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DENSITOMETRIC QUANTITATION OF NEUTRAL LIPIDS ON AMMONIUM SULFATE IMPREGNATED THIN-LAYER CHROMATOGRAMS

JEROME L. HOJNACKI, ROBERT J. NICOLOSI and KENNETH C. HAYES
Department of Nutrition, School of Public Health, Harvard University, Boston, Mass. 02115 (U.S.A.)
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SUMMARY

A procedure is described which extends the densitometric quantitation of phospholipids on ammonium sulfate impregnated thin-layer chromatograms by Gluck *et al.* to include total lipid, free and esterified cholesterol, free fatty acid and triglyceride. Lipids separated on thin-layer plates containing silica gel G impregnated with ammonium sulfate were charred upon heating and absorbance was measured densitometrically. Thus, the necessity of spraying or submersing in a charring agent was eliminated, uniform charring became possible, and quantitation over a wider range of sample sizes than most densitometric procedures was obtained. One linear relationship existed for concentrations of standards over the range of 0.0–3.0 μg and another line from 4.0–50.0 μg. Both accuracy and precision of the method were highly reliable.

INTRODUCTION

Although quantitative photodensitometry of lipid classes separated after thin-layer chromatography (TLC) has seen widespread use[1–10] and offers a simple, fast micromethod for lipid analyses, irreproducibility of the charring process and a narrow range of linearity have limited its application. Preparation of plates for charring has been accomplished previously by spraying or dipping in solutions of sulfuric acid with and without dichromate[4,7] and ammonium bisulfate and bisulfite[10]. The difficulties associated with these methods of application of a charring agent have been background variability due to uneven spraying and inability to assess the precise amount of charring agent incorporated into each plate.

Recently, Gluck *et al.*[1] described a simple procedure for impregnating silica gel H with 5% aqueous ammonium sulfate for quantitating amniotic fluid phospholipids by charring and densitometry that eliminates the need for spraying and greatly enhances the uniformity of background within and between plates. Apparently upon heating the plate, the ammonium sulfate is converted to sulfuric acid, which in turn quantitatively chars the lipid[10].

This communication extends this technique by ammonium sulfate impregnation of silica gel G for quantitating neutral lipids and provides a method which is applicable over a wider range of sample sizes than several reported densitometric

methods[5–8]. In addition, data are presented which indicate that this method is applicable for quantitating total lipid directly from a non-migrating origin if care is taken to maintain a constant spot size and to remove non-lipid impurities of the biological lipid extract.

EXPERIMENTAL

Materials

Reagents. All solvents were either redistilled reagent grade or commercial Nanograde (Mallinckrodt, St. Louis, Mo., U.S.A.).

Standard lipid mixture. Lipid standards were obtained as follows: 1,2-dipalmitoyl-*sn*-glyceryl-3-phosphoryl choline, glyceryl trioleate, and palmitic acid from Sigma (St. Louis, Mo., U.S.A.) and cholesterol and cholesteryl oleate from Applied Science Labs. (State College, Pa., U.S.A.). All compounds migrated as homogeneous spots on TLC plates.

Five milligrams of each of the above reference standards were dissolved in toluene–chloroform (10:1) to a final concentration of 0.1 μg of each lipid class per μl of solution.

Biological samples. Human plasma samples from a normal, fasting individual and a high-density lipoprotein fraction obtained from a 40-h ultracentrifugation[11] at $d < 1.21$ of plasma from a *Cebus albifrons* monkey were extracted via the method of Folch *et al.*[12] for total and individual lipid class quantitation, respectively. Following extraction, samples were dissolved in toluene–chloroform for comparative gravimetric, densitometric, and charring–colorimetric analyses.

Thin-layer chromatography

The following solvents were used: (I) hexane–diethyl ether–glacial acetic acid (140:60:2); (II) petroleum ether (b.p. 39–42°)–diethyl ether (97:3).

The inner wall of the TLC chamber was lined with filter paper to increase saturation for more even migration of lipid and solvent front across the plate.

TLC pyrex glass plates were spread with a slurry consisting of 65 ml of distilled water, 3.25 g ammonium sulfate, and 30 g silica gel G (Merck, Darmstadt, G.F.R.) using a TLC spreader (H. Reeve Angel, Inc., Clifton, N.J., U.S.A.) to a thickness of 0.25 mm. The freshly prepared plates were then dried in a 100° oven for 1½ h and placed in a developing chamber containing distilled petroleum ether to remove organic contaminants. Prior to use, plates were divided into 28 parallel lanes, 7 mm wide, for individual lipid class analyses and activated at 100° for 30 min.

Application of sample. Aliquots of lipid standards and extracted biological sample for both total lipid and individual lipid class analyses were applied to chromatoplates using an automated TLC Multi-Spotter supplied with appropriate microliter syringes (Analytical Instrument Specialties, Libertyville, Ill., U.S.A.) to insure uniformity of spot size and rapidity of sample application. For those samples in which total lipid, only, is to be quantitated, plates need not be laned and only the appropriate total lipid standard mixture is applied along with the biological sample. Samples in which total lipid as well as individual lipid class analyses are to be determined simultaneously are applied to a plate divided in half. The half of the plate for individual lipid class analyses is laned as described and appropriate standards and biological

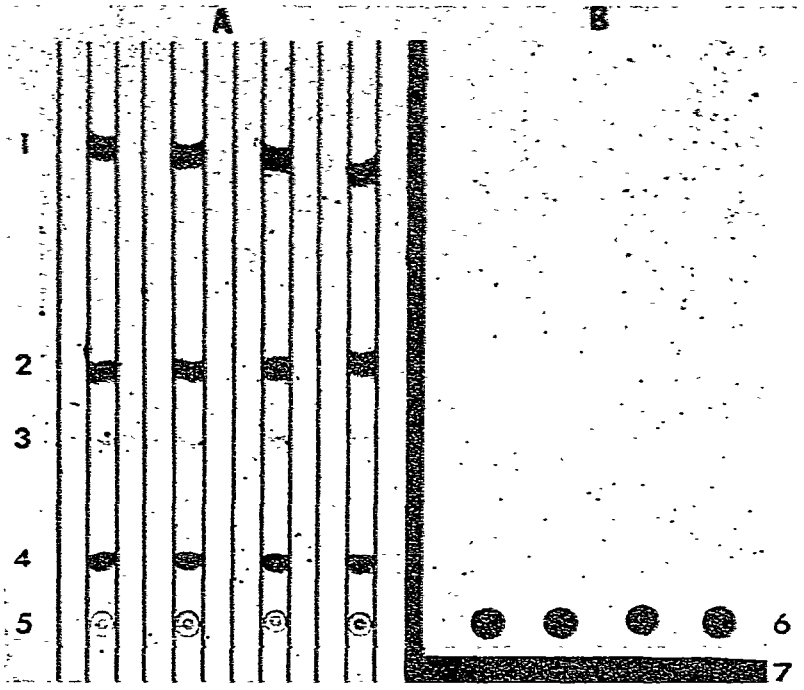

Fig. 1. Thin layer chromatogram for simultaneous analyses of individual lipid classes (A) and total lipid (B). 1 = Cholesteryl ester; 2 = triglyceride; 3 = free fatty acid; 4 = cholesterol; 5 = phospholipid; 6= total lipid of non-migrating origin; 7 = area scraped of silica gel to prevent migration of origin for total lipid quantitation.

sample applied. For total lipid quantitation, the same standard and biological sample are applied to the second half of the plate, and, in addition, the area beneath the standard and biological sample is cleared of silica gel to prevent migration of the spots (Fig. 1).

Development of chromatograms. After spotting, the plate was developed in solvent I to a height 12.5 cm above the origin to separate simple neutral lipids. The plate was then removed, air dried, and placed in a tank containing solvent II and allowed to migrate to the top to separate cholesteryl esters from any contaminants which migrate with the front of solvent I.

The spots were charred by placing the plate on a metal hot plate (Model No. 2200; Thermolyne, Dubuque, Ind., U.S.A.) for 10 min at a surface temperature of 187°. Densitometry was carried out, after cooling, with a Photovolt Model 52-C densitometer supplied with a Varicord Model 42-B recorder (Photovolt, New York, N.Y., U.S.A.).

For individual lipid class analyses, scanning was in the direction of migration because of the 7-mm lanes and the collimating slit of the scanner measured 0.2 mm × 4 mm[7]. Samples for total lipid determination were scanned both parallel and perpendicular to migration since lanes are not required and, therefore, the collimating slit was slightly larger than the spot size for each lipid class[4].

Peak areas for concentrations of samples and standards were determined with

a Model 282 Digital integrator (Buxco, New Haven, Conn., U.S.A.). Quantitation of the sample lipid classes was effected by comparing the peak area in integration units for each class with the standard curves for each lipid class derived from various quantities of standard spotted on the plate.

Aliquots of biological samples were transferred to pre-weighed glass vials, evaporated to dryness under nitrogen and weighed on a Mettler Model B6 balance (Mettler Instrument, Heightstown, N.J., U.S.A.). The weight of the lipid extract was calculated by difference.

Biological extracts and standards were applied to TLC plates, developed in previously described solvent systems, visualized with iodine vapors, scraped into pyrex tubes, and charred and measured colorimetrically for total lipid[13] and individual lipid classes[14]. Results from both methods are compared to the densitometry described here for the same biological sample.

RESULTS AND DISCUSSION

Range of method

One linear relationship existed for the concentration of total lipid and individual lipid class standards over the range of 0.0–3.0 μg and another from 4.0–50.0 μg for analyses of total lipid and each lipid class (Fig. 2). The lower range of linearity for neutral lipids agrees well with other reported densitometric class analyses utilizing spraying or submersing in charring agent[5–8]. The existence of an upper linear

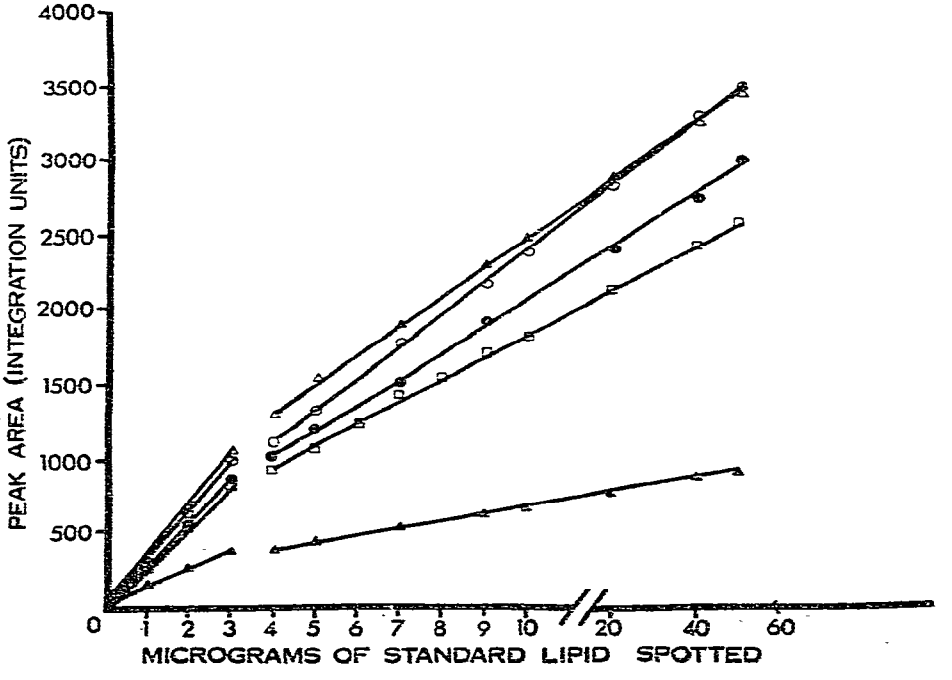

Fig. 2. Standard curves for densitometric analyses of lower and upper ranges of charred total lipid (□), cholesteryl ester (●), triglyceride (○), free fatty acid (▲), and cholesterol (△).

TABLE I

MEASUREMENT OF PLASMA TOTAL LIPID BY VARIOUS METHODS

Sample No.	*Gravimetric*	*TLC*	*Colorimetric**
1	2.15	2.67	2.68
2	2.22	2.47	2.58
3	2.13	2.41	2.70
4	2.12	2.13	2.63
5	2.15	2.28	2.58
6	2.13	2.43	2.43
	2.15 ± 0.02**	2.40 ± 0.07	2.60 ± 0.04

* Marsh–Weinstein procedure[13].

** Values represent mean ± S.E.M. of six replicate aliquots of human plasma lipid extract expressed in μg lipid/μl.

range is consistent with the results of lipid class analyses, *in situ*, fluorometrically[15,16], is suggestive in another densitometric report[9], and permits an application over a wide range of sample size. Since a family of curves is obtained for different concentrations of standard lipid mixture, accurate quantitation of an unknown biological sample requires that a range of standards be applied to each plate. The values for lipid concentrations are then reproducible. It is also noteworthy that all standard curves for the lower range of standard mixtures passed through the origin without the need of hydrogenation as suggested necessary by others[4].

Accuracy and precision

The reproducibility in analyses of replicate aliquots of biological lipid extract for total lipid and the favorable agreement in quantitation of total lipid and neutral lipid classes with more conventional gravimetric and colorimetric procedures indicate that the method has a reliable degree of accuracy and precision (Tables I and II). In addition, quantitation of five aliquots of total lipid by densitometric scanning of the origin (2.53 ± 0.05 mg) was within 1.7% of the results achieved by summation of the lipid classes of a similar number of aliquots allowed to separate into individual lipid classes (2.49 ± 0.08 mg).

TABLE II

COMPARISON OF TLC-DENSITOMETRIC AND COLORIMETRIC MEASUREMENTS OF NEUTRAL LIPIDS

Lipid class	*TLC*	*Colorimetric**
Cholesteryl ester	0.597 ± 0.03**	0.617 ± 0.02
Triglyceride	0.161 ± 0.02	0.176 ± 0.01
Free fatty acid	0.293 ± 0.02	0.252 ± 0.03
Cholesterol	0.145 ± 0.01	0.160 ± 0.004

* Procedure of Kritchevsky *et al.*[14].

** Values represent mean ± S.E.M. of four to seven analyses of monkey high-density lipoprotein lipid extract expressed in μg lipid/μl.

TABLE III

RECOVERIES OF LIPID STANDARDS ADDED TO BIOLOGICAL LIPID EXTRACTS

Lipid class	*Range of per cent recoveries of standard lipids*	*Mean per cent recoveries of standard lipid*
Total lipid	82.1–88.3	87.7
Cholesteryl ester	77.9–92.9	90.5
Triglyceride	85.2–105.8	95.5
Free fatty acid	86.6–88.2	87.4
Cholesterol	84.0–95.4	89.7

Recovery data

Recoveries of different quantities of lipid standards added to known amounts of biological lipid extract are shown in Table III. Adequate comparison with other densitometric procedures[5–7,9] was not possible since, in several cases, published data on recoveries were not available. However, the ranges reported here are similar to those reported for *in situ* fluorimetric analyses of lipids[15,16].

The results of this study suggest that, in addition to the measurement of phospholipid[1], chromatoplates impregnated with ammonium sulfate can also be used to quantitate neutral lipids. In addition, since total lipid is measured by densitometric scanning of a charred non-migrating origin, numerous samples of lipid extract can be applied to any part of the chromatoplate of uniform thickness from which organic contaminants have been removed.

The rapidity of this procedure, its replicability and application over a wide range of sample size and its favorable comparison with other assays indicate that it is a reliable method for the quantitation of individual neutral lipid and total plasma lipid. The method is currently being used for tissue analyses of lipids as well.

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