Two-dimensional thin-layer chromatography of polar lipids from milk and mammary tissue

JOHN G. PARSONS and STUART PATTON

Lipids Laboratory, Pennsylvania State University, University Park, Pennsylvania 16802

SUMMARY A two-dimensional thin-layer chromatographic system that separates all of the known polar lipids of milk and mammary tissue is described. The compositions of phospholipids of milk and of the mammary tissue from which the milk was derived are presented.

KEY WORDS milk · mammary tissue · polar lipids · thin-layer chromatography

ISOLATION OF the individual classes of milk phospholipids has presented many problems. Hawke (1) was not able to separate phosphatidyl ethanolamine and phosphatidyl serine. Recently Morrison, Jack, and Smith (2) have succeeded in resolving most of the polar lipids from bovine milk, but phosphatidyl inositol was not separated in their study. One problem is the inherent difficulty of isolating all the individual components by silicic acid column or one-dimensional TLC. Using two-dimensional TLC (3–6), we have developed a system which permits the complete separation of all known glycolipid and phospholipid components of bovine milk. Application of this procedure to polar lipids of mammary tissue has improved the earlier definition (7, 8) of such lipids.

Fresh milk and mammary tissue were obtained from a Holstein cow producing 20 lb of milk a day. The fat content of this milk averaged 4.9%. Approximately 5 g of lactating mammary tissue were removed by biopsy (courtesy of the Veterinary Science Department, Pennsylvania State University). 2 g of the wet tissue was cut up and homogenized in 20 volumes of chloroform–methanol 2:1. Nonlipid contaminants were removed by the method of Folch, Lees, and Sloane Stanley (9). The solvent was removed from the extract on a rotating evaporator under reduced pressure at 35°C and the total lipid sample was taken up in a small volume of chloroform–methanol 2:1.

Immediately before the biopsy, the quarter to be used as a source of tissue was completely milked. Lipids were recovered from a 20 ml sample of this milk by the Mojonnier extraction.[1] The polar lipids from the milk were obtained in the methanol fraction from a silicic acid column (10). Fig. 1 shows that a small amount of neutral lipid is still present in these polar lipids.

Two-dimensional TLC of the polar lipids was conducted on Silica Gel HR (Brinkmann Instruments,

Abbreviation: TLC, thin-layer chromatography.

[1] This is essentially the same as the Roese-Gottlieb method (Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists). 1960. 9th edition. 190.

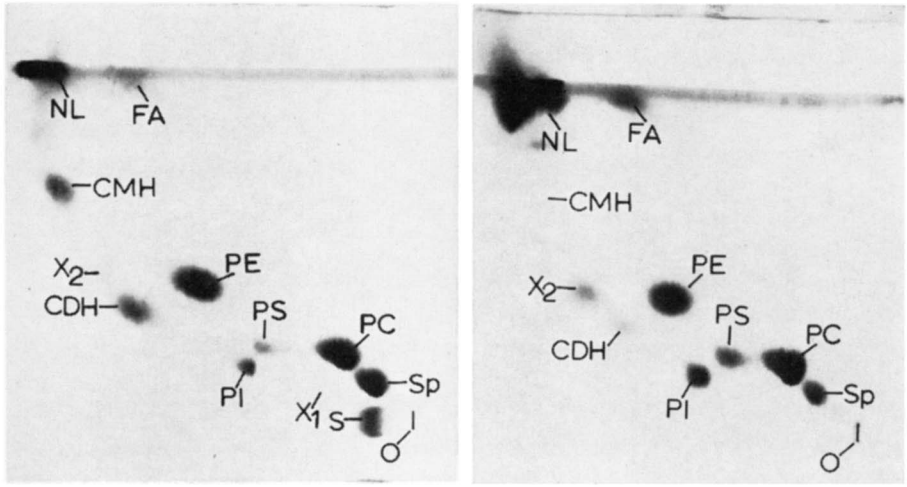

FIG. 1. Two-dimensional TLC of bovine milk polar lipids (left) and mammary tissue total lipids (right). The chromatograms were developed from right to left with chloroform–methanol–water–28% aqueous ammonia 130 : 70 : 8 : 0.5 and then in the vertical direction with chloroform–acetone–methanol–acetic acid–water 100 : 40 : 20 : 20 : 10. *O*, origin; *S*, carbohydrate (lactose) and protein; *Sp*, sphingomyelin; *PC*, phosphatidyl choline; *PS*, phosphatidyl serine; *PI*, phosphatidyl inositol; *PE*, phosphatidyl ethanolamine; *CDH*, cerebroside dihexoside; *CMH*, cerebroside monohexoside; *FA*, free fatty acids; *NL*, neutral lipid; and unknown substances listed as X_1 and X_2. X_2 has been tentatively identified as cardiolipin.

Inc., Great Neck, N.Y.). Thick layers (0.4 mm) were prepared with Research Specialties Co. (Richmond, Calif.) equipment (model 200-11). The air-dried plates were heat-activated for 1 hr at 120°C and cooled, and to them was applied 200–800 μg of lipid extract. The plates were developed in chambers (Research Specialties Co.) lined with solvent-saturated filter paper. The solvent systems were patterned after those of Rouser, Kritchevsky, Galli, and Heiler (3). As a result of many trials for ideal separation, we selected chloroform–methanol–water–28% aqueous ammonia 130:70:8:0.5 in the first dimension followed by chloroform–acetone–methanol–acetic acid–water 100:40:20:20:10 in the second. Development time was 35–40 min in each dimension with 10 min drying in air at room temperature in between.

The spots were made visible by exposure to iodine vapor or by spraying with one of the following reagents: sulfuric acid–potassium dichromate (11), the specific phospholipid spray of Dittmer and Lester (12), and ninhydrin reagent (0.2% in ethanol) for compounds containing amino group. Individual polar lipids were detected by means of spray reagents and were identified by comparing their R_f values with those of purified reference compounds. Further evidence was obtained by cochromatography of reference phospholipids with polar lipid extracts from milk. Authentic cerebroside mono- and dihexosides were a gift of C. C. Sweeley. Phosphatidyl serine and phosphatidyl inositol were obtained from Applied Science Laboratories Inc., State College, Pa. The major milk phospholipids—phosphatidyl ethanolamine, phosphatidyl choline, and sphingomyelin—were previously characterized on thin-layer plates at our laboratory by their IR spectra (13).

Phospholipid phosphorus was determined by the method of Rouser, Siakotos, and Fleischer (14), including their method for aspiration of silica gel from plates and determination of blanks.

Fig. 1 shows representative two-dimensional chromatograms of the polar lipids of bovine milk and mammary tissue. The separation of the polar lipids into the known individual components is complete. Spot X_1 and X_2 are both positive to the Dittmer and Lester spray reagent (12) but have not yet been identified. The phospholipid composition of bovine milk presented in Table 1 is in agreement with the average values reported by Morrison et al. (2), although our results do not include the 6% cerebrosides present (see Fig. 1 and cf. ref. 15). Recovery of lipid phosphorus was about 90%.

TABLE 1 PHOSPHOLIPID COMPOSITION OF BOVINE MILK AND MAMMARY TISSUE

	% Phospholipid by TLC*	
	Milk†	Mammary Tissue‡
Sphingomyelin	17.7 ± 2.2	6.0 ± 1.3
Phosphatidyl choline	33.9 ± 0.4	51.5 ± 0.8
Phosphatidyl serine	2.0 ± 0.3	5.3 ± 0.4
Phosphatidyl inositol	4.0 ± 0.3	7.2 ± 0.4
Phosphatidyl ethanolamine	42.4 ± 1.5	26.3 ± 0.8
X_2 (Cardiolipin?)	—	3.8 ± 0.2

* Average recovery = 90%.
† Mean ± SD, n = 3.
‡ Mean ± SD, n = 4.

Some of this loss may be attributed to minor components not determined and to the fact that only the aspirated spots plus corresponding blanks were removed from the TLC plate. The presence of silica gel during the analysis did not influence phosphorus recovery values.

Variations in room temperature and relative humidity will cause small shifts of the entire chromatogram. The separation of phosphatidyl inositol and especially of phosphatidyl serine is dependent upon the presence of a small concentration of ammonia in the first solvent to prevent their streaking.

We believe that the relatively higher levels of phosphatidyl ethanolamine, sphingomyelin, and cerebrosides in the milk as compared to the tissue may be derived from the plasma membrane of the lactating cell which envelops the milk fat globule in the process of its secretion from the cell (8).

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