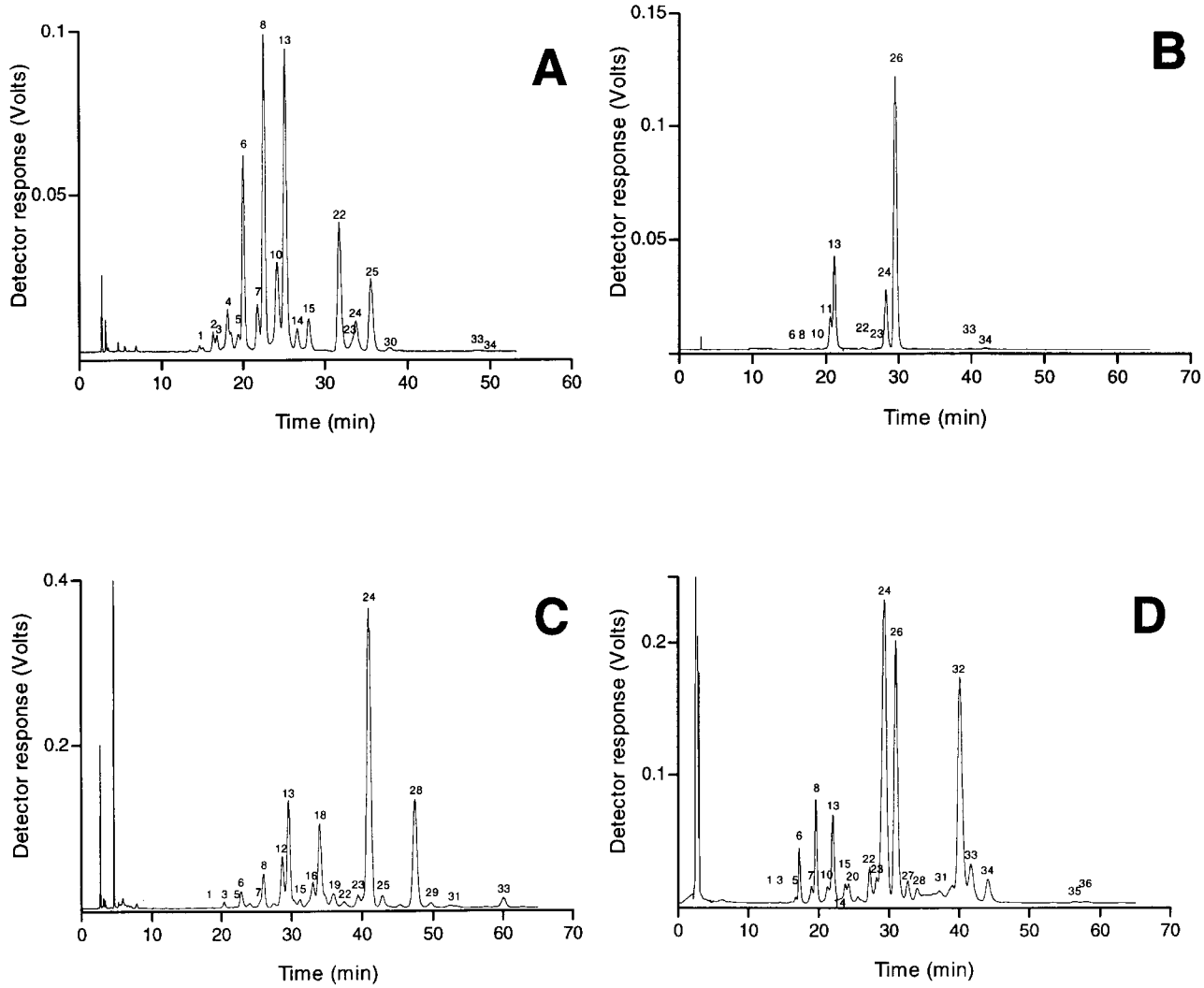

Fig. 5. Separation of PC molecular species by reverse phase HPLC on two 5-μm Lichrosphere 100-RP18 columns in series (total dimensions 4.6 × 500 mm) as detected by light scattering. The solvent system was optimized for each sample and is given below. Peak numbers refer to components given to Table 1. Unnumbered peaks were not identified. Lipids were extracted according to the method of Bligh and Dyer (9) and PC was subsequently isolated by normal phase HPLC according to the method of Letter (10). A: Isocratic elution of intact molecular species of PC isolated from rat liver. The eluting solvent consisted of 2% triethylamine, 40% acetonitrile, and 58% methanol (v/v). A total amount of 50 nmoles was injected. B: Elution of surfactant PC using 5% triethylamine, 50% acetonitrile, and 45% methanol. Note the high amount of the fully saturated species (16:0–16:0)PtdCho (peak 26). C: Separation of PC derived from bovine heart. D: Schistosome PC. Note the occurrence of species containing fatty acids absent from other samples (peaks 20 and 32). The eluting solvent consisted of 4% triethylamine, 35% acetonitrile, and 61% methanol (v/v). All elutions were performed at a flow rate of 1 ml · min^{-1}.

chain elongation from oleic acid (18:1) obtained from the host (22, 23). Small amounts of ether lipids were detected after hydrogenation (Fig. 6C).

DISCUSSION

Separation of molecular species

The use of triethylamine in the separation of molecular species of PC proved to be very efficient in the abolition of aspecific interactions between phospholipid headgroup and column material. This is probably caused by competitive binding of triethylamine to the sites of interaction on the column. The boiling point of triethylamine (89°C) is well within the range of solvents that can be evaporated in an ELSD, making this type of detection applicable in the separation of intact molecular species. Omitting water (causing a high back pressure on reverse phase columns) from the solvent system allowed two RP18 columns to be connected in series without excessive pressure on the column. Depending on the composition of the mobile phase, the pressure

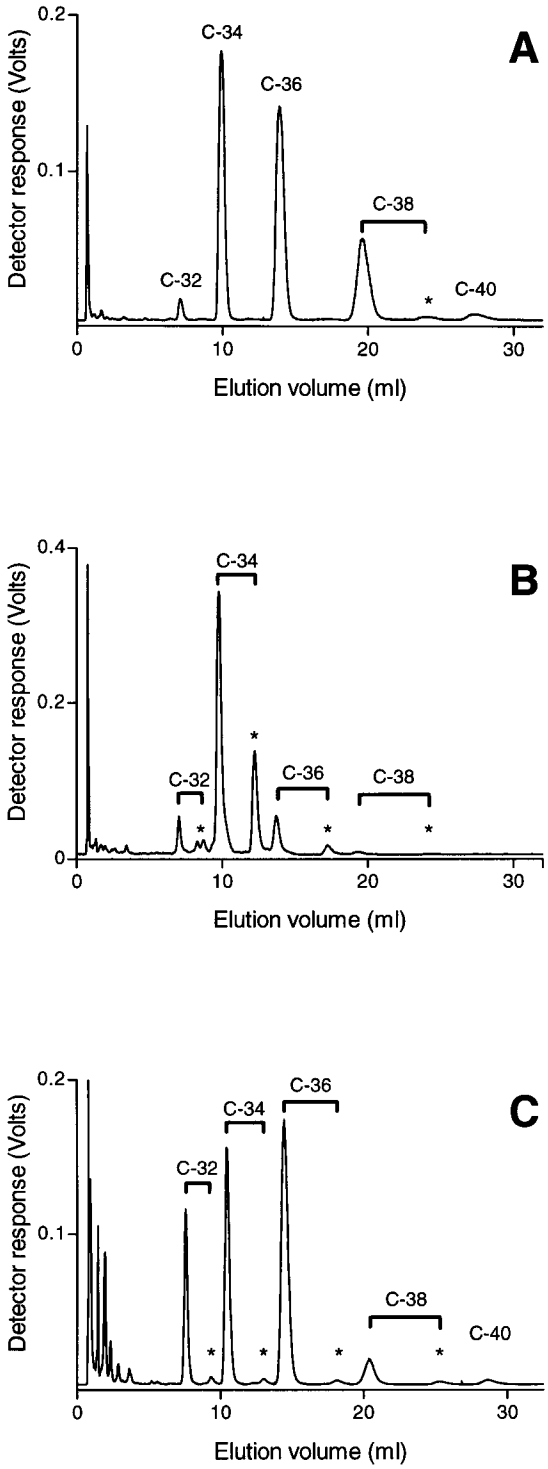

Fig. 6. Separation of alkylacyl and diacyl PC species after saturation of the aliphatic groups. Reduction of C=C bonds was performed with PtO_2 and H_2 as described previously (13). Species were eluted from a single Lichrosphere 100-RP18 column using a solvent system composed of acetonitrile–methanol–triethylamine 20:75:5 (v/v) at a flow rate of 1.5 ml·min^{-1}. Species with an equal number of carbon atoms in the aliphatic groups and similar linkage co-eluted. Compared to diacyl linked species, ether linked species (indicated *) shifted to longer retention times because of the greater hydrophobicity of the ether linkage. The total number of C-atoms in the aliphatic groups is indicated above each peak. Hydrogenated samples from rat liver (A), bovine heart (B), and *Schistosoma mansoni* (C) were analyzed.

drop over the column ranged from 12.0 MPa to 13.6 MPa when the double length column was used. These pressures can easily be delivered by all modern HPLC systems and even allow reduction of analysis time by an increase of flow rate. The connection of two columns in series and the resulting doubling of the number of theoretical plates in the stationary phase is essential for the separation of complex mixtures of molecular species and uses comparatively low amounts of mobile phase.

Both acetonitrile and triethylamine influence retention times (Fig. 2), whereas the concentration of acetonitrile also modifies selectivity. This can be deduced from the slopes in Fig. 2; the difference in retention times of (16:0–16:0)PtdCho and (18:0–18:2)PtdCho is minimal when only 30% acetonitrile is applied (Fig. 2B), but increases with an increasing percentage of acetonitrile. Simultaneously, the difference in retention time of (16:0–18:1)PtdCho and (18:0–18:2)PtdCho decreases with increasing acetonitrile concentrations (Fig. 2B). On the other hand, increasing the percentage of triethylamine decreases the retention of species on the stationary phase, with very little influence on elution order of the components. This can be inferred from Fig. 2A where only parallel lines are observed, indicating that the relative retention time of one component compared to the other is not altered. However, the retention times depicted in Fig. 2 relate to the maxima of the peaks and, especially at short retention times, it might well be that peaks do partially overlap. These mathematical relationships and the knowledge about what components could be expected in a given sample were used to find the optimal solvent composition for the biological samples tested (see below), thus excluding the need for extensive test runs.

Quantification

Light scattering detection, formerly also denoted mass-detection, responds to mass which makes it particularly suitable for the quantification of molecules that do not contain another common property allowing quantification. Therefore, it is not surprising that light scattering detection is more and more used in lipid biochemistry, as the sensitivity of UV detectors for lipids is highly dependent upon the degree of unsaturation, while other detectors have insufficient sensitivity to allow na-

nomolar quantification except when fluorescent PC analogs are used (e.g., ref. 24). However, the response of the detector is not linear over the full range and careful calibration is needed in the range below 20 nmoles (Fig. 4). Saturated species like (16:0–16:0)PtdCho are detected with similar ease as polyunsaturated species (e.g., (16:0–22:6)PtdCho), a large advantage over UV-detection which renders saturated PC species undetectable (3). The (post-column) formation of fluorescent micelles, on the other hand, also enables detection of fully saturated PC species, but the mixing of the column effluent with the fluorescent 1,6-diphenyl-1,3,5-hexatriene results in loss of resolution, while pure species can only be obtained by subsequent post-detection isolation procedures (4). Furthermore, the broad quantification range of PC samples with this new method is notable. Accurate quantification in the range from 50 pmol to 150 nmol (approx. 40 ng–120 μg) can be achieved although the use of the non-linear calibration curve is essential in the range of 50 pmol to 10 nmol (Fig. 4).

Characterization of biological samples

The HPLC-ELSD method described here resolves liver PC into 24 components in 1 h using only 60 ml of solvent. The minor differences in relative quantities of some of the components, observed between literature values and the data in Table 1, can be attributed to differences in the diet, age, and genetic background of the involved rats. The elution order of the PC species, using the method described here, shows great homology to the elution order observed with DAG-dinitrobenzoyl derivatives (16, 25). These derivatives are prepared by dinitrobenzoylation of DAG obtained after phospholipase C digestion of PC, thus eliminating aspecific headgroup interaction. Both methods omit water from the mobile phase and use either methanol or acetonitrile as main solvents. This analogy in elution order further demonstrates the very efficient neutralization by triethylamine of the aspecific headgroup interaction between PC and the column material.

Porcine pulmonary surfactant contains mainly saturated or mono-unsaturated PC species. These phospholipids constitute the air–liquid interface in the lung and are therefore subject to high concentrations of molecular oxygen. A high degree of unsaturation would make these phospholipids vulnerable for oxidative damage, and the high amount of DPPC, as well as its surface tension lowering properties, is considered to be an adaptation to the direct environment of these phospholipids. This highly saturated sample cannot be characterized by UV-detection because of the lack of double bonds in the molecule (3).

The high fusogenicity of the membranes of spermatozoa (a hallmark of these cells which is required for fertilizing egg cells) is reflected in the composition of PC species. These species contain large amounts of highly unsaturated aliphatic chains, giving the membranes a high lateral fluidity. In Fig. 1B it is clearly seen that PtdCho, PlasCho, and AlkCho analogs of (16:0–22:6)PC were completely separated (peak numbers 6, 12, and 16 respectively). The high content of polyunsaturated phospholipids in sperm membranes makes these cells vulnerable for lipid peroxidation which indeed has been identified as a major cause of male infertility (26). The majority of the PC species were ether-linked as has been shown previously (Table 1)(19). In bovine heart, the number of different PlasCho and PtdCho species was considerably higher, but a good separation between species was nevertheless achieved. After hydrogenation, the high amount of ether phospholipids was even more apparent (Fig. 6B).

Schistosomes are unable to synthesize fatty acids (and also sterols) de novo and depend on the host to supply lipid precursors during the growth of the parasite from a small schistosomula to adults of approximately 1 cm length (22). The high amount of schistosome "unique" species was therefore remarkable, but is explained by the capacity of the schistosome to elongate fatty acids obtained from the host. In particular, the conversion of oleate (18:1) to eicosaenoate (20:1) has been well described (6, 22).

In conclusion, we described a new method to separate and detect intact PC species from various biological origins using HPLC and light scattering. Detection is species independent and very sensitive; as little as 40 ng of PC can be detected while the upper limit is determined by the loading capacity of the column. The method can be applied for the preparation of individual PC species in order to detect their biological activities.

Lipids derived from porcine lung surfactant were a kind gift from Edwin Veldhuizen in our laboratory. Electronic preparation of figures and manuscript was greatly facilitated by Cobex Corp. (Roermond, The Netherlands). This work was supported by the Netherlands Foundation for Chemical Research (SON), the Life Science Foundation (SLW) of The Netherlands Organization for Scientific Research (NWO), and by the Royal Dutch Academy of Arts and Sciences (KNAW).

Manuscript received 28 July 1997 and in revised form 16 October 1997.

REFERENCES

1. Exton, J. H. 1990. Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.* **265:** 1–4.
2. Patton, G. M., J. J. M. Fasulo, and S. J. Robins. 1982. Separation of phospholipids and individual molecular species of phospholipids by high-performance liquid chromatography. *J. Lipid Res.* **23:** 190–196.

3. McHowat, J., J. H. Jones, and M. H. Creer. 1996. Quantitation of individual phospholipid molecular species by UV absorption measurements. *J. Lipid Res.* **37:** 2450–2460.
4. Postle, A. D. 1987. Method for the sensitive analysis of individual molecular species of phosphatidylcholine by high-performance liquid chromatography using post-column fluorescence detection. *J. Chromatogr.* **415:** 241–251.
5. Curstedt, T., H. Jörnvall, B. Robertson, T. Bergman, and P. Berggren. 1987. Two hydrophobic low-molecular-mass protein fractions of pulmonary surfactant. Characterization and biophysical activity. *Eur. J. Biochem.* **168:** 255–262.
6. Brouwers, J. F. H. M., I. M. B. Smeenk, L. M. G. van Golde, and A. G. M. Tielens. 1997. The incorporation, modification and turnover of fatty acids in adult *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* **88:** 175–185.
7. Johnson, L. A., J. G. Aalbers, and H. J. G. Grooten. 1988. Artificial insemination of swine: fecundity of boar semen stored in Beltsville TS (BTS) Modified Modena (MM), or MR-A and inseminated on one, three and four days after collection. *Züchthyg.* **23:** 49-55.
8. Harrison, R. A. P., B. Mairet, and N .G. A. Miller, 1993. Flow cytometric studies of bicarbonate-mediated Ca^{2+} influx in boar sperm populations. *Mol. Reprod. Dev.* **35:** 197–208.
9. Bligh, E. G., and W. J. Dyer, 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem Physiol.* **37:** 911–917.
10. Letter, W. S. 1992. A rapid method for phospholipid class separation by HPLC using an evaporative light-scattering detector. *J. Liq. Chromotogr.* **15:** 253–266.
11. Rouser, G., A. N. Siakotos, and S. Fleischer. 1966. Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. *Lipids.* **1:** 85.
12. Wood, R., and T. Lee. 1983. High performance liquid chromatography of fatty acids: quantitative analysis of saturated, monoenoic, polyenoic and geometrical isomers. *J. Chromatogr.* **254:** 237–246.
13. Christie, W. W. 1989. Gas Chromatography and Lipids. The Oily Press, Ayr, Scotland. 83–84.
14. Guiochon, G., A. Moysan, and C. Holley. 1988. Influence of various parameters on the response factors of the evaporative light scattering detector of a number of non-volatile compounds. *J. Liq. Chromatogr.* **11:** 2547–2570.
15. Robins, S. J., J. M. Fasulo, V. F. Robins, and G. M. Patton. 1991. Utilization of different fatty acids for hepatic and biliary phosphatidylcholine formation and the effect of changes in phosphatidylcholine molecular species on biliary lipid secretion. *J. Lipid Res.* **32:** 985–992.
16. Schmid, P. C., E. Deli, and H. H. O. Schmid. 1995. Generation and remodeling of phospholipid molecular species in rat hepatocytes. *Arch. Biochem. Biophys.* **319:** 168–176.
17. Samborski, R. W., N. D. Ridgway, and D. E. Vance. 1990. Evidence that only newly made phosphatidylethanolamine is methylated to phosphatidylcholine and that phosphatidylethanolamine is not significantly deacylated-reacylated in rat hepatocytes. *J. Biol. Chem.* **30:** 18322–18329.
18. Adachi, H., H. Hayashi, H. Sato, and T. Akino. 1989. Characterization of phospholipids accumulated in pulmonary-surfactant compartments of rats intratracheally exposed to silica. *Biochem. J.* **262:** 781–786.
19. Evans, R. W., D. E. Weaver, and E. D. Clegg. 1980. Diacyl, alkenyl, and alkyl ether phospholipids in ejaculated, in utero-, and in vitro-incubated porcine spermatozoa. *J. Lipid Res.* **21:** 223–228.
20. Pugh, E. L., M. Kates, and D. J. Hanahan. 1977. Characterization of the alkyl ether species of phosphatidylcholine in bovine heart. *J. Lipid Res.* **18:** 710–716.
21. Malewicz, B., and W. J. Baumann. 1996. Alk-1-enylacyl, alkylacyl, and diacyl subclasses of native ethanolamine and choline glycerophospholipids can be quantified directly by phosphorus-^{31}NMR in solution. *Lipids.* **31:** 1189–1195.
22. Meyer, F., H. Meyer, and E. Bueding. 1970. Lipid metabolism in the parasitic and free-living flatworms, *Schistosoma mansoni* and *Dugesia dorotocephala*. *Biochim. Biophys. Acta.* **210:** 257–266.
23. Allan, D., G. Payare, and W. H. Evans. 1987. The phospholipid and fatty acid composition of *Schistosoma mansoni* and of its purified tegumental membranes. *Mol. Biochem. Parasitol.* **23:** 123–128.
24. Kasurinen, J., and P. Somerharju. 1992. Metabolism of pyrenyl fatty acids in baby hamster kidney fibroblasts. *J. Biol. Chem.* **267:** 6563–6569.
25. Takamura, H., H. Narita, R. Urade, and M. Kito. 1986. Quantitative analysis of polyenoic phospholipid molecular species by high performance liquid chromatography. *Lipids.* **21:** 356–361.
26. Aitken, R. J., and J. S. Clarkson. 1987. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J. Reprod. Fertil.* **81:** 459–469.