

New colorimetric method for the quantitative estimation of phospholipids without acid digestion

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Summary A unique colorimetric method for the quantitative determination of phospholipids that does not involve the acid digestion of the lipid is described. The phospholipids, after separation by thin-layer chromatography and elution from the silica gel, are heated with a chromogenic solution that is a modification of a spray reagent formulated by Vaskovsky and Kostetsky (1968, *J. Lipid Res.*, 9: 396). The absorbance of the colored complex was read at 710 nm, and it followed Beer's law in the range of 1–10 μg of phospholipid phosphorus.

Supplementary key words chromogenic solution · thin-layer chromatography · egg yolk phospholipids

Existing methods for the estimation of phospholipids, though simple and rapid, invariably involve the digestion of the lipid with acid as an obligatory step for the conversion of organic into inorganic phosphorus prior to the development of a colored complex (1–5). The colorimetric method described here obviates the use of acid digestion for the estimation of phospholipids. This method is also sensitive, accurate, reproducible, and easy to perform.

Experimental

Reagents. All reagents and solvents were of analytical grade and were used without further purification.

Chromogenic solution. This is a modification of the spray reagent described by Vaskovsky and Kostetsky (6).

16 g of ammonium molybdate is dissolved in 120 ml of water to give solution I. 40 ml of concentrated HCl and 10 ml of mercury are shaken with 80 ml of solution I for 30 min to give, after filtration, solution II. 200 ml of concentrated H_2SO_4 is added carefully to the remainder of solution I. To the resultant solution is added solution II to give solution III. 45 ml of methanol, 5 ml of chloroform, and 20 ml of water are added to 25 ml of solution III to give the chromogenic solution, which is stable for at least three months when stored at 5°C.

Reference standards. Chromatographically pure reference standards of phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, lysophosphatidylethanolamine, diphosphatidylglycerol, sphingomyelin, and phosphatidic acid were obtained from V. P. Chest Institute, University of Delhi, Delhi, India.

A Bausch and Lomb Spectronic 20 spectrophotometer with a 1P40 tube and with a red filter was used.

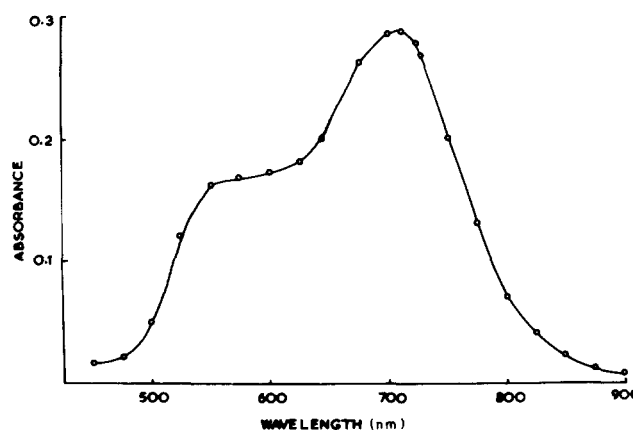


Fig. 1. Spectral curve of Prussian blue phospholipid complex.

Recommended colorimetric method. The lipid sample (1–10 μg P) in chloroform is added to a 15 × 125 mm Corning test tube and the solvent is evaporated. Add 0.4 ml of chloroform and 0.1 ml of chromogenic solution. Prepare a blank with 0.4 ml of chloroform and 0.1 ml of chromogenic solution. Place the tubes in a boiling water bath for 1–1.5 min. After cooling to room temperature, let the tubes stand for 5 min and then add 5 ml of chloroform and shake gently. Transfer the contents of each tube to a small separatory funnel (15-ml capacity). Allow to stand for 30 min and remove the lower chloroform layer. Read the absorbance at 710 nm against a blank. The complex is stable for at least 3 hr. Generally, the upper aqueous layer is quite small (consisting of a few drops) and by carefully manipulating the test tube, it is possible to remove the lower chloroform layer without having to use a separatory funnel.

Calibration curve. Prepare a calibration curve (1–10 μg P) by using any one of the standard phospholipids. Develop the color as described above. Plot the absorbance against μg P.

Results and discussion

The unique feature of the present method is that it does not involve acid digestion of the lipid sample. Instead, the chromogenic solution reacts directly with the phospholipid phosphorus and a Prussian blue complex is formed. That the intact phospholipid reacts with the chromogenic solution is supported by the solubility of the colored complex in chloroform, because the digestion of the lipid sample would result in the conversion of organic to inorganic phosphorus, making the complex water soluble. Addition of 5 μl of an aqueous solution containing as high as 250 μg of phosphorus (as KH_2PO_4) to chloroform and chromogenic solution did not lead to the production of any color in the organic phase when read against the usual blank.

The Prussian blue complex has an absorption maximum at 710 nm (Fig. 1) with an ϵ_{max} of 7500. The com-

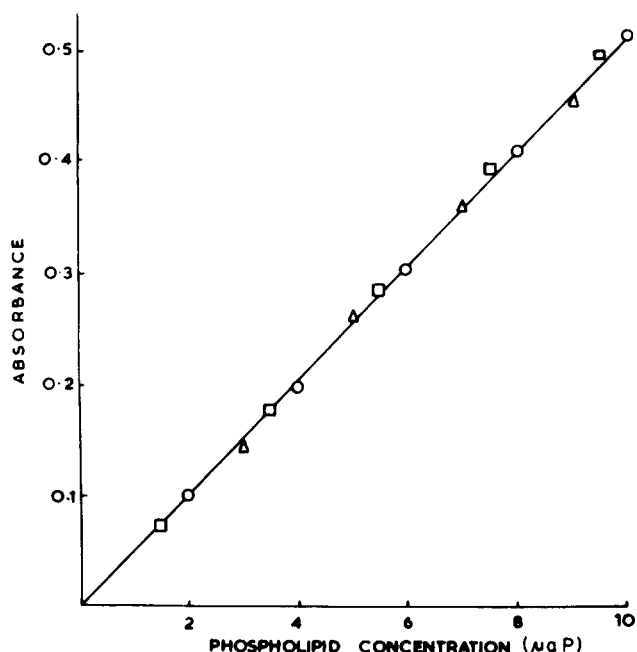


Fig. 2. Calibration curve for phospholipid estimation. O, phosphatidylcholine; Δ , phosphatidylethanolamine; \square , sphingomyelin.

plex obeys Beer's law over the range 1–10 $\mu\text{g P}$. Representative data on three of the phospholipids are given in Fig. 2. Absorbance values of reference phospholipid standards at comparable concentrations of phosphorus showed a variation of $\pm 1\%$.

Effect of excess heating. 0.1 ml of the chromogenic solution was added to known concentrations of phospholipids, and the contents were heated in a boiling water bath for periods of 1–4 min. A heating time of 1–1.5 min was found to be optimal for complex formation; heating beyond this period resulted in a decrease (8–10%) in absorbance.

Effect of neutral lipids. The addition of a large quantity (95–99%) of tripalmitin or the neutral lipid fraction of groundnut to a sample of phosphatidylcholine did not affect the estimation of the phosphorus content by the pres-

TABLE 1. Effect of different concentrations of neutral lipids on phosphatidylcholine estimation

Amount of Phospholipid Taken	Neutral Lipid Added	Amount of Neutral Lipid Added	Approximate Neutral Lipids in Mixed Sample		Phospholipids Recovered
			mg	%	
$\mu\text{g P}$					$\mu\text{g P}$
2.0	Tripalmitin	1.0	95		2.00 ± 0.05
		5.0	99		1.99 ± 0.02
4.0	Tripalmitin	2.0	95		3.99 ± 0.04
		10.0	99		3.99 ± 0.03
3.0	Groundnut non-polar lipids	1.5	95		3.01 ± 0.05
		7.5	99		2.98 ± 0.01
6.0	Groundnut non-polar lipids	3.0	95		6.01 ± 0.06
		15.0	99		5.98 ± 0.03

TABLE 2. Reproducibility of the estimation of phospholipids

Lipid Sample	Amount Analyzed	Mean of Five Determinations	SR ^a	S ^b
		$\mu\text{g P}$		
Phosphatidylcholine	2.4	2.40	0.41	0.010
	4.0	4.02	0.64	0.026
Phosphatidylethanolamine	3.0	3.02	1.12	0.034
	6.0	5.99	0.50	0.030
Sphingomyelin	2.0	2.01	0.74	0.015
	5.5	5.48	0.45	0.025

^aSR, relative standard deviation.

^bS, standard deviation.

ent method (Table 1). This method can, therefore, be directly applied for the estimation of the phospholipid content of oils.

Reproducibility. Data recorded in Table 2 show that this present method is quite accurate and reproducible.

Percentage recovery from thin-layer chromatograms. Aliquots from different phospholipid solutions in chloroform were spotted on 5×20 cm silica gel G (E. Merck) activated plates (110°C for 45 min) with a micropipette. The plates were developed in chloroform–methanol–water 65:25:4 (v/v). The positions of these phospholipids were located by exposure of the plates to iodine vapor, the area corresponding to each phospholipid was removed, and the phospholipids were eluted by the following procedure. Silica gel containing the phospholipid was transferred to a separatory funnel (50-ml capacity). For each gram of silica gel, 10 ml of chloroform and 10 ml of methanol were added and the contents were shaken. After adding 9 ml of water, the contents were again shaken. Two layers were formed; the upper layer consisted of methanol and water and the lower layer consisted of chloroform (exactly 10 ml) that contained all the phospholipids. Silica gel eventually settled at the interface. An aliquot of the lower layer (or the entire chloroform solution) was taken and the phospholipid content was determined as described above. The recovery of phospholipids by this method was higher (Table 3) than a common column chromatographic meth-

TABLE 3. Recovery of phospholipids from thin-layer chromatograms

Lipid Sample	Amount Applied	Amount Recovered	% Recovery
		$\mu\text{g P}$	
Phosphatidylcholine	2.0	1.98 ^a	99.0 ± 0.4
	4.2	4.15	98.9 ± 0.9
Phosphatidylethanolamine	3.0	2.93	97.7 ± 1.2
	6.0	5.92	98.7 ± 0.6
Sphingomyelin	2.0	1.99	99.5 ± 0.5
	7.0	6.91	98.7 ± 0.7
Lysophosphatidylcholine	2.0	2.02	101.0 ± 0.4
	8.5	8.42	99.0 ± 0.9
Average			99.1 ± 0.7

^aEach value is the mean of three determinations.

TABLE 4. Analysis of egg yolk phospholipids. Comparison of the present method with the method of Bartlett ^a

Lipid Components	Present Method		Bartlett Method	
	μg P	% (w/w)	μg P	% (w/w)
Phosphatidylethanolamine	100.0 ^b	17.20	85.5	17.10
Phosphatidylcholine	460.0	79.12	395.9	79.19
Sphingomyelin	16.4	2.82	14.2	2.84
Lysophosphatidylcholine	5.0	0.86	4.3	0.86

^aRef. 1.

^bEach value is the mean of three determinations.

od (7), which gave $85 \pm 1.1\%$ recovery. Seminario De Bohner, Soto, and De Cohan (8), who used the column chromatographic method for the elution of brain phospholipids from silica gel, also reported a low recovery (80%).

The possibility of treating the silica gel containing the phospholipid directly with chromogenic solution without prior elution was also explored. It was, however, concluded that the elution step could not be avoided.

Comparison of the present method with Bartlett's procedure (1). Individual classes of egg yolk phospholipids were separated by preparative thin-layer chromatography using chloroform-methanol-7 N ammonium hydroxide 65:25:4 (v/v). Solvent-free chromatograms were visualized by exposure of the plates to iodine vapor. Zones corresponding to different components were removed, and each component was eluted as described above. Suitable aliquots from these eluted fractions were taken for phospholipid estimation by the present method. The results so obtained were compared with those obtained in a similar experiment in which Bartlett's method was used (1). The

values obtained by the two methods showed good agreement (Table 4). Relative percentages of different egg yolk phospholipids in the present study agree with those reported earlier by Parkinson (9).

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