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Note

Thin-layer chromatography of phospholipids

Separation of major phospholipid classes of milk without previous isolation from total lipid extracts

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The triglyceride content of milk lipids, amounting up to 99 %, causes problems in quantifying and characterizing other lipid subclasses. For instance, the direct enzymatic determination of cholesterol in milk samples is impossible without previous alkaline hydrolysis of triglycerides and subsequent extraction of the non-saponifiable matter[1,2]. With regard to phospholipids, we have been looking for a method for separating and determining single phospholipid classes by thin-layer chromatography (TLC). However, the amounts of lipid extract applied to the plates required for the determination or further analysis of the major phospholipid classes of milk caused unsatisfactory separation owing to overloading by neutral lipids. We therefore investigated several solvent systems, known to move only neutral lipids from the starting region, in order to ensure that they do not influence the recovery in addition to the composition of phospholipids.

Further, the one-dimensional TLC separation of sphingomyelin, lecithin, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine could not be achieved using commercially available pre-coated plates. Phosphatidylserine and phosphatidylinositol either chromatographed together or they could not be resolved properly by any of the solvent systems under investigation[3–6]. We know of only one system that permits the separation of all phospholipid classes we are interested in; almost equidistant spots are obtained by development with chloroform–methanol–acetic acid–water (25:15:4:2) if silica gel plates prepared with 1 m*M* sodium carbonate solution instead of water are used[7].

Owing to the time- and space-consuming technique of preparing thin-layer plates by hand, we first looked for a method for post-impregnating ready-for-use plates with sodium carbonate. Early results were encouraging but eventually the method proved not to be really satisfactory. We therefore tried to find a new solvent system or a combination of solvent systems that could meet our demands for a reliable TLC separation of all of the major phospholipid classes of milk.

EXPERIMENTAL

Materials

Phospholipid standards were obtained from Sigma (St. Louis, MO, U.S.A.)

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and Applied Science Europe (Oud-Beijerland, The Netherlands). TLC plates were purchased from E. Merck (Darmstadt, G.F.R.) and Macherey, Nagel & Co. (Düren, G.F.R.). All chemicals were of analytical-reagent grade (Merck).

Methods

A total lipid extract was prepared by a slightly modified procedure of Bligh and Dyer[8]. Extraction and centrifugation were performed in 140 × 24 mm O.D. sampling tubes (Sarstedt, Nümbrecht, G.F.R.). A 4-ml volume of milk was thoroughly mixed with 10 ml of methanol. After addition of 5 ml of chloroform and thorough shaking, the samples were incubated for 15 min at 37°C, cooled to room temperature and mixed with a further 5 ml of chloroform and 5 ml of distilled water. Before phase separation by centrifugation (15 min at 2500 *g*) the samples were kept in an ice-bath for at least 10 min with intermittent shaking. The organic solvent layer, containing the lipid material, was removed by syringe and cannula. The solvent was evaporated under reduced pressure and the lipids were redissolved in 4 ml of chloroform–methanol (19:1).

TLC was performed using pre-coated silica gel 60 plates. Phospholipid standards (up to 200 μg) or aliquots of the lipid extract (up to 10 mg) were applied as a narrow band (2.5 cm in length) 1.5 cm above the lower edge of the thin layer plate.

The first and second developments were performed using light petroleum (boiling range 40–60°C)–diethyl ether–acetic acid (90:10:1)[9] and acetone, respectively, each to a distance about 15 cm above the starting line. Separation of phospholipid classes was achieved by subsequent development with ethyl acetate–2-propanol–water (50:35:15) to a distance 12 cm above the starting line (third development). Finally, chloroform–methanol–water (75:25:4) was allowed to rise 8.5 cm above the starting line (fourth development).

All developments were carried out in well equilibrated chromatographic tanks with intermediate drying of the plates under an electric fan. Staining was performed either by exposure to iodine vapour or by spraying with a 20% (w/v) solution of molybdophosphoric acid in ethanol and heating the plates for 10 min at 105°C. Phospholipid classes were identified using authentic markers.

Total phospholipids and single phospholipid classes were determined by phosphorus assay as described previously[10].

RESULTS

If run singly, none of the solvent mixtures or organic solvents known to separate neutral lipids and phospholipids by TLC, the latter remaining at the starting line, is able to move all of the neutral lipids to the upper part of the TLC plate. However, development with light petroleum–diethyl ether–acetic acid (90:10:1) followed by a second development with acetone is successful (Fig. 1). Thus it is possible to eliminate all interferences to the TLC separation of phospholipid classes caused by neutral lipids.

Using phospholipid standards we were able to show that no lytic products are generated, that none of the phospholipid classes is lost in considerable amounts, recovery ranging between 95% and 97.5%, and that the composition of the phospholipid fraction is not influenced by this procedure (Table I).

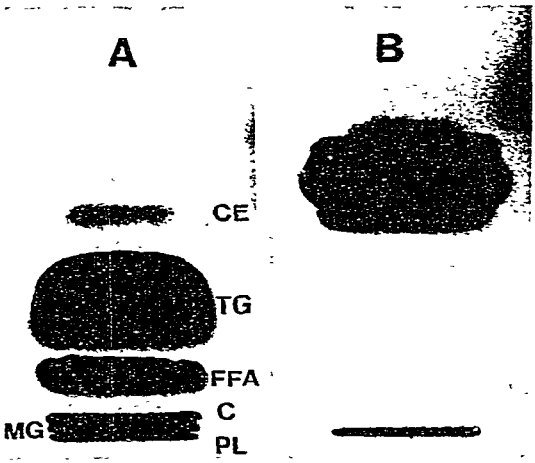

Fig. 1. Elution of neutral lipids from the lower part of the TLC plates. (A) First development with light petroleum–diethyl ether–acetic acid (90:10:1). (B) First development as for (A) and second development with acetone. PL = phospholipids; MG = monoglycerides; C = cholesterol; FFA = free fatty acids; TG = triglycerides; CE = cholesterol esters.

TABLE I

RECOVERY AND RELATIVE AMOUNTS OF SINGLE PHOSPHOLIPID CLASSES AFTER TLC SEPARATION ACCORDING TO THE PROPOSED PROCEDURE

Aliquots of a phospholipid standard mixture were applied to the plates and subjected to TLC separation including all four consecutive developments. Recoveries and relative amounts were calculated from data obtained from quantitative analysis of the single phospholipid classes by phosphorus assay.

Phospholipid	*Recovery after TLC separation (%)*		*Relative amounts (mole-% of total PL)**	
	*Mean**	*Range*	*Standard mixture*	*After TLC separation*
Sphingomyelin	95.5	95.2–95.9	19.7	19.6
Lecithin	96.7	96.4–97.2	20.5	20.6
Phosphatidyl-serine	97.1	96.5–97.5	19.5	19.8
Phosphatidyl-inositol	95.4	95.1–95.8	18.1	18.0
Phosphatidyl-ethanolamine	95.4	95.0–95.7	22.2	22.1

* Mean of five determinations.

Satisfactory TLC separation of the phospholipid classes so far remaining at the starting line can be achieved by consecutive developments with ethyl acetate–propanol–water (50:35:15) and chloroform–methanol–water (75:25:4) to 12 and 8.5 cm, respectively, above the starting line. The former solvent system is able to separate phosphatidylserine and phosphatidylinositol and the latter system accomplishes the separation of sphingomyelin and lecithin and also phosphatidylinositol and phosphatidylethanolamine (Fig. 2). Lyso-lecithin chromatographs beneath sphingomyelin, and lyso-phosphatidylethanolamine between phosphatidylserine and phosphatidylinositol.

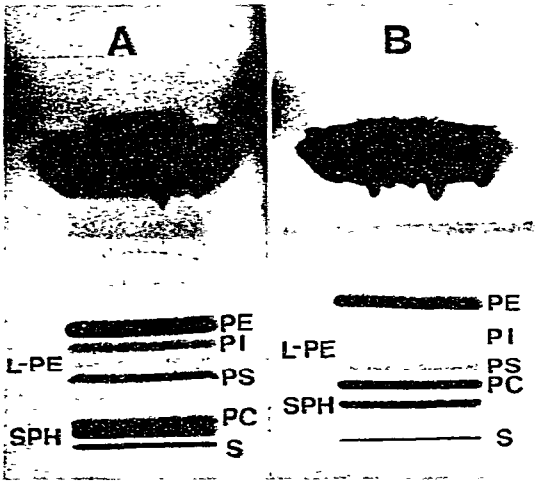

Fig. 2. TLC separation of phospholipid classes from milk. Pre-developments as in Fig. 1. (A) Third development with ethyl acetate–propanol-2–water (50:35:15). (B) Pre-developments and third development as for (A); fourth development with chloroform–methanol–water (75:25:4). SPH = sphingomyelin; PC = lecithin; PS = phosphatidylserine; PI = phosphatidylinositol; PE = phosphatidylethanolamine; L-PE = lyso-phosphatidylethanolamine; S = starting line.

DISCUSSION

The TLC separation of phospholipid classes from lipid extracts containing large amounts of neutral lipids is impossible without previous isolation of total phospholipids, for instance by column chromatography or acetone precipitation[4,5,11–15]. However, the time required, possible reduction of yield, and influences on relative amounts of single phospholipid classes (*e.g.*, refs. 12, 16 and 17) involved in these procedures are inconvenient if large sample series, containing only small amounts of phospholipids, have to be analysed. In addition, we have not been able to find any solvent system described in the literature that was really able to separate the five major phospholipid classes of milk by one-dimensional TLC using pre-coated silica gel plates, the separation of phosphatidylinositol and phosphatidylserine being the main problem.

Problems due to overloading the plates with neutral lipids, by application of lipid extract in sufficient amounts to analyse phospholipid classes quantitatively, could easily be avoided by pre-development with light petroleum–diethyl ether–acetic acid (90:10:1) and acetone successively. However, we had to test many different solvent systems before we achieved a satisfactory one-dimensional TLC separation of all the phospholipid classes under investigation. The proposed combination of four consecutive developments not only makes it possible to apply sufficient amounts of phospholipids to TLC plates without previous isolation from total lipid extracts, even if neutral lipids greatly predominate, but also allows the separation of phospholipids, *e.g.*, from milk, using commercially available pre-coated silica gel plates. If neutral lipids are present in small amounts the first two developments can be omitted without changing the chromatographic behaviour of the single phospholipids.

Moreover, using non-destructive detection methods, the present procedure can also be applied to the direct small-scale preparative isolation of single phospholipid classes from total lipid extracts and therefore might be of interest to many laboratories engaged in analytical work on phospholipids.

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