

Quantitative gravimetric analysis of bovine semen lipids by thin-layer chromatography

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SUMMARY The quantitative thin-layer chromatography technique described has the advantage inherent in TLC of speed plus great separating power. By increasing the thickness of the layer, lengthening the plate, and using two consecutive solvent systems on each plate, the procedure can successfully manage larger quantities of lipid, yet produce excellent separations. The technique affords advantages in preparative analysis.

The exceptional properties of thin-layer chromatography (TLC) allow mixtures of lipids to be readily separated qualitatively (1, 2). Several modifications of the usual TLC procedures have enabled us to make preparative separations of complex mixtures of lipids on a single chromatogram. This was accomplished by using longer plates and thicker layers of absorbent than usual and developing the plates in two solvent systems. A procedure for the recovery of each separated class of lipids from the silica gel and their quantitative estimation has been developed.

Alkaline thin-layer chromatographic plates were prepared by spreading a slurry of Silica Gel G¹ (30 g) in 60 ml of 0.5 N NaOH as a layer 0.5 mm thick on glass plates 20 x 24 cm (1). Interfering contaminants were moved to the top of the previously activated plates by a preliminary development with diethyl ether. Weighed (80–110 mg) samples of lipid extracted with chloroform–methanol

¹ Research Specialties Co., Richmond, Calif.

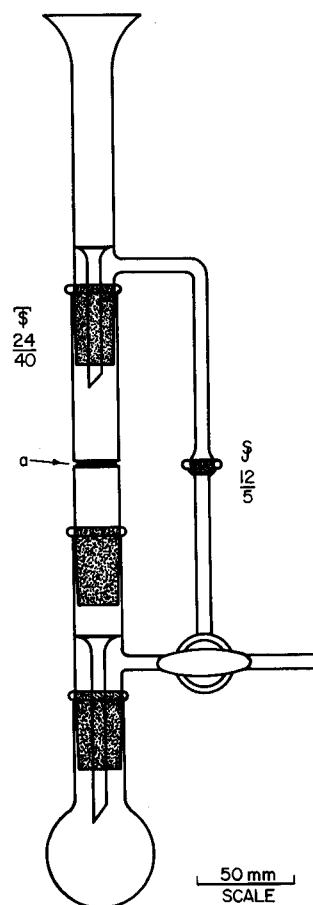


FIG. 1. Filtering apparatus. The apparatus is prepared for filtering by placing glass wool and activated silicic acid (followed by anhydrous CaSO_4 in the case of phospholipids) in the glass column mounted above the filter. *a*, fine fritted glass filter.

2 : 1 (v/v) (3) from bovine spermatozoa and seminal plasma were dissolved in the same solvent, transferred to a small trough, and applied to two plates with a Morgan applicator (4) in a series of closely spaced spots. The lipid remaining in the trough, the applicator and the original tared flask, was recovered and weighed to obtain by difference the amount of lipid applied to the plates. A reference mixture of mono-, di-, and triolein was applied to the plates containing the lipids being analyzed. The plates were developed successively with diethyl ether for approximately 40 min until the solvent front reached a position 15.5 cm from the point of application, and then with hexane–diethyl ether 9 : 1 (v/v) until the solvent front reached a point 0.5 cm from the top of the plate, which required about 80 min.

The development of a single chromatogram with two successive solvent systems resulted in a very distinct improvement and was partially responsible for the practicality of a gravimetric method of quantitation. It accomplished a separation which would have required more manipulation using the more conventional TLC

TABLE 1 PRECISION OF THE GRAVIMETRIC ANALYSIS OF TLC SEPARATIONS OF BOVINE SPERMATOZOA AND SEMINAL PLASMA LIPIDS

Identity	Spermatozoa			Seminal Plasma		
	Range*	S.D. †	CV ‡	Range*	S.D. †	CV ‡
Phospholipids§	71.8-75.6	0.95	1.29	65.1-71.5	1.32	1.92
Cholesterol	13.3-16.1	0.56	3.90	19.5-21.7	0.46	2.31
Diglycerides	6.0-7.2	0.46	6.97	3.3-5.5	0.52	12.92
Triglycerides	2.5-3.8	0.79	27.14	2.7-6.0	0.40	10.10
Wax esters	2.4-3.3	0.33	12.22	2.1-4.9	0.63	19.72

* Range of the average of duplicates, expressed as a percentage of the total recovered.

† Standard deviation of the difference between duplicates.

‡ Coefficient of variation of the duplicates.

§ Free fatty acids are included in this class and represent ca. 1%.

techniques. The use of alkaline silica gel prevented streaking, which usually occurs with these relatively non-polar solvents, by converting the free fatty acids to their salts. The latter do not migrate.

The phospholipid band, which did not migrate and had been located by previous trials, was removed by scraping the Silica Gel G off the plate with a razor blade. The glass area underneath the band was rinsed by holding the plate at an angle and carefully directing a stream of suitable solvent from a wash bottle on to the plate. The solvent was allowed to run down the plate and off the corner into a filtering device. The other lipid bands were removed in the same manner. This procedure was found to be necessary in order to obtain maximum recovery of the lipids. The remaining lipid bands were visualized by spraying the plates with bromothymol blue (5) and were seen as yellowish-green and greenish-blue bands on a deep blue background. The phospholipids were removed from the plate before spraying because the solvents used to extract the phospholipids also extract bromothymol blue. The plates were air-dried for 1 hr and placed in a desiccating cabinet overnight.

The lipids were eluted from the Silica Gel G by extracting with various lipid solvents. Erroneously high values were first obtained due to the passage of dissolved CaSO_4 through the filters even though dry solvents were used. A filtering apparatus was devised to remove the CaSO_4 and silica gel from the lipid sample. Internal desiccants were placed in the apparatus to remove traces of water from the solvent before it passed through the filter. The filtering apparatus² (Fig. 1) consisted of a column mounted above a fine fritted glass filter, constructed so as to permit suction to be applied both below the column and to the filter. The apparatus was prepared for filtering the phospholipid band by placing a glass wool plug in the bottom of the column followed by 1 cm each of activated silicic acid and anhydrous CaSO_4 . It was similarly prepared for filtering the neutral lipids, except that CaSO_4 was omitted. The silica gel scrapings containing

² This apparatus will be available from Arthur H. Thomas Company, Philadelphia, Pa.

each lipid class were placed on the prepared filtration apparatus and eluted with six to eight 5-ml portions of diethyl ether for the neutral lipids, or six 5-ml portions of chloroform-methanol (2 : 1) followed by eight 5-ml portions of methanol for the phospholipids. Absolute methanol was needed as a second extracting solvent to quantitatively remove and recover phospholipids from silica gel. Solvents used for both developing and elution were carefully purified and dried. After removing the accumulated solvent by means of a rotary vacuum evaporator at 45°, the lipids were transferred to small glass weighing flasks (tare wt 1-2 g), designed to prevent creeping of the lipids during evaporation of solvent. The solvents used in transferring were evaporated with a stream of dry nitrogen at 40°. The flasks were placed in an evacuated desiccator overnight and then weighed on a microbalance.

Figure 2 illustrates a typical separation of the total lipids obtained from spermatozoa. Identities of the bands starting from the bottom of the plate are as follows: phospholipids (plus traces of free fatty acids, ca. 1%), cholesterol, diglycerides, triglycerides, and wax esters. A band may include small quantities of other compounds

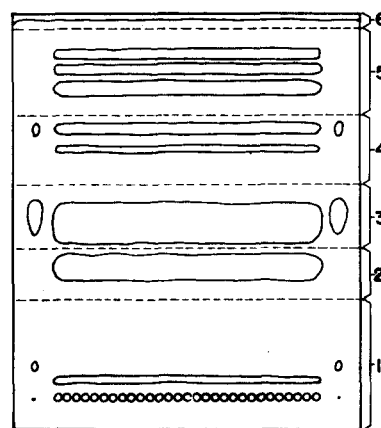


FIG. 2. Thin-layer chromatogram of lipids of bovine spermatozoa. Developed in diethyl ether and then in hexane-diethyl ether 9 : 1, on alkaline silica gel. Bands were grouped in the following classes: 1, phospholipids; 2, cholesterol; 3, diglycerides; 4, triglycerides; 5, wax esters; 6, silica gel contaminants.

of similar polarity. Identification of each group was made by infrared spectrophotometry, similar migration of reference compounds on TLC, tests for glycerides (6), Liebermann-Burchard reaction for cholesterol, and phosphorus analysis. The thin-layer separations were tested by rechromatographing each band. No overlapping or contamination by other lipid classes was found.

Samples ranging from 80 to 110 mg were analyzed quantitatively for the major lipid classes by this technique with relatively good precision (Table 1). The recovery for 18 determinations was 92.7% (SD 4.72) and ranged from 86.2 to 101.0%. Although gravimetric analysis of the small quantities often encountered present obvious difficulties, the technique described provides a measurement which can be applied to the varied compounds in lipids from biological sources.

The Morgan applicator allowed uniform distribution of the lipid mixture in a band at the base of the plate which, in spite of the relatively small difference in R_F values, resulted in uniform, easily separable, straight bands. If a lipid mixture different from those encountered in semen lipids is to be analyzed with this technique, alterations in polarity of the solvent or solvent mixtures may be necessary to obtain satisfactory resolution of lipid classes.

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