Quantification of major classes of Xenopus phospholipids by high performance liquid chromatography with evaporative light scattering detection

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Abstract **Lipid signaling has become a major research area of cell biology and there is a need for methods that accurately and easily measure substrates and products of lipases involved in cell signaling. In this report, we provide new methodology for separation of more than 10 lipids in one chromatographic run by high pressure liquid chromatography (HPLC) and detection with an evaporative light scattering detector (ELSD). There is no significant loss of sphingomyelin and no large baseline change, no peak obscures another, and acidic phospholipids are cleanly separated. We have optimized the procedure for a two-pump HPLC, an inexpensive silica column without the use of a column heater jacket and for low grade nitrogen. An application of the procedure separates lipids from *Xenopus laevis* cells. These cells are commonly used in the study of various lipid signaling paths in cell division, fertilization, and after expression of exogenous membrane receptors.**—Stith, B. J., J. Hall, P. Ayres, L. Waggoner, J. D. Moore, and W. A. Shaw. **Quantification of major classes of *Xenopus* phospholipids by high performance liquid chromatography with evaporative light scattering detection. *J. Lipid Res.* 2000.** 41: **1448–1454.**

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Major phospholipids vary greatly in their polarity and this has made separation of the major classes difficult (1). Because phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are substrates for phospholipase D and phosphatidic acid (PA) is a product, separation techniques for these lipids are of interest (2). Phosphatidylserine (PS) acts a cofactor for protein kinase C (3), phosphatidylinositol (PI) is a precursor for two lipids known to be important in lipid signaling (e.g., phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 3′-phosphate) (4). Sphingomyelin (SM) is a substrate for sphingomyelinase and this enzyme has been shown to be involved in cell regulation (5). The method described below separates PC, PE, PS, PI, and SM. We also report resolution of ceramide, triglycerides (TG), cholesterol (CH), free fatty acids (FA), phosphatidylglycerol (PG), cardiolipin (CA), monoacyl phosphatidylethanolamine (LPE), and monoacyl phosphatidylcholine (LPC).

Most solvent systems used for separation of lipids begin with either *n*-hexane–2-propanol–water (6) or chloroform–methanol (7).

Even with improvements of the hexane–2-propanol solvent system, there are problems with solvent viscosity and separation of PI, PS, and SM [see summary in ref. 8; note the lack of sphingomyelin peak in ref. 9; negligible amounts of PS or sphingomyelin are reported for salmon eggs (10)]. Modifications include use of dangerous solvents (6) or tetrahydrofuran [Christie and Urwin (11) and others recommend against its use because of oxidation].

Taking these criticisms into consideration, we chose the chloroform–methanol solvent system. However, the laboratories at the University of Colorado-Denver and at Avanti Polar Lipids (Alabaster, AL) independently found that the method of Becart, Chavalier, and Biesse (7) produces a varying baseline and does not separate certain phospholipids well (e.g., PI or PS from PC).

We have modified the Becart, Chavalier, and Biesse (7) procedure to analyze lipids from *Xenopus laevis* cells. In addition, while many methods separate small amounts of phospholipid standards, cellular samples demand greater separation of elution times. That is, because large PE and PC peaks can obscure small PI and PS peaks (8 and 12), we altered

Abbreviations: CA, cardiolipin (1,1′,2,2′-tetramyristoyl-acyl-cardiolipin); CH, cholesterol; FA, free fatty acids; LPC, monacyl phosphatidylcholine (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidylcholine); LPE, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidylethanolamine; PA, 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid; PG, 1,2-dimyristoyl-*sn*-glycero-3-phospho(*rac*-1-glycerol); PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PG, phosphatidylglycerol; PI, bovine liver phosphatidylinositol; PS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; SM, chicken egg sphingomyelin; TG, triglyceride (1,2,3-trioleoylglycerol).

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the elution times of phospholipids so that PI and PS peaks are separated by 4 min or more from the larger PC and PE. The new method also corrects the varying baseline that occurs with the Becart, Chavalier, and Biesse (7) method.

Evaporative light scattering detectors (ELSD) are universal detectors because they quantify any solutes less volatile than the solvents (6, 13). The use of the ELSD eliminates lipid derivatization, produces a quantitative response (6, 7), and allows the use of chloroform (this nonpolar solvent improves lipid solubility, especially that of sphingomyelin). The ELSD is not sensitive to solvent flow rate or ambient temperature (6).

In contrast, thin-layer chromatography (TLC) separation is sensitive to loading, has a relatively narrow linear response, and many have found that even the use of a scanner did not produce sufficient "accuracy" (7). Refractive index detectors are less sensitive, and their baseline changes with variation in temperature, pressure, and flow rate and with different solvents. Because of the latter problem, gradient separation with these detectors is restricted to a few pairs of solvents that have virtually identical refractive indices.

UV absorption (typically, at 203–214 nm) is contraindicated because chloroform absorbs below about 245 nm. In addition, UV absorption is largely dependent on the number of double bonds present in the lipid (absorbance by naturally occurring functional groups in lipids is negligible; refs. 14 and 15). Thus distearate phosphatidylcholine (no double bonds) has an extinction coefficient (M^{-1} cm^{-1}) of 200 whereas that of dilinoleate phosphatidylcholine (4 double bonds) is 12,900 M^{-1} cm^{-1} (16). Derivatization of lipids can result in higher levels of sensitivity than the ELSD but the procedure is subject to problems such as the use of "dry" reagents, and varying levels of derivatization from preparation to preparation and from lipid to lipid (6).

MATERIALS AND METHODS

Standards and chemicals

Standards (all obtained from Avanti Polar Lipid; >99% chromatographically pure) were as follows: PA, 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid; TG, 1,2,3-trioleoylglycerol; CH, cholesterol; CA, 1,1′,2,2′-tetramyristoyl-acyl-cardiolipin; PG, 1,2-dimyristoyl-*sn*-glycero-3-phospho(*rac*-1-glycerol); LPE, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidylethanolamine; LPC, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidylcholine; PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; SM, sphingomyelin from egg (percentage of molecular species: 78% for 16:0, 7% 18:0, 2% 20:0, 4% 22:0, 4% 24:1, 3% 24:0, 2% 22:6); PI, bovine liver phosphatidylinositol (2.7% 16:0; 48.4% 18:0; 14.5% 18:1; 8.8% 18:2, 9.2% 20:3; 13.4% 20:4); soy PI (29.5% 16:0, 8.18% 18:0, 5.7% 18:1, 47.26% 18:2, 7.18% 18:3), PS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine. All solvents were from J. T. Baker (Phillipsburg, NJ) or Fisher (Fairlawn, NJ) (high performance liquid chromatography [HPLC] grade) (choroform stabilized by 0.75% ethanol). It is important to obtain solvents with the lowest particulate value ("ppm") because the ELSD can detect these particulates.

The relationship between the amount of standards and the ELSD peak size was examined by linear or polynomial regression analysis, using Sigmaplot 4.0 for Windows (SPSS, Chicago, IL).

Extraction of lipids

Excess liquid was removed and 80 *Xenopus* oocytes were rinsed and washed once with O-R2 (83 mM NaCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.9). After all excess O-R2 is removed, 1 ml of chloroform–methanol 1:2 was added and cells were homogenized with 20 strokes with a Dounce homogenizer filled with nitrogen. After transferring the extract to a test tube, the mortar and pestle were washed with 0.5 ml of chloroform followed by 0.5 ml of 1 M NaCl. Use of acid (instead of 1 M NaCl) to negate charge and drive phospholipids into the organic phase resulted in significant lyso formation (as detected by an enzymatic *sn*-1,2-diacylglycerol [*sn*-1,2-DAG] assay) (2).

The extract and washes were combined and then centrifuged (clinical centrifuge; setting of high, 2 min) and the organic layer was removed and stored at −20°C. All lipid solutions were kept under nitrogen and were stored in chloroform to minimize oxidation and polymerization.

Just before use, cell extracts were dried under nitrogen and reconstituted in chloroform–methanol 2:1. We examined whether this step resulted in loss of standards; less than 2% of PE, PI, PC, or SM was lost at this step but the loss of PS amounted to 14.6%.

HPLC and ELSD equipment

To separate lipids, the analytical laboratory at Avanti Polar Lipids used an Agilent Technologies (Palo Alto, CA) model 1050 quaternary pump system and an Astec diol normal-phase silica column (250 mm × 4.6 mm i.d., 5-μm spherical particle size; Advanced Separations Technologies, Whippany, NY) heated to 50°C. Injection volume was 20 μl and no flow splitter was used.

At the University of Colorado-Denver, a model HPXL Rainin-Varian (Emeryville, CA) HPLC was connected to a normal-phase Microsorb silica column (25 cm long, 5-μm particle size, 100-Å pore size, 4.6 mm i.d.; Rainin-Varian). These silica columns are one-third the cost of bonded columns (such as the diol column) but bonded columns offer greater stability and more rapid re-equilibration times. A splitter sent 20% of the flow to a fraction collector and 80% to the ELSD (data were corrected for this loss of 20% of the sample). Injection loops of either 20 or 200 μl were used. In an attempt to simplify and economize, this laboratory did not use a column heater jacket, a more expensive diol silica column, or highly purified nitrogen gas for the ELSD, and we modified the solvent gradient for a two-pump HPLC system.

In both laboratories, there was a flow rate of 1 ml/min, and typical column pressures were about 1 kpsi (older columns produced as much as 2 kpsi).

Guard columns saturate solvents with silica to reduce silica loss from the analytical column. Because the cost of a guard column can exceed $100 (an analytical Microsorb normal-phase column costs $125), guard columns can cause peak spreading (17), and a normal-phase column can last a period of months, guard columns were not used.

In both laboratories, the ELSD (Sedex model 55; Richard Scientific, Novato, CA) was set at a detector temperature of 40–42°C, nitrogen flow pressure of 1.7–2.2 bar (1.7×10^5 to 2.2×10^5 Pa), and a gain of 7. This make of ELSD has a relatively low rate of nitrogen gas usage, and can operate at lower temperatures without accessories. Note that the laboratories of Avanti Polar Lipids used grade 5 nitrogen (99.999% pure) whereas the University of Colorado-Denver laboratory used industrial grade (99.9% pure). In the University of Colorado-Denver laboratory, ELSD data were collected and analyzed with the Dynamax Method Manager (Rainin-Varian) whereas Avanti laboratories used an Agilent (New Castle, DE) A/D converter to perform electronic integration of detector peaks. With the Dynamax software (version 1.4.1), intermediate ordinate values are not printed.

TABLE 1. HPLC gradient

Time	Solution 1	Solution 2	Solution 3
min	%	%	%
0–5	100	0	0
5–15	0	100	0
20	0	100	0
30	0	0	100
45	0	0	100
50	100	0	0
60	100	0	0

HPLC gradient

To improve phospholipid separation, we altered solvent gradients, added a third solvent, changed the amount of methanol, and doubled the percentage of ammonium hydroxide recommended by Becart, Chavalier, and Biesse (7). The gradient used by Becart, Chavalier, and Biesse (7) for phospholipid separation is a linear change from chloroform–methanol–30% ammonium hydroxide 80:19.5:0.5 to chloroform–methanol–water–30% ammonium hydroxide 60:34:5.5:0.5. Concentrated ammonium hydroxide (EM Science, Cherry Hill, NJ) was kept in a −20°C freezer, and new containers are used as often as possible.

Although the laboratories of Avanti Polar Lipids were able to take advantage of a four-pump system, the following method was developed for the more commonly available two-pump HPLC system (such as that at the University of Colorado-Denver). With a switch that allowed the use of two different solvents by pump B, this procedure was used (**Table 1**). Solution 1 (pump B) (chloroform–methanol–30% ammonium hydroxide 80:19:1) is the starting solvent and it is run for at least 5 min (this first 5-min period enhanced PS separation because it maintained a minimal column reequilibration time between runs). Over the next 10 min, there is a linear gradient changing from solution 1 (pump B) to solution 2 (pump A) (chloroform–methanol–30% ammonium hydroxide 60:39:1). Solution 2 is run for 5 min and then there is a 10-min linear gradient changing back to pump B, which now has solution 3 (chlorform–methanol–water–30% ammonium hydroxide 60:34:5:1). Solution 3 is run for 15 min. To set up the HPLC for the next run, a 5-min linear gradient switching back to solution 2 on pump A is then run. The switch on the lines to pump B is thrown so that solution 1 is "on line" and there is a 5-min linear gradient back to pump B. Thus, the switch on pump B is changed between 15 and 20 min, and again at 50 min.

Daily, we would wash the normal-phase column with 80% methanol for 20 min, followed by 100% methanol for 20 min. The column was stored overnight in 100% methanol. Each day would begin with a period of time during which solution 1 was run through the column, then a blank run (no sample injected) was performed, and finally a run with standards was conducted. Only after these runs would a cell extract be injected.

RESULTS AND DISCUSSION

The method of Becart, Chavalier, and Biesse (7) was used to separate phospholipids from a *Xenopus* egg extract (**Fig. 1**). Separation was variable but these results were typical. Note the changing baseline, the inability to clearly separate PI and PS, and that the elution times were relatively close (so that large peaks of PE and PC tend to overlap others).

Using our new method, a typical separation of phospholipid standards performed at the University of Colorado-Denver (**Fig. 2A**) shows good separation of peaks. A column jacket is recommended if PS analysis is to be emphasized because there is a decrease in PS peak size and peak sharpness when using a column at room temperature (the elution of PI, PC, and PE was not significantly affected by increasing column temperature to 50°C) (Fig. 2B). Separation of many different standards is shown in a chromatogram from Avanti Polar Lipids (Fig. 2C) (note that they do not use the first 5 min of the run described in Table 1). Neutral lipids (e.g., diacylglycerol, triglycerides), ceramide, and cholesterol elute very early (about 3 min).

Standards produced rectilinear results over the range of 10 to 50 μg (for a lipid with a molecular weight of 700, this would be about 1.4 to 8.6 nmol) (**Fig. 3**) and these lines were used in the analysis of cellular samples. Regression using second- and third-order polynomials did not improve the regression coefficient. Christie (6, 11) reported a linear ELSD response over 10–200 μg. There are other reports of ELSD linearity over limited ranges of 10–40 μg (18) or 15–98 μg (19).

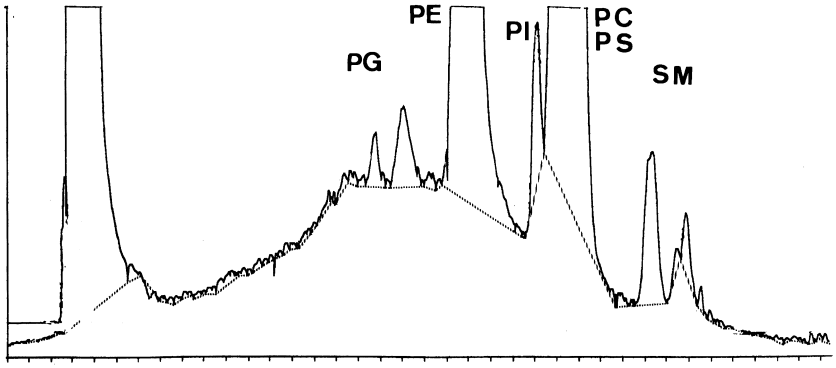

Fig. 1. Initial separation of phospholipids. Lipids from an extract of *Xenopus* cells were separated by the method of Becart, Chavalier, and Biesse (7). The abscissa is time (min) and the ordinate is the ELSD response in microvolts (thus, electronic integration of peaks is reported elsewhere as μV-sec). In addition to the elution of those lipids shown, neutral lipids eluted at about 3.7 min (large peak far left).

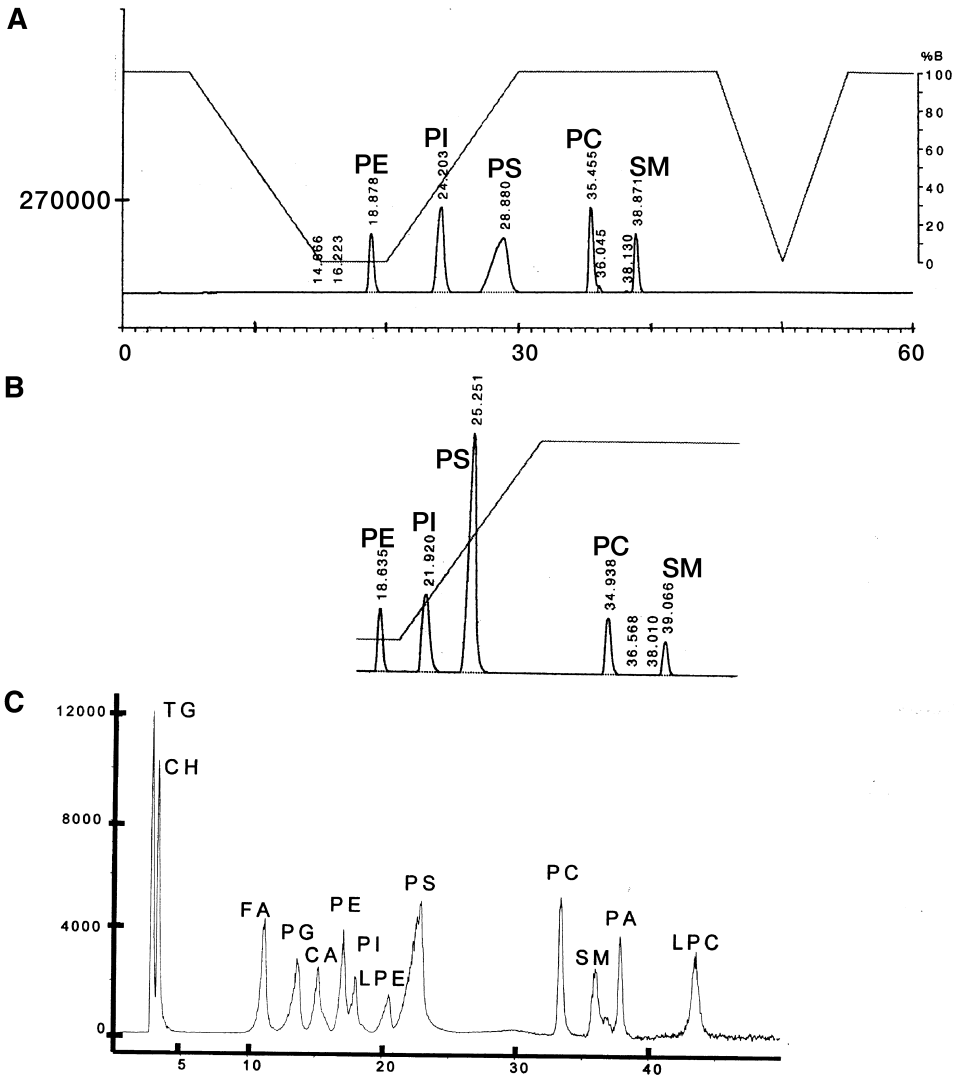

Fig. 2. Separation of lipid standards from two laboratories. (A) A mixture of standards (in a total volume of 20 μl; 20 μg of PE, 50 μg of PI, 80 μg of PS, 20 μg of PC, and 30 μg of SM) was separated on an inexpensive silica column with a two-pump HPLC system at the University of Colorado-Denver. The gradient of solvents is diagrammed as a straight line (representing the percentage or contribution of flow due to pump B). As is typical, sphingomyelin splits into two peaks; both peaks are combined for use in the standard line. Not shown: ceramide elutes at 3.5 min. (B) After a column jacket heated the silica column to 50°C, the standards were run again. Note that the elution of PI and PS was earlier and that the PS peak was much larger and sharper. As compared with the separation in (A), there were no or minimal changes in PE, PC, and SM peaks. (C) Separation of lipid standards by the heated diol column and three-pump HPLC system in the Avanti Polar Lipids analytical laboratories. The abscissa represents time (min) whereas the ordinate presents the ELSD response (μV). TG, CH, FA, PG, CA, PE, soy PI, LPE, PS, PC, SM, PA, and LPC (20 μg of each standard in a total volume of 20 μl) were injected. Note that, as is commonly found, neutral lipids produce a larger ELSD response than phospholipids or SM.

Because of properties of both the solvent and the solute (16), the ELSD produces different slopes for the standard lines for phospholipids. In spite of the use of different solvent systems or detectors, different laboratories find that acidic phospholipids (e.g., PI, PS, and PA) produce smaller detector signals (**Table 2**). Most ELSD detectors available before about 1990 produced standard lines with slopes of less than 100,000 (6, 18, 20).

To examine lower limits of detection, we used PI for further study. This lipid was chosen because it produces a lower ELSD response, and Avanti Polar Lipids have found that is sensitive to oxidation and is unstable at room temperature. The use of this lipid would be a good test of sensitivity, lipid handling, and storage procedures.

A wider range of PI standard (1–80 μg) versus the ELSD response was nonlinear. A double log plot produced a regression line that was accurate only at midrange standard values [although, overall, the r^2 was 0.973; the regression line was y = (0.327)x − 1.653]. A second-order polynomial proved to be a better fit for all values (r^2 =

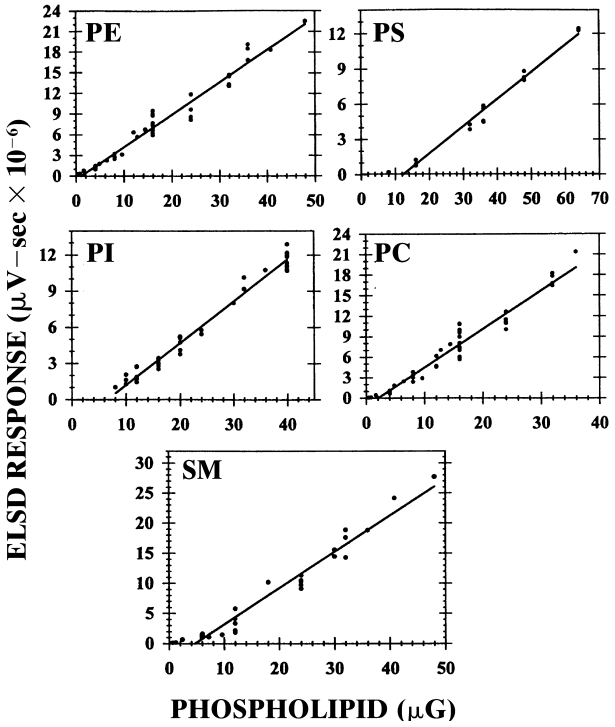

Fig. 3. Standard lines for lipids. On the basis of data from runs such as in Fig. 2A, the ELSD detection was recorded and graphed versus the amount of phospholipid or SM injected. Standards were typically injected as a mixture and peak areas were corrected for a flow splitter that diverted 20% of the sample to a fraction collector (not to the ELSD). Regression lines for the standards (regression coefficient is in parentheses): PI, y = (0.347)X − 2.24 (r^2 = 0.98); PE, y = (0.470)X − 0.47 (r^2 = 0.95); PC, y = (0.561)X − 1.08 (r^2 = 0.94); PS, y = (0.231)X − 2.79 (r^2 = 0.98); SM, y = (0.599)X − 2.83 (r^2 = 0.95).

0.989) but a third-order regression produced the best fit (r^2 = 0.994) (**Fig. 4**). A fourth-order regression line was similar in accuracy to the third order and a fifth-order line was not accurate. If the highest (80 μg) data were not included, a power function produced a regression line that

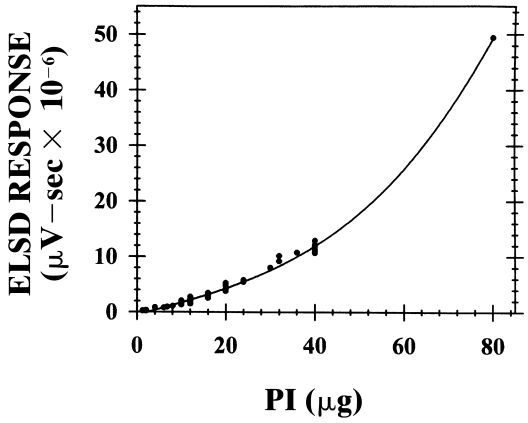

Fig. 4. Analysis of PI standard line. Using a wider range of PI standard, the best regression fit was a third order polynomial. The correlation coefficient (r^2) was 0.994, and the regression line was as follows: $y = (-0.5639) + (0.2266)x + (0.000687)x^2 + (0.000070512)x^3$.

was similar in accuracy to the second-order polynomial [r^2 = 0.984; $y = (0.0600)x^{1.432}$] but if the range was extended, the power function did not produce an accurate regression. Using standard values of 1–25 μg of various phospholipids, regression analysis by Bunger and Pison (12) produced a power function with approximately 1.7 as the exponent. A similar regression analysis of a wide range of PE standard (range, 0.3 to 48 μg) resulted in a second-order polynomial producing the best results (it was better than a linear regression and a third-order polynomial produced results equivalent to those of the second-order regression). A wide range in the amount of phospholipid typically produced a nonlinear standard line (9, 20, 21).

With a PI molecular weight of 909.12, the lower limit of the PI standard line (1 μg) equates to 1.1 nmol. Christie also found a lower practical limit of about 1 μg (6, 11). We did not need to optimize our ELSD settings for maximal sensitivity or increase the gain beyond midrange, but, on the basis of our signal-to-noise ratios, we would suggest that the limit of detection would be about 0.1–0.2 μg (~0.3 nmol). This is similar to the lower limit (about 0.2 μg of lipid) noted by Picchioni, Watada, and Whitaker (21). For lipids that produce higher ELSD responses (PE, PC), Becart, Chavalier, and Biesse (7) estimated lower detection limits of about 0.02 μg. These lower limits of detection can be compared with UV detection: Maximum

TABLE 2. Comparison of slopes of standard lines[a]

	Christie and Urwin (1995)	Silversand and Haux (1997)	Present Article (Fig. 3)	Homan and Anderson (1998)	Picchioni et al. (1996)
PE	720,000	780,000	470,000	229,000	250,000
PC	560,000	720,000	561,000	453,850	250,000
PI	420,000	450,000	347,000	153,750	200,000
PS	ND	330,000	184,615	161,550	ND

These are estimates of relative response; variation in slope from one laboratory to another could be due to detector, solvent, phospholipid source, or the amount of standard. Note Christie and Urwin (11) used a Varex Model III, Picchioni, Watada, and Whitaker (21) used a Varex IIA, whereas Silversand and Haux (10) used a Sedex 45 (all these laboratories used gradients involving hexane, isopropanol, and acetic acid). Homan and Anderson (9) used a Sedex 55 (data from their Table 2; they used an isooctane–tetrahydrofuran, acetone, dichloromethane, propanol, water, acetic acid ternary gradient). In higher standard ranges, the slopes presented by Homan and Anderson (9) appear to be similar to those in the present article. ND, no data presented.

[a] Microvolts per second.

TABLE 3. Reproducibility of ELSD detection

Amount of PI	ELSD Peak Size: Average ± SE (n)	ELSD Peak Size: Coefficient of Variation
μg	*μV-sec*	%
12	1.997 ± 0.103 (6)	12.60
16	3.322 ± 0.289 (7)	9.6
20	5.558 ± 0.322 (5)	12.94
24	5.792 ± 0.322 (4)	11.13
40	12.410 ± 0.692 (4)	11.14

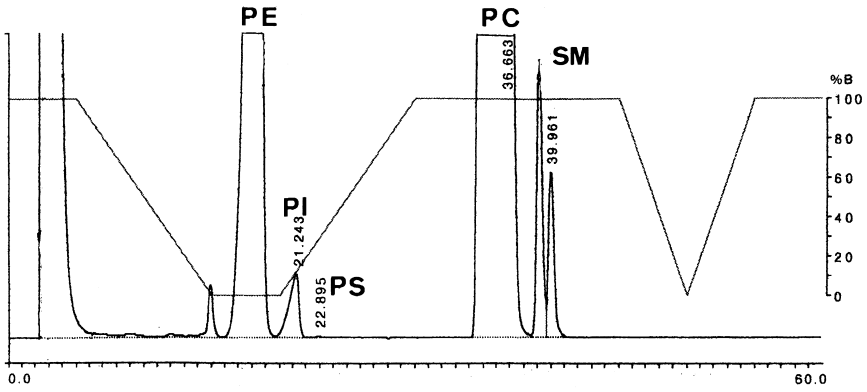

Fig. 5. Analysis of an extract from *Xenopus* eggs. The lipids from 80 *Xenopus* cells were extracted and injected onto the HPLC for separation. Neutral lipids eluted by 4 min and phospholipid elution times are noted. The solvent gradient is shown with straight lines (%B pump is noted on the right).

sensitivity is about 1 nmol of phosphatidylcholine if one double bond is present in the phospholipid (14). Lower limits of detection are possible after phospholipid derivatization and the use of UV absorbance: 0.3–0.4 ng or 10–13 pmol of phospholipids can be detected (22).

As a measure of reproducibility, PI eluted from the silica column used at the University of Colorado-Denver at 23.675 ± 0.1135 min (n = 16). Peak size for various amounts of PI varied as shown in **Table 3.** These data represent standard lines obtained over a period of 1 year, with different phospholipid standard lot numbers, with three different operators who made up solvent mixtures often, and with three Microsorb columns (results with one column, over a small period of time, produced coefficients of variation less than 5%).

Analysis of a cellular extract

The true test of a method is whether separation of a cellular extract produces clearly differentiated peaks. Cellular extracts contain molecular species of phospholipids, lipids are not synthetic short-chain standards, and the amount of each phospholipid varies dramatically. Using an extract from *Xenopus* oocytes, successful separation was noted (**Fig. 5**). Becart, Chavalier, and Biesse (7) suggested that lysophosphatidylcholine (lysoPC) can be used as an indicator of hydrolysis and poor storage. LysoPC elutes at about 45 min; the negligible peak here denotes that our extraction and storage conditions did not result in extensive hydrolysis.

The relative amounts of phospholipids obtained from *Xenopus* eggs (**Table 4**) are similar to those obtained after ^{32}P labeling and TLC separation of phospholipids using *Xenopus* oocytes (23).

Low concentrations (2–20 mg/100 ml) of 2,6-di-*tert*-butyl-*p*-cresol (BHT) can be added as an antioxidant (the BHT evaporates with the solvent), but we found no difference between peak sizes in cellular extracts that had BHT and those that did not.

We conclude that the method described here produces excellent resolution of phospholipids, including acidic phospholipids and sphingomyelin.

TABLE 4. Phospholipids in a *Xenopus* oocyte

Lipid	Average ± SEM (n)
	μg/oocyte
PE	4.41 ± 0.42 (9)
PI	2.43 ± 0.22 (14)
PS[a]	0.40 ± 0.03 (6); with 50°C column: 0.65 ± 0.06 (6)
PC	15.95 ± 1.49 (9)
SM	1.26 ± 0.16 (17)

[a] PS values corrected for 14.5% loss (see Materials and Methods).

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