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Determination of phospholipids on two-dimensional thin-layer chromatographic plates by imaging densitometry

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ABSTRACT

Data acquisition and image analysis by a flying-spot-based densitometer linked with a personal computer for the determination of compounds separated by thin-layer chromatography (TLC) were studied. This method was applied to the reflectance imaging densitometry of phospholipids separated two-dimensionally on a TLC plate. Phospholipids were determined with high accuracy and reproducibility, showing that the proposed method is very useful for the determination of compounds separated by two-dimensional TLC.

INTRODUCTION

Densitometry has been widely used for the quantitative assay of zones of light-absorbing compounds or fluorophores that have been separated on a supporting material, such as an electrophoretic gel or a thin-layer chromatographic (TLC) plate. Two techniques are available for densitometric scanning of separated zones. One is linear scanning with a slit-shaped light beam, which is widely used clinically for determining serum proteins. In this instance, the density distribution of the substance examined is assumed to be virtually uniform in the direction perpendicular to the scanning axis, and so the slit illumination should give the correct absorbance signal. However, on two-dimensional electrophoresis or TLC, the density distribution of a substance in a zone is usually not uniform, and the density is distributed randomly in the two-dimensional plane. In such instances, the alternative technique of two-dimensional scanning, rather than linear scanning, should be used.

Several types of densitometers that scan sample bands mechanically in two dimensions with a small light spot have been shown to be effective in eliminating errors due to irregular distribution of the sample [1–3]. Recently, new techniques of imaging densitometry using image detectors and computer image analysis techniques have been reported to be useful for the quantification of gel electropherograms or thin-layer

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chromatograms [4–10]. These techniques facilitate fast scanning and easy data handling, but have the common disadvantages of a narrow dynamic range of the video camera, difficulty with uniform illumination and lack of optical contrast on the image plane due to mutual optical interference of pixels. Taube and Neuhoff [11] reported methods for two-dimensional data acquisition using a scanning photometer and image processing of bands separated by chromatography or electrophoresis.

We have reported the technique of dual-wavelength, zig-zag scanning [12], in which a small light spot scans the sample bands two-dimensionally in a zig-zag manner so that a wide dynamic range of signal detection and high optical contrast are achieved. However, with this type of mechanical scanning system, a long time is required for scanning the whole TLC plate.

The densitometric determination of phospholipids on TLC plates, mainly those separated one-dimensionally, has been reported [13–17].

To improve the quantification, imaging densitometry using a black-and-white video camera has recently been applied in two-dimensional TLC [8]. However, this method seems to have the limitation of a narrow dynamic range. This paper describes an improvement of our mechanical scanning system for imaging densitometry and the application of this system to the determination of phospholipids separated two-dimensionally on TLC plates.

EXPERIMENTAL

Reagents

Soybean phospholipid was purchased from Wako (Osaka, Japan) and dissolved in chloroform to give a 0.05 g/ml solution. The phosholipids, L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE) and L-α-phosphatidylinositol (PI), all from soybeans, and synthetic L-α-phosphatidic acid (PA) were purchased from Sigma (St. Louis, MO, U.S.A.) and dissolved together in chloroform. Other reagents were of analytical-reagent grade.

Thin-layer chromatography

TLC was performed on silica gel high-performance TLC plates (10 × 10 cm) from E. Merck (Darmstadt, Germany). Mixtures of chloroform–methanol–7 *M* ammonia (65:30:4) and chloroform–methanol–acetic acid–water (170:25:25:6) were used for development in the first and second dimension, respectively. After development, the plates were allowed to dry, sprayed with copper(II) sulphate solution in 8% phosphoric acid and then charred by heating at 160°C for 20 min. Densitometry was carried out at 400 nm only, because the absorbance of the charred zones on the TLC plate was non-specific.

Flying-spot densitometry

A dual-wavelength flying-spot scanner (Shimadzu, Model CS-9000) linked with a personal computer was used for data acquisition and image processing. A minute monochromatic light beam (0.4 × 0.4 mm) was generated by an assembly of a fixed slit and spiral slit on a rotating disk located in the exit portion of a monochromator, as shown in Fig. 1. By reciprocating movement of the rotating disk, the light beam moved up and down, and was projected onto a TLC plate by collimating mirror optics to

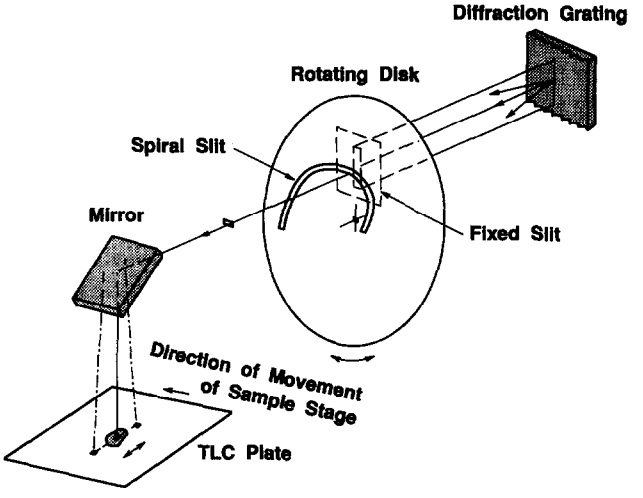

Fig. 1. Principle of flying-spot scanning.

illuminate an area of 0.4 × 0.4 mm on the plate surface and reciprocated for 10 mm in a stepwise manner at 0.2 mm pitch (Fig. 1). The sample stage was moved in the longitudinal direction with a 0.2 mm pitch at the end of each reciprocating movement of the light spot (= flying spot) so as to generate raster. For scanning the whole TLC plate, this raster scan of 10-mm width was repeated in the longitudinal direction by shifting the sample stage laterally in steps of 10 mm.

The diffusely transmitted portion of light under the plate was picked up by the photomultiplier PM_t and the other portion of light reflected from the plate was picked up by the photomultiplier PM_r, as shown in Fig. 2. The signals were converted to those

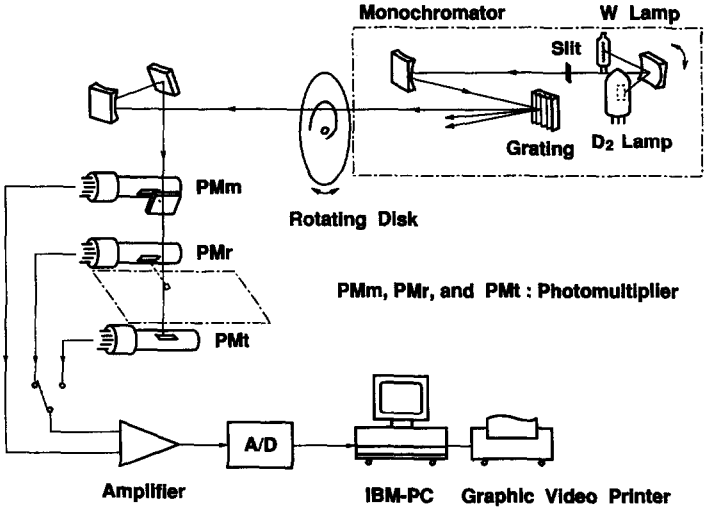

Fig. 2. Optical and signal conversion systems of the flying-spot densitometer.

of transmitted intensity (I_t) and reflected intensity (I_r). The photomultiplier PM_m detected a fraction of the irradiation light, and its signal I_m was used to compensate for fluctuations of the light source in terms of the ratios I_t/I_m and I_r/I_m.

The logarithmic signals, log (I_m/I_t) and log (I_m/I_r), were converted into binary values. These had the dynamic range of -0.8 and 4.0, which was digitized with 12-bit resolution.

Determination of phospholipids on TLC plates

For application of the above methods, we determined phospholipids on TLC plates by diffuse reflectance measurement. Zones on a TLC plate usually have specific absorption peaks in either the visible or ultraviolet region, or both. Therefore, the use of a monochromator that allows the selection of any desired wavelength is useful in the densitometric determination of zones on a TLC plate. Our scanning device offers continuous wavelength selection of monochromatic lights of 10-nm bandwidth over the range 200–700 nm. Therefore, it allows the selection of a optimum wavelength according to the optical characteristics of the sample compound.

The minute light spot moves laterally on the sample surface, as shown in Fig. 1, and so the angle and position of the incident beam on the photocathode of the photomultiplier also vary. Consequently, the local sensitivity differences of the photocathode of photomultipliers, PM_m, PM_r and PM_t (Fig. 2) must be compensated for each signal of either log (I_m/I_r)or log (I_m/I_t) for the sample compound. The data for correction were obtained by scanning a blank area of the TLC plate in the lateral direction for a 10-mm distance in 0.2-mm steps. These data were stored in the memory as a locality correction table and subtracted from subsequent real-time signals at the corresponding lateral positions. There was a *ca.* 20–30% change in local sensitivities on the photocathode, but this was compensated for by subtraction of the correction data.

Signals obtained by the procedure described above were led to an IBM PC-AT external computer, as shown in Fig. 2, and stored in a RAM disk as image data. The RAM disk had an 8 Mbyte capacity, which was sufficient to memorize sixteen full images from a plate of 100 × 100 mm area. The computer had an installed coprocessor to increase the calculation speed and a "mouse" for easy operation. In zones of interest diluted with a high background noise, it was sometimes necessary to smooh raw data. A Laplacian 3 × 3 two-dimensional smoothing function was used to help in eliminating noise and enhancing zone detection. This smoothing was repeated when necessary. The effect of smoothing on a zone of interest is exemplified in Fig. 3. Image data stored in the memory were subsequently processed to detect each zone and sum the absorbance values in the zone area.

Each zone could be detected either automatically or manually. In the automatic mode, the absorbance difference from one pixel to the next was calculated and each zone was detected through a computer algorithm according to either the discrimination level selected earlier or the sensitivity of curvature. In the manual mode, each zone was outlined by use of a "mouse"-controlled pointer on the CRT screen. Use of mapping images obtained at different discrimination levels and three-dimensional projection images or sectional images made manual assays easier. The pattern around the zone and its sectional images corresponding to the x-axis and y-axis cursors are shown in Fig 4. For accurate integration of the absorbance value in each zone, the

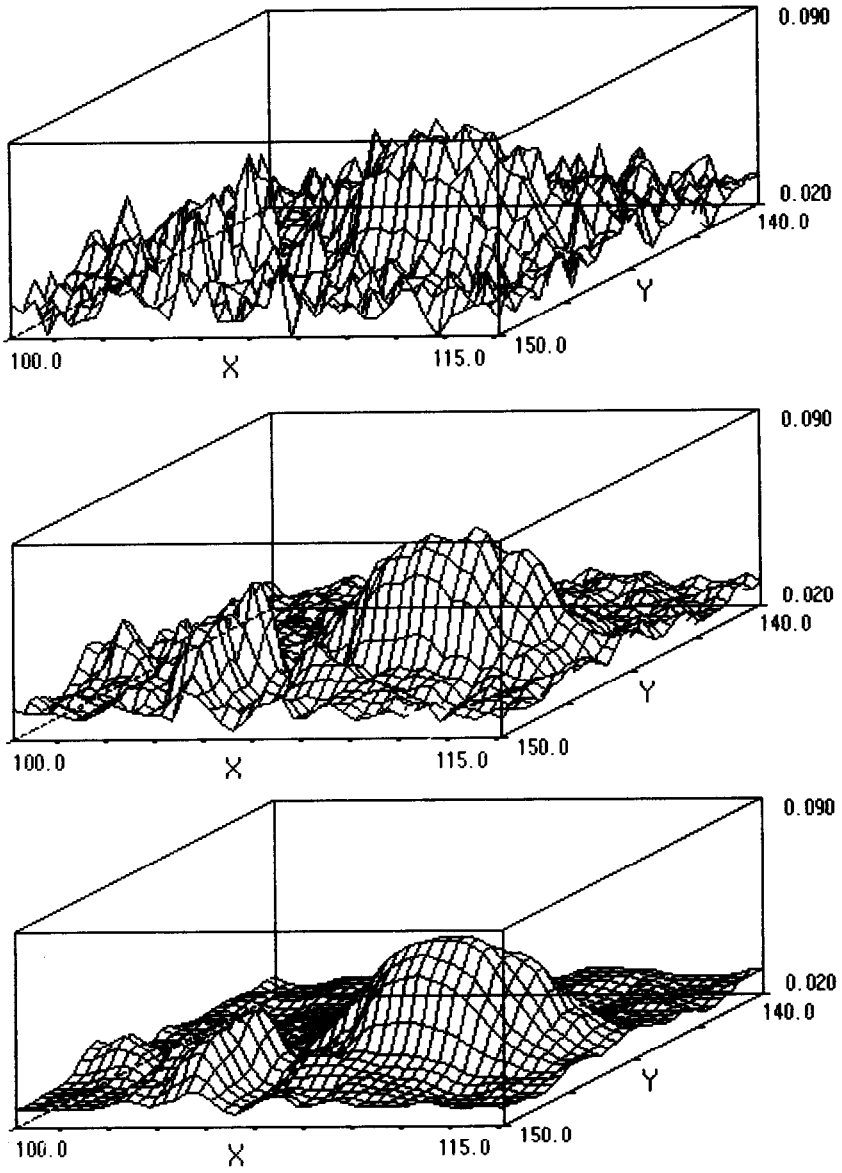

Fig. 3. Effect of Laplacian 3 × 3 smoothing. (a) Original data; (b) results smoothed once; (c) results smoothed five times.

background absorbance was subtracted. Three levels of background subtraction could be selected: the background absorbance could be taken as zero or the mean level on the border, or could be chosen by the operator. The background value, calculated as the product of the background absorbance and the area, was subtracted from the integrated absorbance value inside the border of each zone. Selection of the level of

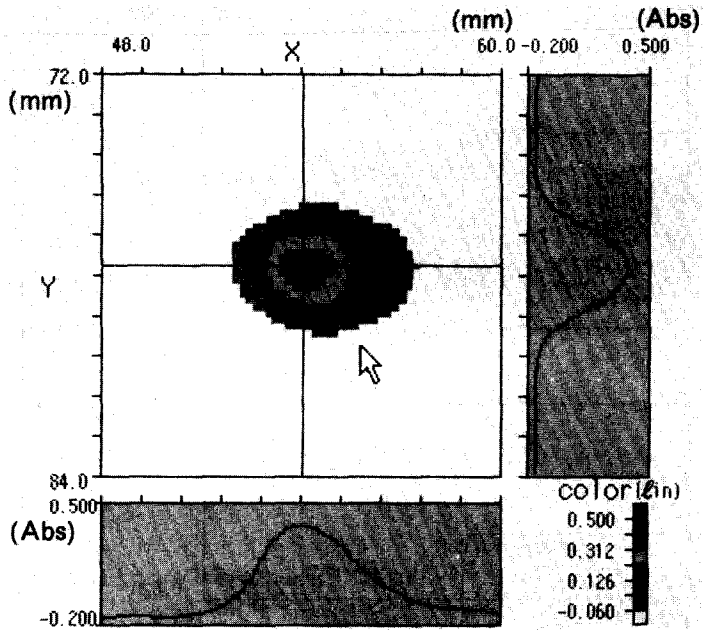

Fig. 4. Two-dimensional pattern around the zone and its sectional images corresponding to *x*- and *y*-axis cursors.

background subtraction depended on whether there were adjacent zones and on whether the background absorbance varied markedly.

RESULTS AND DISCUSSION

It is very important to minimize the data acquisition time for obtaining image data. The present method of flying-spot densitometry required about 15 min to scan a total area of 100 × 100 mm with a resolution of 0.2 mm in both the longitudinal and lateral directions, including the real-time locality correction process. Once the data had been stored in the memory, the time required for image processing of the 100 × 100 mm area was *ca.* 2 min, and this time could be greatly shortened by use of a faster computer.

Soybean phospholipids were separated by two-dimensional TLC, as shown in Fig. 5, then the whole area of the plate was scanned and image data were stored in the memory of the computer. Each zone was displayed as a contour map by preselecting a threshold level for the absorbance value. Detection of each zone and integration of absorbance in a zone were usually performed automatically by the computer algorithm alone. However, for the analysis of adjacent zones, manual determination was adequately adapted. Therefore, another borderline was drawn manually for a chosen discrimination level, which was determined by reference to sectional views corresponding to *x*- and *y*-axis cursors in which the start and end points of the peaks were found one-dimensionally (Fig. 4). The three-dimensional distribution of density was

observed visually so the borderline could be drawn to surround the entire zone. The contour line level for the mapping image was varied by 0.001 absorbance unit as the smallest unit when this borderline was being checked.

Two-dimensinal TLC of soybean phospholipid was performed in five runs, and densitometric determinations of four major components, PE, PC, PI and PA, and four minor components stearoylglucoside (SG), phosphatidylglycerol (PG), cerebroside (Ce) and X (not identified), as shown in Fig. 5, were carried out on each plate. TLC of standard phospholipids PC and PE was also performed. To eliminate errors caused by differences in the charring conditions for different plates, integrated values for the eight components were summed and the total value for each plate was taken as 100%, then the percentage densitometric values for each of the eight components and average values for the five TLC plates were calculated. These values are summarized in Table I. The relative standard deviations (R.S.D.) for the four major phospholipids PE, PC, PI and PA were in the range *ca.* 2–5%. This range is similar to that observed in one-dimensional TLC densitometry [14], indicating that this method is suitable for two-dimensional TLC densitometry.

Next we carried out two-dimensional TLC of mixtures of various amounts of PA, PE, PI and PC, from 0.25 to 3.0 μg, on six TLC plates. In this experiment, 5.0 μg of PC were applied separately to each plate as a standard. Fig. 6 shows the results of

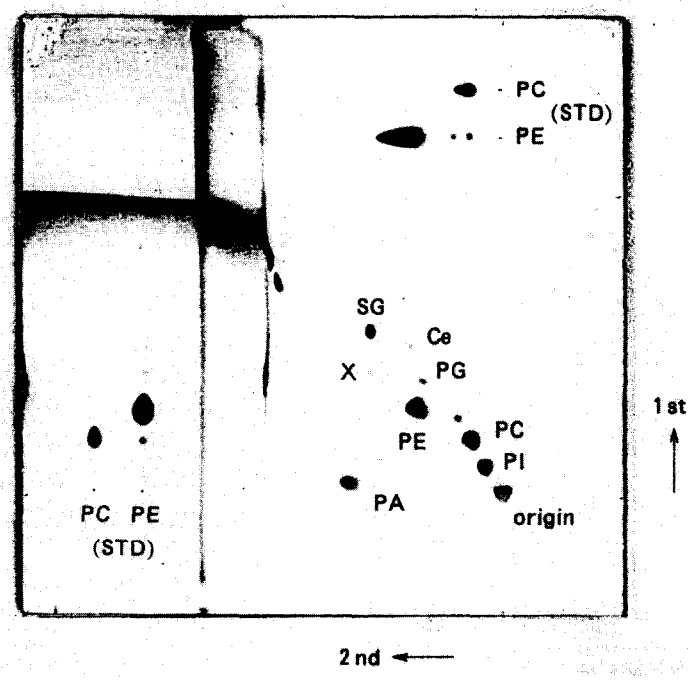

Fig. 5. Two-dimensional TLC of soybean phospholipids. PE = Phosphatidylethanolamine; PC = phosphatidylcholine; PI = phosphatidylinositol; PA = phosphatidic acid; SG = stearoylglucoside; PG = phosphatidylglycerol; Ce = cerebroside; x = unidentified substance.

TABLE I

INTEGRATED ABSORBANCES OF PHOSPHOLIPIDS FROM TWO-DIMENSIONAL HIGH-PERFORMANCE TLC PLATES DETERMINED BY FLYING-SPOT SCANNING[a]

Plate No.	Relative absorbance (%)[b]								
	PE	PC	PI	PA	SG	PG	Ce	X	Total
1	30.36	26.09	15.15	16.11	7.13	1.94	1.74	1.48	100
2	28.48	25.07	16.44	16.56	7.02	2.61	2.04	1.78	100
3	28.80	25.59	15.24	15.91	6.86	3.09	2.70	1.81	100
4	27.44	25.07	15.06	16.69	7.85	2.91	2.67	2.31	100
5	28.99	25.43	14.49	17.69	8.01	2.24	1.68	1.47	100
Average	28.80	25.45	15.28	16.59	7.37	2.56	2.17	1.77	100
S.D.	1.05	0.43	0.71	0.69	0.52	0.48	0.49	0.34	
R.S.D. %	3.65	1.67	4.67	4.17	7.06	18.56	22.85	19.31	

[a] Soybean phospholipid was separated by two-dimensional TLC. The plates were then charred and integrated absorbances of phospholipids were determined by flying-spot densitometry. Their values are shown as percentages of the total absorbance.

[b] PE = Phosphatidylethanolamine; PC = phosphatidylcholine; PI = phosphatidylinositol; PA = phosphatidic acid; SG = stearoylglucoside; PG = phosphatidylglycerol; Ce = cerebroside; X = unidentified substance.

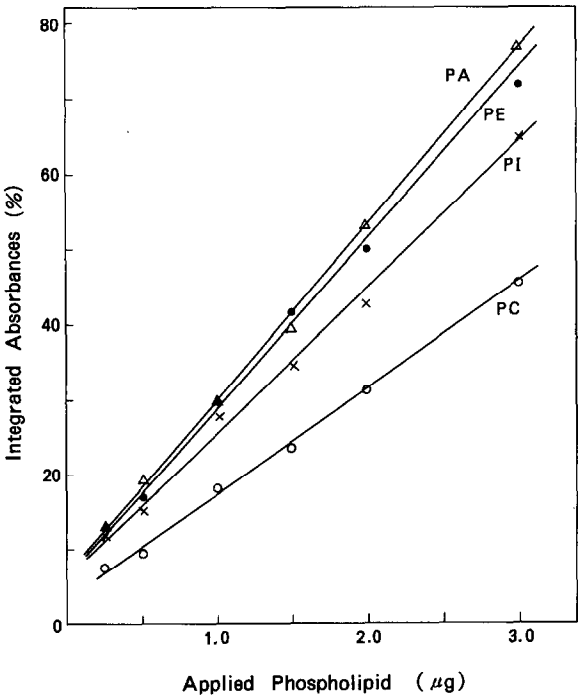

Fig. 6. Linear relationships between integrated absorbances of phospholipids and their amounts applied to two-dimensional TLC plates. Integrated absorbances are expressed as percentages of that of the standard sample of PC. Abbreviations as in Fig. 5.

TABLE II

LINEAR RELATIONSHIP BETWEEN INTEGRATED ABSORBANCES AND AMOUNTS OF PHOSPHOLIPIDS APPLIED ON TWO-DIMENSIONAL TLC[a]

Phospholipid	Linear relationship[b]	γ[c]
PA	$y = 23.18x + 7.07$	0.999
PE	$y = 21.69x + 7.43$	0.999
PI	$y = 14.03x + 3.13$	0.998
PC	$y = 18.93x + 7.06$	0.997

[a] Two-dimensional TLC of various concentrations of samples of PA (phosphatidic acid), PE (phosphatidylethanolamine), PI (phosphatidylinositol) and PC (phosphatidylcholine) was carried out on six plates. The plates were then charred and flying-spot densitometry was performed. A typical chromatogram is shown in Fig. 5.

[b] Results of regression analyses of the relationships between the amounts (in μg) of phospholipid applied (x) and the integrated absorbances relative to that of standard PC (y).

[c] Correlation coefficient.

densitometry represented by integrated absorbances relative to that of the standard PC (%) as functions of the amounts of phosholipids applied. As can be seen, with all four phospholipids the integrated absorbance was linearly related to the amount of phospholipid applied, although the plots did not pass through the origin. The calibration graphs obtained by densitometry of charred zones using one-dimensional TLC have also been reported not to pass through the origin [18–20]. The results of regression analyses of these results are summarized in Table II: very significant linear relationships were obtained between the integrated absorbance and the amount of phospholipid applied for all the phospholipids, indicating that the present method is suitable for the determination of phospholipids.

To confirm this, we next carried out densitometry of the two-dimensional TLC plates to which known amounts of several phosholipids had been applied with 5.0-μg samples of PC and PE as references. The results were quantified according to the relationships in Table II and are summarized in Table III. The differences between the

TABLE III

DETERMINATION OF PHOSPHOLIPIDS BY TWO-DIMENSIONAL TLC AND FLYING-SPOT DENSITOMETRY ACCORDING TO THE RESULTS OF REGRESSION ANALYSES SHOWN IN TABLE II

Phospholipid[a]	Amount of phospholipid (μg)			
	Sample 1		Sample 2	
	Applied	Determined	Applied	Determined
PA	1.00	0.92	3.00	3.08
PE	3.00	3.09	2.00	1.93
PI	2.00	1.97	2.00	1.85
PC	2.00	1.81	1.00	0.95

[a] PA = Phosphatidic acid; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PC = phosphatidylcholine.

calculated amounts and amounts applied in different runs were always less than 10%, confirming that the calculated values were within acceptable limits for practical use of the method [17,18,20].

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