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Behaviour of vegetable phospholipids in thin-layer chromatography

Optimization of mobile phase, detection and direct evaluation[a]

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SUMMARY

By systematic studies using a mobile solvent composition triangle, the optimum chloroform–methanol–water mobile phase for the separation of phospholipids by thin-layer chromatography on 5 × 5 cm silica gel was found. By using acetate buffer (pH 4) instead of water in the mobile phase and by impregnation of the silica gel layer with phosphoric acid it was possible to optimize the separation of phospholipids so that baseline separation was obtained, which is necessary for direct evaluation. Another effect of impregnation with phosphoric acid is the higher stability and intensity given with Dittmer–Lester reagent, which is specific for phosphorus. It reacts with the phosphate group common to all phospholipids so that the same signals were obtained with a measuring wavelength of 720 nm for all phosphatidylcholines of soybean, rape-seed and sunflower seed lecithin. The other phospholipids (phosphatidylethanolamines, phosphatidylinositols and phosphatidic acids) could be quantified by this procedure. The signal intensity was the same for all phospholipids as the molecular weights are very similar, although the patterns of fatty acids are different.

INTRODUCTION

Little information is available in the literature on the separation of vegetable

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phospholipids by thin-layer chromatography (TLC)[1–3]. Most papers have dealt with the separation of phospholipids of animal or human origin[4–13].

The main constituent of vegetable lecithins, such as the raw lecithins of soybean, rape-seed and sunflower seed, are phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidic acid. These phospholipids, isolated from raw lecithin of different origins, differ in their fatty acid patterns (Table I). Owing to these different patterns, it is necessary to quantify phospholipids by means of a standard for each plant species and to use conversion factors.

Our experience with the H-chamber[14,15] led us to carry out the separation and the densitometric evaluation of vegetable phospholipids on a 5 × 5 cm silica gel plate. Because the separation takes place over a very short distance of 3.5 cm, the mobile solvent first had to be optimized, which was done using a mobile phase composition triangel (Fig. 1).

The selection of suitable analytical reagents turned out to be difficult with regard to quantification. Detection of phospholipids on thin-layer plates has been reported by many workers using different reagents, *e.g.*, molybdophosphoric acid[16], copper(II) sulphate[16–18], copper(II) acetate[4,5,16,18,19], 2,7-dichlorofluorescein[16] and sulphuric acid[16]. Sometimes reagents are used that react with certain functional groups, *e.g.*, Dragendorff reagent[20] for the quaternary ammonium group in phosphatidylcholine, ninhydrin[20] for the ammonium group in phosphatidylethanolamine and various reagents for the phosphate group contained in all phospholipids[12,21–25].

For our purpose we used the group-specific Dittmer–Lester reagent[21], which reacts only with the phosphorus that is common to all phospholipids. In order to be able to use the reagent as an immersion reagent and thus to enhance the quantitative evaluation, we added ethanol.

TABLE I

PATTERNS OF FATTY ACIDS IN PHOSPHOLIPIDS

Phospholipid	*Source*	*Fatty acid*					
		16:0	*18:0*	*18:1*	*18:2*	*18:3*	*Others*
PC	Soybean	14.5	4.2	8.9	59.6	6.3	4.5
	Rape	9.6	1.2	44.5	28.5	4.1	8.6
	Sunflower	11.1	3.7	12.3	63.3	5.5	3.5
PE	Soybean	19.1	3.0	7.7	58.9	6.4	3.1
	Rape	9.5	0.7	45.1	37.8	4.4	2.2
	Sunflower	13.3	3.4	9.2	71.2	0.3	2.5
PI	Soybean	2.7	5.7	5.6	47.0	6.8	1.9
	Rape	17.2	1.6	37.7	35.1	5.6	2.7
	Sunflower	32.6	4.7	6.9	47.2	5.1	2.9
PA	Soybean	20.4	3.6	10.8	55.9	5.7	3.1
	Rape	9.0	0.9	48.3	35.5	4.1	1.5
	Sunflower	12.3	3.8	10.3	67.8	0.9	4.7

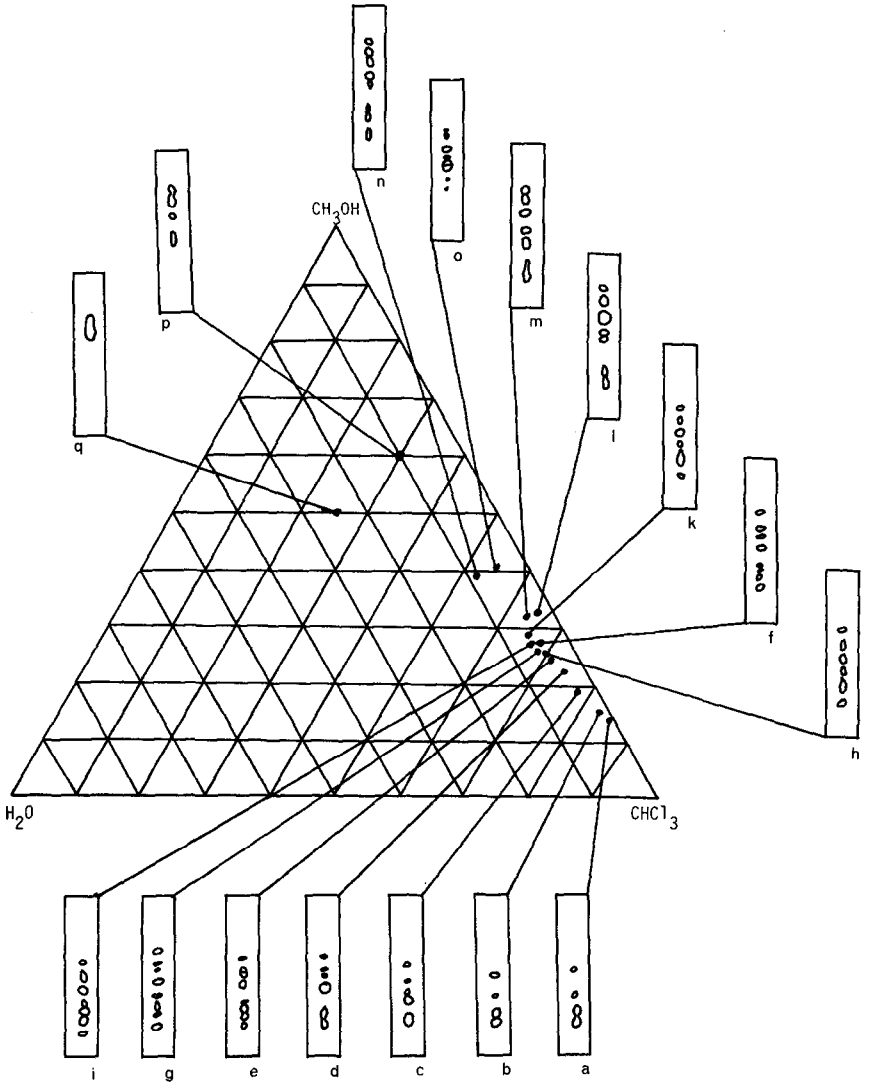

Fig. 1. Mobile phase composition triangle for chloroform–methanol–water.

EXPERIMENTAL

Standards

Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA) were enriched by preparative high-performance liquid chromatography from the lecithins of soybean, rape-seed and sunflower seed and isolated by means of low-pressure column chromatography in pure form (94–98%)[26].

Thin-layer chromatography

The application solvent was 2.5 mg of phospholipid per 10 ml of chloroform. Merck silica gel 60 HPTLC plates (5 × 5 cm) were impregnated by dipping into a 0.5% solution of phosphoric acid in 70% methanol and then heated for 20 min at 120°C. The mobile phase was chloroform–methanol–0.2 *M* acetate buffer (pH 4) (65:25:4.3, v/v/v). A Desaga-H-Kammer TLC chamber was used. Detection was effected with modified Dittmer–Lester reagent (see below), with measurement at 720 nm.

Composition of the Dittmer–Lester immersion reagent. A stock solution is prepared as follows: (a) 40.11 g of molybdenum(VI) oxide were dissolved in 1 l of 12.5 *M* sulphuric acid by heating; (b) 1.78 g of molybdenum were dissolved by heating in 500 ml of solution (a); equal volumes of (a) and (b) were mixed. For the immersion solution, 1 part of the stock solution was mixed with 2 parts of water and 3 parts of ethanol.

The immersion solution must be prepared freshly when required whereas the stock solution can be kept for several months. Following the immersion, the phospholipids become visible without further treatment of the plate as blue spots on a light blue background which decolorizes almost entirely within 30 min, during which process the intensity of the blue phospholipid spots increases; 30 min after immersion, direct evaluation can be carried out.

Measuring parameters. A Zeiss PM Q II chromatogram spectrophotometer was used with a slit of dimensions 6 mm × 0.1 mm, a recorder voltage of 5 mV, plate feed at 30 mm/min and paper feed at 120 mm/min.

RESULTS AND DISCUSSION

For the separation of vegetable phospholipids on a thin layer of silica gel, we started with a known composition of the chloroform–methanol–water mobile phase and optimized the composition using the triangle shown in Fig. 1. Each corner represents 100% by volume of one of the solvents. The percentage by volume on the three connecting lines shows the compositions of binary mixtures. The surface of the triangle represents all possible percentages by volume of the three solvents.

As shown in Fig. 1, even very slight variations of the water content (chromatograms g and f) has a large influence on the separation. Other workers[15,27,28] also observed that the water conent of the mobile phase has a large effect on the R_F values of phospholipids.

By using acetate buffer (pH 4) instead of water, an even better separation of the phospholipids was obtained owing to the dependence of the phospholipid separation on pH[29,30]. In addition, by impregnation of the layer with phosphoric acid of different concentrations, we tried to obtain a baseline separation. As shown in Table II, the R_F values of all the phospholipids increase with increasing amount of phoshoric acid in the layer. This increase is particularly noticable for PA. The best separation was obtained with 0.5% phosphoric acid, as shown in Fig. 2.

As the R_F values of the phospholipids of each class from the lecithins of soybean, rape-seed and sunflower seed are almost identical, it can be assumed that the chromatographic behaviour of phospholipids depends on the groups esterified with the phosphoric acid rather than on the fatty acid pattern.

In previous studies[30] we showed that, by using the Dittmer–Lester reagent,

TABLE II

R_F VALUES OF THE PHOSPHOLIPIDS ON A PHOSPHORIC ACID-IMPREGNATED SILICA GEL LAYER

Mobile phase: chloroform–methanol–acetate buffer (pH 4) (65:25:4.3).

Phosphoric acid (%)	*PI*	*PC*	*PA*	*PE*
10	0.54	0.53	0.69	0.60
1	0.29	0.41	0.58	0.58
0.5	0.24	0.34	0.45	0.56
0.2	0.23	0.33	0.39	0.51
0	0.19	0.21	0.23	0.47

which is specific for phosphorus, the signal obtained for each class of phospholipids is independent of the origin of the soybean, rape-seed and sunflower seed, *i.e.*, independent of the fatty acid pattern. This is possibly due to the fact that all phospholipid classes have similar molecular weights. Thus each phospholipid class is characterized by a similar content of phosphorus (Table III).

By using the Dittmer–Lester immersion reagent mixed with ethanol on a non-impregnated silica gel layer, the analytical evaluation has to be carried out

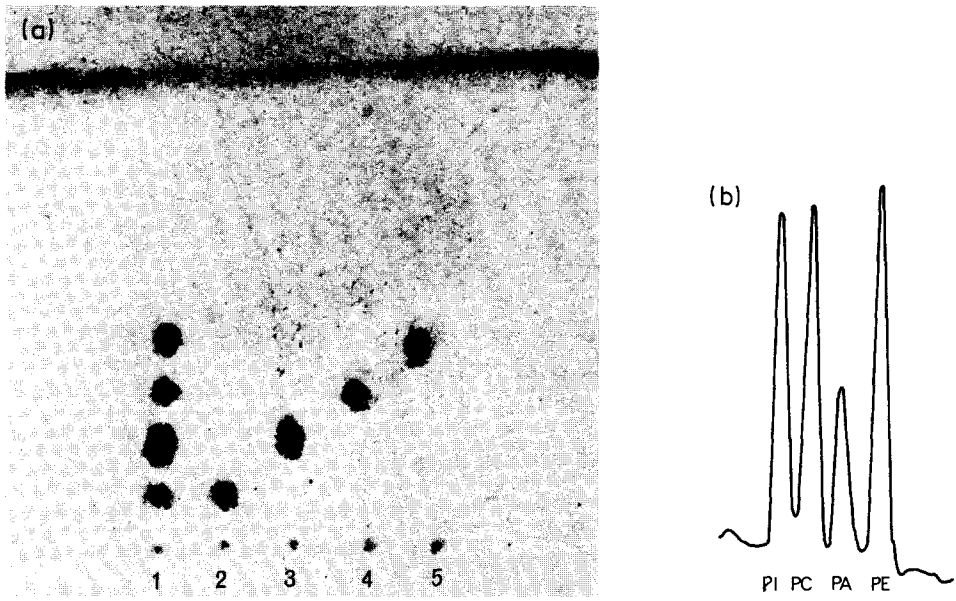

Fig. 2. Chromatograms of phospholipids. (a) Thin-layer chromatogram. 1 = Mixture of phospholipids; 2 = phosphatidyl inositol (PI); 3 = phosphatidyl choline (PC); 4 = phosphatidic acids (PA); 5 = phosphatidyl ethanolamine (PE). Stationary phase: silica gel impregnated with 0.5% phosphoric acid. Mobile phase: chloroform–methanol–acetate buffer (pH 4) (65:25:4.3). Detection: Dittmer–Lester reagent. (b) Chromatogram obtained with Zeiss PM Q II. For conditions, see Experimental.

10 min after immersion because, depending on the phospholipid class, the intensity of the spots decreased by 10–30% within 30 min after immersion and by 40–50% after 5 h (Fig. 3a). Gustavsson[12] showed that the pigmentation depends on the water content of the plate. The intensity of faded spots could be increased by using water vapour.

Impregnation of the silica gel layer with phosphoric acid turned out not only to enhance the separation of phospholipids, but also to improve the stability of the colours and the detection with Dittmer–Lester reagent. The maximum intensity of the blue spots was observed not earlier than 3 h following the detection and then remained more or less stable for about 3 h. The dependence of the measured intensities of the blue phospholipid spots on time is shown in Fig. 3b.

The quantitative measurement of the non-impregnated silica gel layer should be carried out 10 min after detection; although the signal intensity becomes relatively stable after about 1 h, by this time the signals are weak and difficult to measure. When detecting phospholipids on a phosphoric acid-impregnated layer, the intensity of the signal increases within the first 3 h and then remains almost unchanged for the next

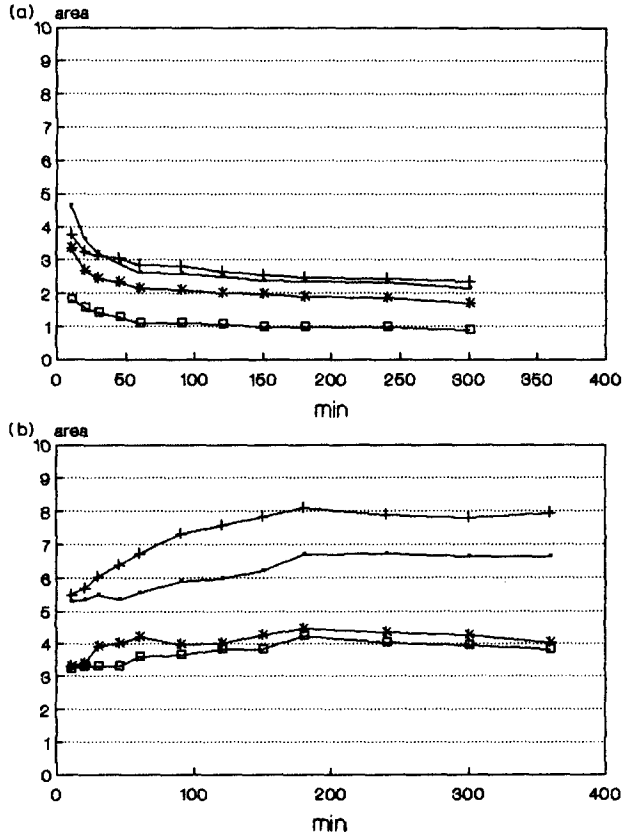

Fig. 3. Intensities of colours of phospholipids after detection with Dittmer–Lester reagent. (a) On the silica gel thin-layer surface; (b) on the phosphoric acid-impregnated silica gel thin-layer surface. ● = PC; + = PE; ∗ = PI; □ = PA.

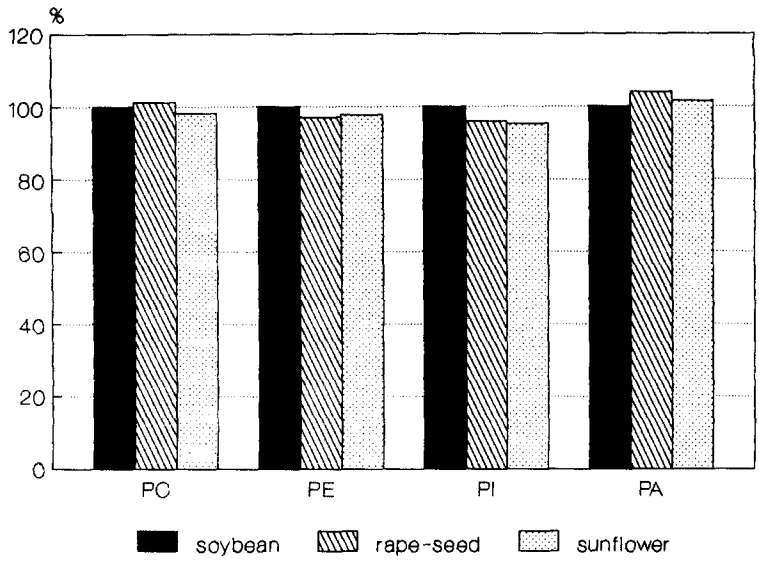

Fig. 4. Relative peak areas of the phospholipids.

TABLE III

AVERAGE MOLECULAR WEIGHTS OF PHOSPHOLIPIDS

Phospholipid class	*Soybean*	*Rape-seed*	*Sunflower seed*
PC	770	768	778
PE	724	735	736
PI	834	850	843
PA	687	691	695

TABLE IV

RELATIVE PEAK AREAS OF PHOSPHOLIPIDS

Phospholipid class	*Soybean*	*Rape-seed*	*Sunflower seed*
PC	100	101.4	98.1
PE	100	97.0	97.7
PI	100	96.0	95.4
PA	100	104.0	101.5

3 h (Fig. 3b). The quantitative evaluations can be carried out about 30 min after detection, as the intensity of the blue spots is then sufficiently stabilized. There is no need to wait before measuring until the maximum intensity of the colours has been achieved, because in spite of the higher molar absorptivity the standard deviation does not improve.

Subsequently we examined whether, after impregnation with phosphoric acid, the intensities of the colours were the same for the phospholipid classes with the Dittmer–Lester reagent irrespective of their origin. As soybean phospholipids are used as reference substances for the routine analysis, their peak areas were set as 100. The peak areas of the phospholipids of rape-seed and sunflower seed were calculated in proportion to the values for the soybean phospholipids. The results obtained for each phospholipid are shown in Table IV and Fig. 4.

There are only slight differences in the intensities of each phospholipid of one class; the differences are all within the range of the standard deviation of 4%.

By using this method of detection we were able to determine the phospholipid contents of lecithin specimens from soybean, rape-seed and sunflower seed with phospholipid standards (PC, PE, PI and PA) isolated only from the most frequently used soybean lecithin.

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