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A UNIVERSAL REAGENT FOR PHOSPHOLIPID ANALYSIS*

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SUMMARY

A simple procedure for preparing a stable stock reagent and working reagents for the detection and determination of phospholipids is proposed, and a simple, rapid and accurate thin-layer chromatographic technique is suggested. The results obtained for egg yolk phospholipids are presented as an example.

INTRODUCTION

The determination of phosphorus using phosphomolybdenum blue (PMB) is widely used in chemistry and biochemistry, four papers in particular being most frequently cited[1–4].

Many different methods are used for the determination of phosphorus in lipids[3–15]. Aminonaphtholsulphonic acid[3,5,8], ascorbic acid[4,6,7], hydrazine sulphate[9], amidol[10], iron(II) sulphate[12] and tin(II) chloride[13] are the most common reducing agents used for PMB formation. Recently, new reductants have been suggested[14,15]. However, there is a very interesting group of reagents for phosphorus analysis that have only limited application in phospholipid quantitation. These are reagents containing mixtures of molybdenum(VI) and molybdenum(V) (Zinzadze[16], Hahn and Luckhaus[17] and Lucena-Conde and Prat[18] reagents), and are well known to lipid biochemists as phospholipid sprays[19–23]. Hooghwinkel and Van Niekerk[24] recommended the Zinzadze reagent for the determination of phosphorus in lipids.

Previously, we used one of those reagents[18] for phospholipid analysis[25]. Recently, we have thoroughly investigated the conditions for the formation of PMB in order to find out if it was possible to simplify the procedure[26]. Here we report a procedure for preparing stock molybdate reagent together with a technique for

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obtaining working reagents and sprays. The application of these reagents is illustrated by the analysis of egg yolk phospholipids using standard and micro-thin-layer chromatography (TLC).

MATERIALS AND METHODS

Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), hydrazinium chloride and hydrochloric, perchloric and sulphuric acids were of analytical-reagent grade and used without additional purification. Egg yolk phospholipids were extracted by a minor modification of the method of Folch *et al.*[27]. Phosphatidylcholine (PC) was isolated from the extract by column chromatography on Silica gel. TLC and micro-TLC were performed as described previously[28].

Preparation of stock reagents and investigation of their stability

A solution of hydrazinium chloride (60 mg) in 4 *N* hydrochloric acid (2.0 ml) was added to a solution of sodium molybdate in 4 *N* hydrochloric acid (8.0 ml). The mixture was heated in a boiling water-bath for 20 min. After cooling, calculated volumes of acid and water were added to give a final volume of 15 ml. Working reagents were prepared both from freshly prepared reagents and those stored for 6 months at room temperature.

Investigation of working reagents

Aliquots of stock reagents were diluted with calculated amounts of acids and water, and portions (4.7 ml) of the resulting solutions were added to pyrex test-tubes containing 0.1 ml of dipotassium hydrogen orthophosphate solution (25 μg/ml of phosphorus) and 72% perchloric acid (0.2 ml) or water (0.1 ml) and perchloric acid (0.2 ml). The contents of the test-tube were thoroughly mixed with a vibrator mixer, and the tubes were then heated in a boiling water-bath for 15 min and subsequently cooled; absorbances were measured at 815 nm on a Spekol spectrophotometer with an EKA attachment. Three samples and three blanks were prepared in parallel from the working reagents for each assay.

Investigation of spray reagents

Amounts of 1.0, 2.5 and 10.0 μg of PC were spotted on to thin-layer plates in 5-mm diameter areas. Aliquots of the stock stable reagent were diluted with calculated volumes of sulphuric acid and water. The colouring rate, spot intensities, their stabilities, background colour and alteration were fixed for the sprays obtained.

Recommended procedures

Stable stock reagent. Add a solution of hydrazinium chloride (400 mg) in 4 *N* hydrochloric acid (14 ml) to a solution of sodium molybdate (10 g) in 4 *N* hydrochloric acid (60 ml). Heat the mixture in a boiling water-bath for 20 min; after cooling, add concentrated sulphuric acid (14 ml) with cooling and mix. Adjust the volume to 100 ml with water. Keep the reagent in a dark bottle at room temperature.

Working reagent I. Add 1 *N* sulphuric acid (48 ml) to the stock reagent (4 ml) and adjust the volume to 100 ml with water. The reagent is stable at room temperature for at least 1 week.

Working reagent II. Add 1 *N* sulphuric acid (26 ml) to the stock reagent (5.5 ml) and adjust the volume to 100 ml with water. The reagent is stable at room temperature for 1 week.

Spray reagents. A. Dilute a volume of stock reagent with three volumes of water. B. Dilute one volume of stock reagent with seven volumes of 10 *N* sulphuric acid.

Determination of phospholipids in extracts and fractions. Evaporate an aliquot of lipid solution in a Pyrex test-tube on a boiling water-bath or with a stream of nitrogen. Add to the residue 0.2 and 0.05 ml of 72% perchloric acid for the standard and micro-procedures, respectively. Digest the samples by heating in an electrically heated metal block at 180–200° for 20 min, allowing most of the tubes to project outside the block so as to prevent evaporation of perchloric acid. After cooling, add reagent I (4.8 ml) for the standard procedure, or reagent II (0.45 ml) for the micro-procedure; mix thoroughly with a vibrator mixer and heat in a boiling water-bath for 15 min. After cooling, measure the absorbance at 815–830 nm against a blank. When the absorbance is too high, the samples and blanks can be diluted with 1 *N* sulphuric acid 2–10 times.

Determination of phospholipids by TLC. Detect the phospholipid spots after development with concentrated sulphuric acid–methanol (1:9) and subsequent heating at 180° for 10 min, or with one of the phospholipid sprays. Scrape the silica gel zone containing the phospholipids (using a spatula made from a razor blade) into a Pyrex test-tube. Add 72% perchloric acid (0.2 ml) for all spots from standard TLC and the largest spots from micro-TLC, or perchloric acid (0.05 ml) for the remaining spots from micro-TLC. Take the blanks from plate areas without lipids. Perform the digestion and subsequent operations as described above. After PMB formation, sediment the silica gel by centrifugation for 10 min at *ca.* 2000 *g* and measure the absorbance of the supernatant.

RESULTS

As shown in our previous work[26], there is a definite region of molybdenum [Mo(VI): Mo(V) = 3:2] and acid concentrations in the reaction mixture. High yields of PMB are produced in that region, the formation of molybdenum blue being negligible, *i.e.*, the blank values do not exceed 0.040 (see Table I and Fig. 1). On the basis of these data, it is possible to calculate the composition of a working reagent, which, after addition to the sample digested with acid, gives a reaction mixture of optimal composition for PMB formation. Such a reagent should be sufficiently stable during storage. As can be seen from Fig. 1, the acid concentration in such a working reagent must be within the range 0.5–2.0 *N*. Reduced molybdate reagents are known[18] to be very stable when their acid concentrations are not less than 8 *N*. Hence it is reasonable to divide the procedure for obtaining the reagent into the following two stages: preparation of stable stock reagent, and its dilution with acid to give a suitable working reagent. As it is easy to decrease the molybdenum: acid concentration ratio in the solution by diluting with acid, we selected for the stock reagent a concentration of sodium molybdate equal to 100 mg/ml (about 40 mg/ml of molybdenum), and hydrochloric, perchloric and sulphuric acids were used as stabilizing reagents. The best results were obtained with sulphuric acid; hydrochloric acid precipitates molybdate when its concentration in the reagent reaches 5 *N*, and the presence of a high propor-

TABLE I

INFLUENCE ON ABSORBANCES AND BLANK VALUES OF THE RELATIONSHIP BETWEEN MOLYBDENUM AND ACID CONCENTRATIONS IN FINAL REACTION MIXTURES

The numerator gives the true absorbance $\times 10^3$ and the denominator the blank absorbance $\times 10^3$; both are expressed as mean values and standard deviations for six determinations.

Mo (V)/(VI) in final solution (mg/ml)	*Acid normality of final solution*										
	0.25	*0.5*	*0.75*	*1.0*	*1.5*	*2.0*	*2.5*	*3.0*	*3.5*	*4.0*	*4.5*
8.0							Blue	445±3 127±3	363±3 62±3	292±4 33±1	0±0 0
7.0						Blue	456±2 217±7	450±4 71±0	440±3 70±4	230±3 29±3	8±1 8±1
6.0						426±4 367±2	455±2 90±1	440±2 50±1	392±3 32±1	120±1 18±0	
5.0					Blue	448±4 109±3	436±5 59±3	433±1 41±2	394±4 36±1		
4.0					422±2 290±4	445±4 47±2	453±4 30±1	410±0 30±0			
3.0			Blue	Blue	439±3 78±2	444±4 27±1	446±2 27±1	250±2 10±1			
2.0			429±3 98±4	429±3 98±4	453±3 14±0	450±4 13±1	233±1 7±0				
1.0	430±3 85±3	430±3 85±3	457±2 16±2	457±2 16±2	333±2 13±1	53±2 0	5±0 0				
0.5	384±2 118±4	430±2 14±1	200±5 10±1	38±2 7±1							

tion of perchloric acid in the stock reagent decreases PMB formation. There were no differences in the results and stabilities between stock reagents with acid concentrations of 8, 10 and 12 *N* (see Table II). We therefore chose as a stock reagent the reagent containing 8 *N* acid and 40 mg/ml of molybdenum, and termed it the universal stable reagent, as it can be used for easily preparing any working reagent for phosphorus analysis or phospholipid spray.

For the determination of phospholipids by micro-TLC, it is essential to take 0.05 ml of concentrated perchloric acid for digestion, as this is the minimum amount required in order to wet the entire silica gel sample scraped off. This amount of the acid gives a normality of 1.2 in the final 0.5 ml of the reaction mixture. Inasmuch as a reduced molybdate gives molybdenum blue in solutions with acidity less than 0.3 *N*[29,30], we compared a series of working reagents with a molybdenum concentration 2.22 mg/ml (0.45 ml of such reagents gives a molybdenum concentration of 2.0 mg/ml in the final 0.5 ml) and acid normalities from 0.4 to 0.8. The results are given

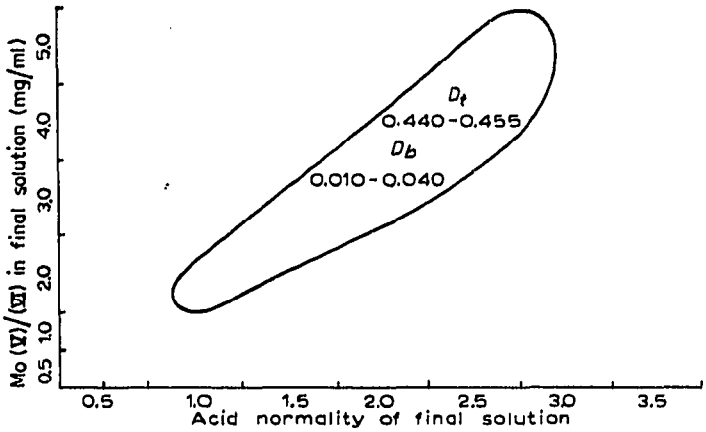

Fig. 1. Region of optimal conditions for PMB formation (maximum absorbances and minimal blank values). D_t = true density; D_b = blank density.

TABLE II

STABILITY OF CONCENTRATED REAGENTS WITH DIFFERENT ACID NORMALITIES AT A CONCENTRATION OF MOLYBDENUM OF 40 mg/ml

Acid normality of conc. reagent	*Acid normality of diluted reagent*	*Acid normality of final reaction reagent**	*First day***			*Sixth day****		
			D_0	D_b	D_t§	D_0	D_b	D_t§
8	0.6	1.74	470	31	439 ± 2	471	31	440 ± 2
10	0.6	1.74	471	31	440 ± 3	470	29	441 ± 3
12	0.6	1.74	472	32	440 ± 3	471	30	441 ± 3
8	0.7	1.83	468	29	439 ± 3	469	29	440 ± 2
10	0.7	1.83	466	27	439 ± 3	468	28	440 ± 2
12	0.7	1.83	467	27	440 ± 3	466	27	439 ± 2
8	0.8	1.92	468	29	439 ± 3	470	30	440 ± 3
10	0.8	1.92	465	27	438 ± 4	469	30	439 ± 3
12	0.8	1.92	467	28	439 ± 3	468	29	439 ± 3

* Mo concentration in final solution = 2 mg/ml.
** D_0 = overall absorbance $\times 10^3$; D_b = blank absorbance $\times 10^3$; D_t = true absorbance $\times 10^3$.
*** After 6-months storage of concentrated reagent similar results were obtained.
§ Standard deviation for six determinations.

TABLE III

STABILITY OF WORKING REAGENT AT A MOLYBDENUM CONCENTRATION OF 2.22 mg/ml AND VARIABLE ACID NORMALITY

Abbreviations as in Table II.

Acid normality of diluted reagent	*Acid normality of final solution*	*First day*			*Sixth day*		
		D_0	D_b	D_t	D_0	D_b	D_t
0.4	1.56	568	138	430 ± 4	—	510	—
0.5	1.65	470	38	432 ± 2	—	210	—
0.6	1.74	461	26	435 ± 3	465	28	437 ± 1
0.7	1.83	462	25	437 ± 1	462	25	437 ± 1
0.8	1.92	464	27	437 ± 1	469	30	439 ± 2

TABLE IV

STABILITY OF WORKING REAGENTS AT VARIABLE ACID NORMALITIES AND MOLYBDENUM CONCENTRATIONS

Abbreviations as in Table II.

Molybdenum concn. in reagent (mg/ml)	*Acid normality of diluted reagent*	*Acid normality of final solution*	*Molybdenum concn. in final solution (mg/ml)*	*First day*			*Sixth day*		
				D_0	D_b	D_t	D_0	D_b	D_t
1.04	0.54	1.0	1.0	460	20	440 ± 4	474	38	436 ± 3
1.56	0.8	1.25	1.5	460	20	440 ± 3	463	23	440 ± 3
2.08	1.06	1.5	2.0	465	28	437 ± 2	468	29	439 ± 2

TABLE V

INFLUENCE OF Mo AND H^+ ION CONCENTRATIONS IN MOLYBDATE REAGENT ON QUALITY OF DEVELOPMENT OF PHOSPHOLIPID SPOTS IN TLC

Point No.	*Mo (mg/ml)*	*Acid normality*	*Time of development of maximum colouring (min)*	*Intensity of spot colouring**	*Background at moment of development of maximum colouring*	*Spot stability*	*Retention time for light background (min)*
1	20	4	4–5	+	White	2–3 h	4–5
2		6	6–7	+	Bluish	2–3 h	6–7
3		8	1–1.5	+ + +	Greenish	2–3 h	2–3
4		10	1–1.5	+ + +	Green	After 25 h	2–3
5		12	1–1.5	+ + +	Green	no changes	2–3
6	15	4	3–4	+ + +	White	2–3 h	5
7		6	2	+ + +	White	After 20–	5–6
8		8	2	+ + +	Greenish	25 h no	5–6
9		10	1	+ + +	Greenish	changes	
10		12	2	+ +	Green		Blues instantly
11	10	2	2	+ + +	White	24 h	30
12		4	2	+ + +	White	24 h	30
13		6	2–2.5	+ + +	White		4–5
14		8	3	+ + +	White	After 24 h	4–5
15		10	4	+ + +	Greenish	no changes	4–5
16		12	2	+ +	Green		Blues instantly
17	7.5	2	3	+ + +	White	10 h	30
18		4	3	+ + +	White	10 h	35
19		6	4	+ + +	White	After 25 h	35
20		8	4	+ + +	White	no changes	35
21	5	2	3	+ +	White	15 min	35
22		4	2	+ +	White	15 min	35
23		6	6	+ +	White	15 min	35
24		8	3–4	+ +	White	After 20–	35
25		10	3–4	+ +	White	25 h	60
26		12	3–4	+ +	Greenish	no changes	60

* +, Weak; + +, medium; + + +, strong colouring.

TABLE VI

RESULTS OF PHOSPHOLIPID QUANTITATIONS IN EGG YOLK LIPIDS BY STANDARD TLC (PLATE 13 × 13 cm)

Abbreviations as in Table II.

*Phospholipid**	D_0	D_b**	D_t	*Phosphorus content (μg)*	*Phospholipid content (%)*
Phosphatidylcholine***§	625	20 ± 0.1	605	10.8	73.47 ± 0.15
	632		612		
	622		602		
	631		611		
	628		608		
	632		612		
	627		607		
	623		603		
Phosphatidylethanolamine***	262	30 ± 0.1	232	2.82	19.2 ± 0.12
	272		242		
	269		239		
	277		247		
	262		232		
	272		242		
	277		247		
	267		237		
Lysophosphatidylcholine§§	130	40 ± 0.2	90	0.35	2.38 ± 0.06
	140		100		
	145		105		
	133		93		
	140		100		
	155		115		
	135		95		
	140		100		
Lysophosphatidylethanolamine§§	146	40 ± 0.2	106	0.39	2.69 ± 0.05
	156		116		
	146		106		
	156		116		
	159		119		
	149		109		
	149		109		
	159		119		
Sphingomyelin§§	129	40 ± 0.2	89	0.33	2.26 ± 0.05
	134		94		
	129		89		
	134		94		
	141		101		
	131		91		
	131		91		

* Recovery of phospholipids: about 99%.

** Mean values for 8 blanks ± standard deviations.

*** Determined using 0.4 ml of perchloric acid and 9.6 ml of working reagent.

§ Reaction mixtures were diluted with 1 *N* sulphuric acid to 15 ml before centrifugation.

§§ Determined using 0.2 ml of perchloric acid and 2.8 ml of working reagent.

TABLE VII

RESULTS OF PHOSPHOLIPID QUANTITATIONS IN EGG YOLK LIPIDS BY MICRO-TLC

Abbreviations as in Tables II and VI.

*Phospholipid**	D_0	D_b	D_t	*Phosphorus content (μg)*	*Phospholipid content (%)*	*Phospholipid**	D_0	D_b	D_t	*Phosphorus content (μg)*	*Phospholipid content (%)*
Phosphatidyl-	660	25 ± 0.1	635	3.48	73.5 ± 0.24	Lysophosphatidyl-	220	30 ± 0.1	190	0.12	2.5 ± 0.06
choline**	660		635			ethanolamine	252		222		
	676		651				248		218		
	663		638				252		222		
	640		615				253		223		
	666		641				263		233		
	650		625				255		225		
	638		613				220		190		
Phosphatidyl-	193	25 ± 0.1	168	0.93	19.38 ± 0.18	Sphingomyelin	230	30 ± 0.1	200	0.11	2.27 ± 0.08
ethanolamine**	190		165				265		235		
	200		175				225		195		
	189		164				223		193		
	199		174				220		190		
	194		169				238		208		
	190		165				220		190		
	181		156				190		160		
Lysophosphatidyl-	217	30 ± 0.1	187	0.10	2.23 ± 0.04	Phosphatidylinositol	55	30 ± 0.01	25	0.01	0.3 ± 0.05
choline	227		197				54		24		
	227		197				56		26		
	212		182				55		25		
	227		197				55		25		
	233		203				54		24		
	227		197				55		25		
	200		170				55		25		

* Recovery of phospholipids: about 98%.

** Determined using 0.2 ml of perchloric acid 4.8 ml of the working reagent.

in Table III. It can be seen that the 0.4 and 0.5 *N* reagents are not stable and we therefore chose a working reagent with an acid normality of 0.7. In the final reaction mixture, which also contains 0.05 ml of concentrated perchloric acid, the reagent gives a molybdenum concentration of about 2 mg/ml and an acid normality of about 1.8. Hence the reagent corresponds to the optimal conditions of PMB formation (see Fig. 1).

In the most typical cases of the determination of phospholipids (in extracts, in fractions after column chromatography and in spots after standard TLC), perchloric acid (0.2 ml) is used for the digestion of samples, and the reaction of PMB formation is carried out in a final volume of mixture of 0.5 ml. The perchloric acid makes a contribution to the normality of the final reaction mixture of about 0.5. As is apparent from Fig. 1, one can obtain many working reagents with different molybdenum and acid concentrations that give optimal conditions in the final reaction mixture.

We compared three such reagents, which gave in the reaction mixture molybdenum concentrations of about 1.0, 1.5 and 2.0 mg/ml, the acid normalities being 1.0, 1.25 and 1.5, respectively. The results are shown in Table IV. All three reagents give similar results, but the reagent of minimum acid concentration is less stable. Hence we chose as a working reagent the one with a molybdenum concentration of 1.6 mg/ml and an acid normality of 0.8.

Previously[20,22], we found that a good phospholipid spray could be obtained with wide ranges of molybdenum and acid concentrations and degree of molybdenum reduction. In order to choose an optimal procedure for preparing a phospholipid spray from the stock reagent, we decided to check again the influences of different factors on the quality of such a spray reagent. We examined molybdenum concentrations from 5–20 mg/ml of the spray and acid normalities from 2–12. The time of spot development, intensity of colour, background colour and alteration were compared. The results in Table V show that the reagent detects phospholipids satisfactorily over both of these ranges. However, maximum sensitivity with a relatively stable background colour can be attained at definite molybdenum and acid ratios in the reagent. Hence, we suggest that two sprays should be prepared from the universal stock reagent, one containing 10 mg/ml of molybdenum and 2 *N* in acid, and the other with 5 mg/ml of molybdenum and about 10 *N* in acid.

The practical application of the recommended reagents and procedures was demonstrated by the phospholipid analysis of egg yolk. The results of a standard TLC determination (plates 13 × 13 cm) are shown in Table VI, and those obtained by micro-TLC are given in Table VII.

DISCUSSION

A wide variety of methods for the determination of phospholipids appears even in the recent literature, giving an indication of their limitations. Most of the methods have common disadvantages: the stability of reagents is low and, fairly often, they have to be prepared from dry substances.

We focused our attention on pre-reduced molybdate reagents, which are very stable and give reproducible results[31]. As our data on the optimal conditions of PMB formation indicate, it is possible to calculate the composition of a single working reagent for addition to a reaction mixture after digestion.

We first developed a procedure for preparing a stock molybdate reagent. We obtained the best results with both readily soluble sodium molybdate and hydrazinium chloride in hydrochloric acid[26]. Such a reduced reagent is stabilized by adding concentrated sulphuric acid[18]. We checked some batches of the starting material for the reagent and obtained good results in all instances. The stock reagent is very stable, being stored in our laboratories without deterioration for more than 6 months.

There are two principal steps in the usual procedure for the determination of phospholipids, namely digestion and PMB formation. Different methods of digestion have been used[3,4,9,13,32–34]; however, the most convenient method is digestion with 70–72% perchloric acid[5–8,10,24,35–38], which is a more powerful oxidant than sulphuric acid and gives a lower acidity in the reaction mixture after mineralization. Its sole disadvantage is an explosion hazard. However, when operating with lipid samples that require only a few hundred microlitres of the acid, the hazard is small.

We have found that perchloric combustion is completed in most instances in 15–20 min, although Böttcher *et al.*[35] reported that when the reaction mixture became colourless within 12–18 min only 94–97% of the phosphorus in the sample could be determined as orthophosphate, and that it was necessary to heat the mixture with perchloric acid for 40 min for complete digestion. Combustion is accelerated by the addition of a small amount of molybdate[37,39,40]. From the perchloric acid digest, nitrogen can be determined in addition to phosphorus[36].

In preliminary experiments[26], we established that perchloric acid had no influence on the results of phosphorus determinations over a wide range of concentrations, provided that the total acid concentration in the reaction mixtures was not changed.

Previously, after lipid digestion, water, a molybdate solution and a reducing reagent were generally added successively to the residue. In order to reduce the number of reagents and operations, a pre-mixed solution of water plus molybdate[3] or molybdate plus reducing reagent were added[9]. The use of a single reagent for the determination of phosphorus has also been suggested[4,41–45]. However, all of these reagents proved to be unstable and had to be prepared each day or several times a day. In this respect, our working reagents are superior to others in both stability and simplicity of preparation.

We have not considered in detail the development of lipids in TLC, but it should be noted that at present two-dimensional TLC[6,7,15,44–47] is increasingly replacing the one-dimensional procedure[48–55], although some recent papers have suggested the use of the latter[56,57]. Most of the natural complex lipid mixtures can be separated by the solvent systems recommended by Rouser[58].

In quantitative TLC, phospholipids are mainly detected with iodine vapour[15,44–46,53,57,59,60] and with sulphuric acid plus heating[6,7,51,52,56]. Other reagents have also been used[37,47–50,61]. In our opinion, iodine vapour, the most widely used reagent, is not satisfactory. It is toxic, detection is not sensitive and the procedure is time consuming. In order to detect phospholipids, we applied our phospholipid spray or sulphuric acid in methanol with subsequent heating. The molybdate spray locates phospholipid spots at room temperature in a few minutes; it not only doesn't interfere in further analyses, but also promotes phospholipid digestion. Molybdate sprays were also recommended for use in phospholipid determinations by other workers[37,61]. Sulphuric acid excels the molybdate spray in sensitivity. It is convenient to heat a

plate after spraying with phospholipid reagent, using sulphuric acid as a char agent, in order to detect minor components of lipid mixtures and check the resolution of phospholipids from other lipids.

Silica gel is scraped from the plates and transferred with a spatula into test-tubes for digestion, a razor blade being most commonly used[15,44,45,50–53,57,59], or is collected with a special device under vacuum[5,6,46]. We prefer the first procedure, especially in micro-TLC, because it is simpler and decreases the possibility of losing microparticles of silica gel.

For the digestion of phospholipids in a mixture with silica gel, the same methods are applied as used for phospholipid digestion without silica gel: with sulphuric acid[43,50,53,60], with mixtures of sulphuric and perchloric acids[44,45,47] or with perchloric acid[6,7,15,37,46,49,51,52,54,57,59]. Kahovcová and Odavić[56] suggested combining the procedures of detection and digestion and to accomplish the run on a thin-layer plate heated at 180° for 60 min after spraying with 50% sulphuric acid. They considered this method to give useful savings in time, yet this appears to be erroneous. Fung and Kalant[62], who used this procedure, increased the time of phospholipid digestion on plates to 10 h.

For PMB formation after phospholipid digestion with silica gel, the Bartlett method[3] or that of Rouser and co-workers[6,7] is generally used. Other procedures have also been employed[15,43,44]. Kahovcová and Odavić[56] used the Hahn and Luckhaus reagent[17], which is most closely related to our stock reagent both in its preparation and in composition, but they added water and the concentrated reagent to the reaction mixture separately.

We checked the recommended reagents and procedures for egg yolk phospho-

TABLE VIII

EGG YOLK PHOSPHOLIPID COMPOSITIONS

Phospholipid content (%)*					*Methods used*	*References*
PC	*PE*	*Sph*	*LP*	*Other phospholipids*		
73.0	15.0	2.5	5.8		Column chromatography	63
69.1	23.9	1.0		DPG, 3.24	One-dimensional TLC; phosphorus determinations in spot eluates	64
68.6	18.9	9.1	3.4		One-dimensional TLC and densitometry	65
69.0	24.0	3.0	3.0		One-dimensional TLC; phosphorus determination without elution	66
76.3	17.4	2.3	2.2	PI, PA, PG, or DPG traces	One-dimensional TLC	67**
77.7	15.7	2.4	2.6			
73.5	19.2	2.3	2.4	LPE, 2.7	Standard TLC	Present work
73.4	19.4	2.3	2.2	LPE, 2.5; PI, 0.3	Micro-TLC	Present work

* PC = phosphatidylcholine; PE = phosphatidylethanolamine; Sph = sphingomyelin; LPC = lysophosphatidylcholine; DPG = diphosphatidylglycerol; PI = phosphotidylinositol; PA = phosphatidic acid; PG = phosphatidylglycerol; LPE = lysophosphatidylethanolamine.

** Eggs from different hen strains were studied.

lipid analyses. Previously, the composition of these lipids was investigated by different methods[63–67]. Our results (see Tables VI–VIII) proved to be in good agreement with the latest data of Christie and Moore[67]. However, the use of two-dimensional TLC allowed us to measure lysophosphatidylethanolamine, which has been determined by other workers together with PC.

The proposed reagents and procedures, especially those based on micro-TLC[28], are very simple, rapid and cheap and give reproducible results, and they should therefore be useful in phospholipid analyses.

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