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IMPROVED REPRODUCIBILITY AND QUANTITATIVE ANALYSIS OF PHOSPHOLIPIDS BY FLAME IONIZATION DETECTION

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SUMMARY

Quantitative analysis of phospholipids by flame ionization was improved by careful application of samples with a Hamilton syringe and use of a sealed dual tank system. Chromarods developed more consistently with reproducible scanning times or R_F values (coefficient of variation of 1%) and with sharper peaks if development was carried out in a sealed dual tank system. The Chromarods were placed in the inner tank, which was the standard ground glass topped tank furnished with the Iatroscan TH-10 system. This inner tank was placed inside a larger thin-layer chromatography tank which was sealed with silicone grease and the lid held in place with a lead brick. Both tanks were lined with absorbent paper and contained the same solvent system. Biological samples quantified with these procedures and measured in amounts between 1 and 30 μg had coefficients of variation between 0.2 and 6%. An efficient method of completely separating neutral lipids from phospholipids and allowing quantitative determination of cholesterol is desribed. Scanning times and R_F values of various phospholipids are compared to determine the best separation of the major phospholipids found in 3T3-L1 and leukocyte membranes.

INTRODUCTION

Progress in quantifying phospholipids obtained from biological systems has been impeded by time consuming methods of sample separation, identification and quantitation. Phospholipids are often separated by thin-layer chromatography (TLC), eluted and quantified by determining phosphorus content. This process requires complete digestion of the sample with strong oxidants such as perchloric acid and is time consuming[1,2]. Measurement of phosphorus content is generally limited by these procedures to values greater than 1 μg which requires the sample size to be approximately 25 μg of total lipid weight[3]. High-performance liquid chromatography (HPLC) with quantification by ultraviolet absorption has also been used to separate and isolate the various phospholipids, but reproducibility is dependent upon constant fatty acid content or colormetric determination of phosphorus[4,5]. Bessman[6] has developed an automated system coupling HPLC and phosphorus detection to speed up

this process, but it does not provide an appreciable increase in sensitivity. The Ia-troscan, which uses the principles of TLC for lipid separation and flame ionization for sample detection provides additional sensitivity and rapid detection for phospholipid determination[7,8]. A large proportion of the literature using this technique has centered on the development of neutral lipid separation and detection. Efficient analysis of this class of lipids is possible by gas–liquid chromatography (GLC) and the separation of neutral lipids using both techniques has been evaluated[9,10]. Phospholipids, on the other hand are not detectable by GLC without extensive molecular alteration. Data obtained with the Iatroscan has been reported to give standard deviations as large as 16% from the mean[10,11]. It is the purpose of this study to demonstrate that phospholipids isolated from biological samples can be accurately measured by flame ionization detection if care is employed in each step of the procedure.

METHODS

Verification of lipid species and standard quantitative curves were made with known lipid samples purchased from Supelco (Bellefonte, PA, U.S.A.). Lipids were extracted from crude membranes obtained from human polymorphonuclear leukocytes, and purified plasma membranes from 3T3-L1 fibroblasts as previously described[12,13]. Extraction was by the Bligh and Dyer procedure[14]. Reagent grade solvents were redistilled before use. Solvents for developing systems were HPLC grade and were also redistilled before use (J. T. Baker, Phillipsburg, NJ, U.S.A.). The extracted lipid samples were conentrated by evaporation in a water bath at 37°C under a stream of nitrogen. The samples were washed to the bottom of glass conical tubes with chloroform–methanol (2:1). Lipids were then transferred quantitatively to 100-μl conical glass vials (Supelco). The sample was completely dried, was reconstituted with a known volume of solvent, and sealed with PTFE lined cap just prior to application of the sample to Chromarods (Iatron Labs., Tokyo, Japan). The amount of solvent used to reconstitute the lipid sample was determined by the amount of protein measured in the biological sample. Protein content was used as a standard to adjust lipid sample size so that 1–4 μg of sphingomyelin would be present in each sample applied to the Chromarods. Prior to sample application the Chromarods were activated by passing them through the Iatroscan TH-10 flame (Newman-Howells Assoc., London, U.K.). Samples (0.5–4 μl) were spotted on rods with an appropriate type of applicator [Hamilton syringe (Hamilton, Reno, NV, U.S.A.), Oxford micropipettor (Lancer Division of Sherwood Medical, St. Louis, MO, U.S.A.), or Drummond automatic pipettor (Drummond Scientific, Broomall, PA, U.S.A.)]. The first and last rods on the rack were used to spot known lipid standards. Application was done carefully allowing the dispensed solvents to evaporate as applied to concentrate the sample in a small area on the rod. With the Mamilton syringe about 8 to 10 drops per microliter were applied to each rod. The rack of Chromarods were then placed in a humidifing chamber (85%) for 10 min as suggested by the manufacturer (Newman-Howells Assoc.), after which the rods in the rack were developed in an appropriate solvent system.

The development system was contained in two separate tanks, one placed inside the other. The smaller tank which was capped by a ground glass lid (Newman-Howells Assoc.), was placed inside a larger TLC tank whose lid was sealed with

Lead Brick Weight

Sealed Lid (silicone grease)

Ground Glass Lid

Tanks Lined With
Absorbent Paper

Rack With Chromarods

Inner Chromatography
Tank
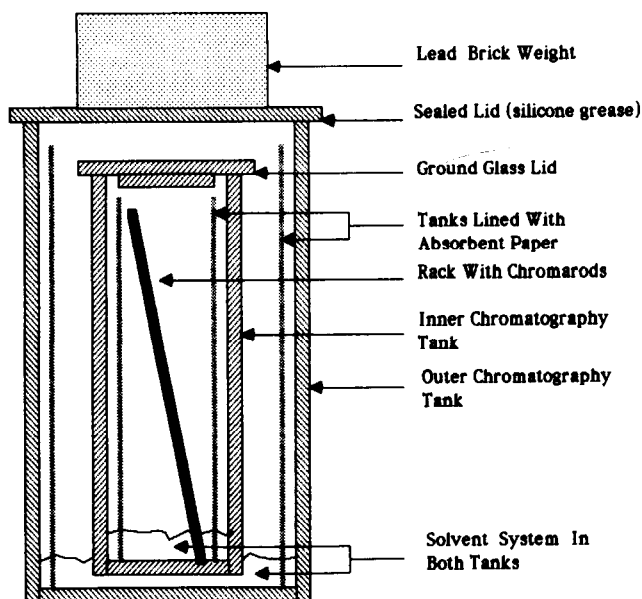
Outer Chromatography
Tank

Solvent System In
Both Tanks

Fig. 1. Dual tank system. This schematic drawing demonstrates the design of the dual tank system used
to develop the Chromarods for this study. The outer larger tank is sealed with silicone grease and weighed
with a lead brick. The inner tank houses the Chromarods during development and is covered with a
ground glass lid. Both tanks contain the solvent system and are lined with absorbent paper.

silicone grease (silicone high vacuum grease purchased from Dow Corning, Midland,
MI, U.S.A.) and lead bricks. Both tanks were lined with absorbent paper and con-
tained the same solvent systems (Fig. 1). The solvent system was allowed to equili-
brate overnight in this tightly sealed system and each two or three days a fresh solvent
system was added.

A number of solvent systems were employed. The solvents used and their con-
centrations are found in the result section. As the rate of solvent migration on the
rods could not be visualized in the dual tank system, time of development was care-
fully controlled. In most cases development was carried out for 1 h but occasionally
two separate 1-h separations were used. Chromarods were allowed to dry 30 min at
room temperature before being placed in the Iatroscan or before being reintroduced
into a second solvent system. The entire rack of Chromarods that had been developed
and air dried, was placed in an Iatroscan TH-10 which volatilizes the sample and
quantitatively determines the ionizated products. The instrument was set to scan the
Chromarods at a speed of 0.39 cm/sec with the hydrogen flow control set at 0.75
kg/cm^2 and air flow at 1800 ml/min. The signal from the detector was passed to a
Model 3390A Hewlett-Packard integrator. The integrator was started manually pre-
cisely as the Iatroscan started to scan each Chromarod. Starting the integrator in this
manner on each rod achieves reproducible retention times on each run.

Chromarods were matched and stadardized for each lipid that was mea-
sured.The Chromarods were cleaned in an acid bath solution monthly or as residues
which interfered with lipid separation accumulated.

Proteins were determined according to the procedure of Lowrey et al.[15].

RESULTS AND DISCUSSION

The most frequent source of error was found to be the sample application to the Chromarods. Fig. 2 contains the developed and scanned results of sample application with a 5-μl Hamilton syringe and with an adjustable Drummond micropipettor, with sample amounts between 0.5 to 4 μl. There were 40 applications for each point to compare the coefficient of variation between the two applicators with varying volumes of sample applied to the Chromarods. Sample applications smaller than 1 μl had larger errors with both types of applicators. Comparisons between detector response and the amount of sample applied to rods with either a Drummond or Hamilton pipettor demonstrated that the Drummond pipettor had a larger standard deviation from the mean (S.D.M.) than did the Hamilton syringe. The more consistent results obtained with the Hamilton syringe are attributed to the ease of control possible while expelling the sample because it is not necessary to apply constant pressure on the dispensing handle as is necessary in the spring loaded applicators. The small bore needle on the Hamilton syringe allowed a smaller volume of sample to be applied than with the larger bore sizes found on the Drummond or Oxford micropipettors. This small bore aided in maintaining a smaller spot on the Chromarod during sample application. The sharp point on the needle can scrape the rod surface, therefore, only the side of the needle where the orifice is found was allowed to make contact with the rod.

In some cases, duplicate samples were quantitatively applied to four separate rods and evaluated with the Iatroscan. The results of the four determinations were averaged as a single point. When this technique was employed the coefficient of variation was lowered markedly with the Drummond micropipettor so that the data matched that obtained with the Hamilton syringe. Data collected with this quadrupole sample method did not improve the coefficient of variation with the Hamilton syringe. Oxford micropipettors were also used and results were similar to those obtained with the Drummond micropipettor.

Each applicator type was checked for its ability to deliver reproducible aliquots (0.5–4 μl) of [^{14}C]lipids directly into scintilation vials, compared to applying unla-
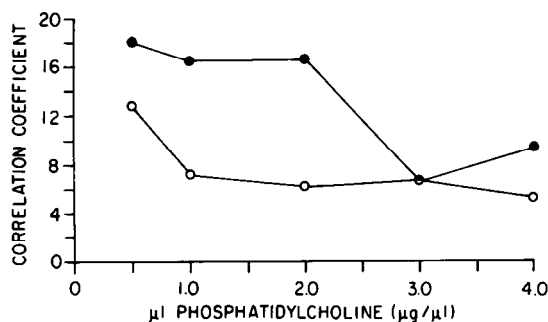

Fig. 2. Sample applicators tested. Phosphatidylcholine was applied to Chromarods in 0.5- to 4-μl volumes with either a Hamilton microsyringe (10 μl) (O) or a Drummond digital microdispenser (●). Following sample application the rods were developed in a system containing chloroform–methanol–water (30:20:2) for 1 h. Phosphatidylcholine was measured using an Iatroscan TH-10 and the correlation coefficient was determined ($n = 40$).

beled lipids to Chromarods and quantitating them by flame ionization with the Ia-troscan. Both applicator types appeared to deliver samples more reproducibily to vials than to Chromarods. Precautions were taken to prevent drops of solvent drying at the applicator tip. Large beads were not allowed to form on the tip of the needle and the plunger was not forced in until the needle was in close proximity to the Chromarod so that the solvent would flow directly to the rod from the syringe.

For the remainder of the study, quantitative application was carried out routinely with either a 5- or 10-μl Hamilton syringe with the sample volume applied to the Chromarod ranging between 2 and 4 μl. The same sample was applied to two separate rods. This procedure increased the accuracy of quantitating lipids with the Iatroscan. This Iatroscan method was faster, safer and more sensitive than the thin-layer technique employing phosphorus determination.

Type S and type SII Chromarods used in this study demonstrated little differ-ence in results between the two types. There appeared to be as much varibility be-tween lot numbers of the same type of Chromarod as between the different types of rods. The varibility between paired rods[8] was lessened by developing samples in duplicate and averaging their results.

Chromarods developed in a dual tank system (Fig. 1) had better sample sep-aration and sharper peaks. Use of this dual tank system, coupled with careful sample application, has removed the need to focus samples after application to Chromarods as reported by other workers[16]. Maintaining a small spot when applying samples to the rods and maintaining consistent vapor pressure with the dual tank system elim-inated the problem of double peaks caused by sample application[17] both in phos-pholipids and neutral lipids. Care was taken with the dual tank system not to allow silicone grease to contaminate the inner tank. Silicone grease contamination migrated on the rods and interfered with peak separation.

Table I contains the results obtained using a number of solvent systems to separate phospholipids and cholesterol. The results in the table are measurements of scanning time (ST) recorded by the integrator and R_F values for each lipid sample. Cholesterol was used to indicate the solvent front for calculating R_F values and was assigned an R_F value of 100. Some workers have reported that R_F values vary by as much as 70% from run to run[9]. It has been our observation that ST and/or R_F values vary about 1% with the dual tank system. Examination of data obtained from 20 Chromarods over a number of days demonstrates the stability of the ST (or R_F values) of a number of lipids: phosphatidylethanolamine, 12.6 $\pm$ 0.6; phosphatidyl-choline, 37.1 $\pm$ 0.4; sphingomyelin, 40.4 $\pm$ 0.5; cholesterol, 21.5 $\pm$ 0.7; $n = 100$ $\pm$ S.D.M. Phospholipids from biological unknowns and from known standards were used to determine the above data. All phospholipids were separated with a system of chloroform–methanol–water (30:20:2) for 1 h. Cholesterol was separated from the phospholipids in a system of isopropoyl ether–acetic acid (96:4) for 30 min (see Table I). These data clearly demonstrate the consistency with which lipids migrate up the Chromarods in the dual tank system. Separation of mixed phospolipid standards is highly reproducible, but examination of phospholipids isolated from biological sam-ples shows a more complex situation. Two sources of dispersion observed with bio-logical samples were large variations in quantities of the lipid present and variable fatty acids associated with the phospholipids. The sample size applied to the Chro-marod must be sufficiently large to measure, but very large quantities separate poorly

TABLE I

INTEGRATOR SCANNING TIME (ST) AND R_F VALUES OBTAINED WITH IATROSCAN DETECTION OF LIPIDS USING DIFFERENT SOLVENT SYSTEMS

Lipid separation is indicated by scanning time (ST) and by R_F values. Scanning times were recorded with a Hewlett-Packard integrator which was started at the exact moment the instrument began scanning each Chromarod. Scanning time is defined as the elapsed time for the Iatroscan to scan from the top of a Chromarod to that point to which a specific lipid has migrated. Scanning time is analogous to retention time in GC and is dependent on the scanning speed of the Iatroscan. Scanning speed was set at 0.39 cm/sec for all the values in this table. R_F values were calculated from cholesterol migration. The distance that cholesterol migrated was assumed to be equal to the solvent front.

Lipid	ST/R_F						Isopropyl ether–acetic acid*
	Chloroform–methanol–water						96:4
	30:20:2 (1 h)§	30:20:2 (2 × 1 h)	65:25:4** (1 h)	39:15:2.6 (1 h)	80:25:3**** (1 h)	80:25:3**** (90 min)	(30 min)
Cholesterol	6/100	3/100	9/100	6/100	15/100	13/100	21/100
Phosphatidic acid	8/95	4/98	14/87	13/83	19/88	24/68	46/4
Phosphatidylglycerol	10/90	5/95	17/79	16/76	20/84	22/73	46/4
Phosphatidylethanolamine	12/85	8/89	17/79	11/88	24/72	20/79	46/4
Phosphatidylserine	19/68	12/80	20/71	22/61	29/56	29/53	46/4
Phosphatidylinositol	19/68	11/82	26/55	25/54	33/44	30/50	46/4
Lysophosphatidylethanolamine	31/61	24/52	18/76	28/46	40/22	30/50	46/4
Phosphatidylcholine	37/24	32/34	34/34	29/44	39/25	36/32	46/4
Sphingomyelin	40/17	37/23	40/18	37/24	43/13	41/18	46/4
Lysophosphatidylcholine	42/12	41/14	36/29	40/17	44/9	42/15	46/4

* See ref. 3.
** See ref. 18.
*** See ref. 19.
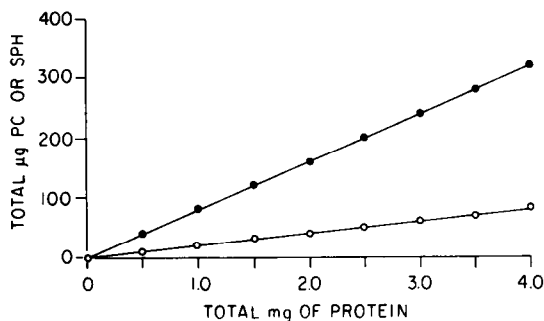§ Development time in solvent system.

Fig. 3. Lipid-to-protein ratio. To assure that the proper quantity of isolated phospholipids from a bio-
logical source is placed on a Chromarod the optimum amounts of sphingomyelin (O) and phosphatidyl-
choline (●) is calculated according to membrane protein. The optimal amount of sphingomyelin applied
to a rod has been determined to be between 1 and 4 μg and phosphatidylcholine between 3 and 15 μg.
Using these amounts separation and quantitation is best. The total biological lipid sample was reconsti-
tuted to a volume which assured that the optimal amounts of lipids were applied to each Chromarod.
Polymorphonuclear leukocyte membranes were used as the biological sourse to collect data for this figure.

from the other lipids. The low-high range of lipids that allows for best sample sep-
aration, detection and quantification is between 1–12 μg. Standardization of the
amount of lipid applied to a Chromarod was made in our studies by determining
protein content of the biological sample at the time of lipid extraction. Correlation
between protein and the amount of sphingomyelin and phosphatidylcholine present
in leukocyte membranes allows adjustment of the proper sample size applied to rods
is shown in Fig. 3. The lipid-to-protein ratio changes in different biological samples
and it could be necessary to construct a new phospholipid to protein curve for each
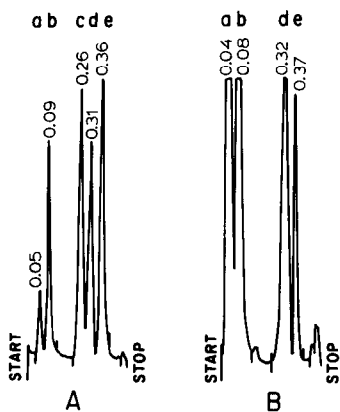

Fig. 4. Separation of different types of phosphatidylcholine from other phospholipids on Chromarods.
Scanning times are marked at the apex of each lipid peak and are characteristic of each lipid. In panel A
standard phospholipids were applied on a Chromarod and then developed in chloroform–methanol–water
(30:20:2) for two separate 1-h periods in a dual tank system. Compounds separated include: a, less polar
compounds; b, phosphatidylethanolamine; c, phosphatidylcholine diarachidonoyl; d, phosphatidylcholine
dimyristoyl; e, sphingomyelin. Panel B is a chromatogram of membrane lipids isolated from human po-
lymorphonuclear leukocytes (PMNs) with phospholipids containing unknown fatty acid composition.
Isolated lipids included: a, neutral lipids; b, phosphatidylethanolamine; d, phosphatidylcholine; e, sphin-
gomyelin.

biological source used in order to assure that the proper amount of lipids are placed on the Chromarods.

The fatty acid species of phospholipids influence the elution distance of that phospholipid on a Chromarod (Fig. 4). Phospholipids from biological samples having a large variety of fatty acid combinations may cause shoulders on some phospholipid peaks in a chromatogram. These shoulders should be examined in multiple solvent systems to insure that other phospholipid species are not present. An inherent problem with the Chromarod is that its length limits the number of phospholipid species it can adequately separate. Knowledge of the lipids in a sample allows one to choose the best solvent system or systems to adequately separate those found in a biological source.

Neutral lipid analysis was carried out by first developing the biological samples in isopropyl ether–acetic acid (96:4)[3] for 30 min to move the less polar lipids from the origin (Fig. 5). The developed samples on the Chromarods were placed in the Iatroscan and were automatically scanned. The Iatroscan was adjusted to scan only the top 8 cm of each rod leaving the bottom 2 cm of each rod unscanned[8]. Following volatilization of the neutral lipids in the Iatroscan, the intact phospholipids (found on the bottom 2 cm of the Chromarods when they are developed in the above system) were separated by redevelopment of the rods in a different system using chloroform–methanol–water (30:20:2). The Chromarods were rescanned to measure those phospholipids present in the samples. Cholesterol was easily separated both from other neutral lipids and phospholipids (Table I). Phospholipid measurement was then made possible without interference by the neutral lipids present in the bio-
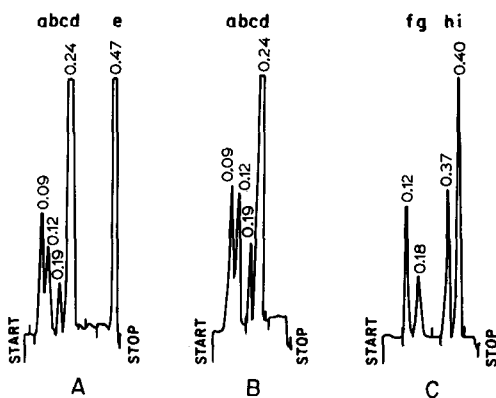

Fig. 5. Separation of neutral lipids from phospholipids. This series of chromatograms illustrates the separation of neutral lipids from phospholipids. Scanning times are marked at the apex of each lipid peak. Panel A is a chromatogram of both neutral lipids and phospholipids that have been completely burned from the rods. The following lipids were separated in a solvent system of isopropyl ether–acetic acid (96:4): a, triglycerides; b, fatty acids; c, diglycerides; d, cholesterol; e, combination of phospholipids that have remained at the origin. Panel B contains neutral lipids that were separated in the same solvent system used in panel A. The Iatroscan was allowed to only volatilize neutral lipids found at top of the Chromarod. The lower section of the Chromarod was not scanned leaving the undeveloped phospholipids intact to allow them to be separated in a different solvent system. Panel C is a chromatogram of phospholipids separated in a solvent system of chloroform–methanol–water (30:20:2) after neutral lipids had been earlier removed. Isolated phospholipids include: phosphatidylethanolamine (f); combined peak containing phosphatidylserine and phosphatidylinositol (g); phosphatidylcholine (h); sphingomyelin (i).

TABLE II

PERCENT OF PHOSPHATIDYLCHOLINE DETERMINED AT VARIOUS SCANNING SPEEDS

Scanning speed (cm/sec)	µg Lipid added to Chromarods		
	2	5	10
0.3	100	100	100
0.39	100	99.3	99.1
0.46	100	96.3	95.6

logical samples. If more extensive analysis of neutral lipids is required, a diethyl ether (9:1) solvent system can be used[3], but, cholesterol does not separate from the phospholipids in this system.

The ability of the Iatroscan to totally remove samples from the Chromarod in a single pass through the flame was studied. Tables II and III contain the results of these experiments in determining both the scanning speed and the amount of lipid that could be applied safely to a Chromarod and assure adequate removal by the flame. The scanning speed normally used for these experiments was 0.39 cm/sec. Approximately 99% of the lipid samples were volatilized at this speed. When samples from 2 to 30 µg of lipid were employed more that 99% of the sample was volatilized. If larger amounts of phosphatidylethanolamine (40 µg) were applied, less than 98.6% of the sample was volatilized.

As reported by other workers[8,20], it is necessary to construct quantitative curves for each phospholipid measured. The results determined for phosphatidylcholine, phosphatidylethanolamine and sphingomyelin are found in Fig. 6. These lipids were quantitatively measured in amounts ranging from 0.5 to 30 µg. Using biological samples, determination of 1 to 25 µg was satisfactorily reproduced (correlation coefficients less then 6%). The ideal range was between 2 and 15 µg.

Successful use of the Iatroscan instrument depends on careful attention to details such as sample handling, adjusting sample size applied to the Chromarod, development in an adequate system that is maintained in a sealed tank and proper adjustment of the Iatroscan.

TABLE III

PERCENT OF THREE PHOSPHOLIPIDS DETERMINED AT SCANNING SPEED 0.39 cm/sec

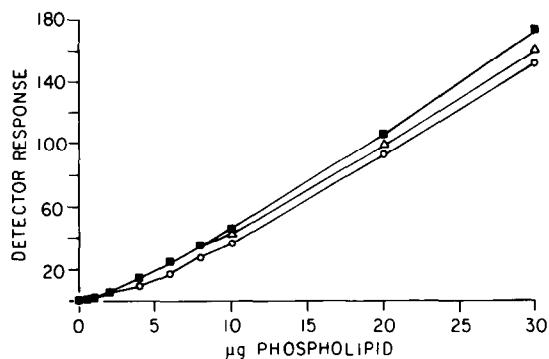| Lipid	Amount of lipid (µg)	Volatilized (%)
Phosphatidylethanolamine	30	99.1
	40	98.6
Phosphatidylcholine	30	99.6
	40	99.3
Sphingomyelin	30	99.6
	40	99.4

Fig. 6. Standard quantitative lipid curves. Standard curves were determined with sphingomyelin (■), phosphatidylcholine (△) and phosphatidylethanolamine (○) in amounts of 0.5 to 30 μg. Quantitative analyses of samples gave best results when 2- to 15-μg amounts were present.

ACKNOWLEDGEMENTS

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