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# SIMULTANEOUS FLUOROMETRIC ANALYSIS OF FIVE LIPID CLASSES ON THIN-LAYER CHROMATOGRAMS

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## SUMMARY

Cholesteryl ester, triglyceride, free fatty acid, sterol, and phosphatidyl choline were separated on thin-layer chromatograms and estimated fluorometrically by means of a recording TLC scanner after spraying with Rhodamine 6G. Quantitation was effected by comparing the fluorescence of each sample spot with that of several concentrations of a similar fraction from a standard lipid mixture separated on the same chromatogram. One linear relationship existed for concentrations of standards over the range of 0.0 to 3.0 $\mu$g and another line from 3.0 to 130 $\mu$g. The accuracy of the method with standard lipids was $\pm$ 0.2 $\mu$g at the 99.8% confidence level, and the precision of analyses of biological lipid extracts was $\pm$ 0.2 $\mu$g from the mean value at the 99.5% confidence level.

## INTRODUCTION

Methods for estimating submilligram amounts of lipid classes require either elution of the separated spots from a thin-layer chromatogram (TLC) followed by chemical analysis or direct quantitation of the spots by densitometry. Direct quantitation on thin-layer chromatograms eliminates the tedious recovery procedures necessary in elution methods[1].

Recently, steroids have been quantitated directly on a thin-layer chromatogram by fluorometry[2,3]. Fluorescence measurements have been reported to be most economical of time and effort in estimating, as well as identifying, microgram amounts of complex compounds in test mixtures[1,2].

Compared to densitometric methods fluorometric quantitation: is generally more sensitive[2,4]; eliminates the effects of light scattering[4]; is independent of the chromatogram adsorbent[4]; and, permits the use of absorption- and/or fluorescence-spectra on the same plate when it becomes necessary to localize zones of a compound[2,4].

This communication describes a technique for the direct and simultaneous fluorometric analysis of five lipid classes separated on thin-layer chromatograms, a method which is applicable over a wider range of sample sizes than several reported

densitometric analyses[5,6] and which permits the quantitation of those phospholipid classes which can be induced to fluoresce in the UV range.

## EXPERIMENTAL

### *Materials and methods*

#### *Reagents*

All solvents were reagent grade and redistilled prior to use.

#### *Standard lipid mixture*

Highly purified (99%) lipids were obtained as follows: cholesterol and glyceryl trioleate from Applied Science Laboratories, State College, Pa.; 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine from Mann Research Laboratories, New York, N.Y.; stearic acid from Calbiochem, Los Angeles, Calif.; cholesteryl laurate from Dr. P. Smith, Dept. of Microbiology, Univ. of S. Dakota. All compounds migrated as homogeneous spots on TLC plates.

Ten milligrams of each of the above reference standards were dissolved in methylene chloride–methanol (2:1) to a final concentration of 1 $\mu$g of each lipid per $\mu$l of solution. The 2:1 solvent ratio of methylene chloride–methanol provided optimal solubility for the five lipid classes.

An aliquot of the solution was evaporated to dryness and resuspended in methanol–methylene chloride (9:1) for hydrogenation by the micro-hydrogenation procedure of FARQUHAR[7]. Hydrogenation was carried out for 4–5 h using 30 mg of $PtO_2$ as catalyst. After hydrogenation the catalyst was removed by filtration, the solution evaporated to dryness under nitrogen and the residue redissolved in methylene chloride–methanol (2:1).

### *Biological samples*

*Extraction.* Lipid extracts from tissue cultures (low-line Mouse Fibroblasts, NCTC No. 2555; embryonic chick fibroblasts; embryonic chick and duck kidney epithelial cells; and pigeon aorta cells) and a tissue homogenate (chicken liver) were prepared according to the method of FOLCH *et al.*[8]. Following extraction, the lipid solution was evaporated to dryness and brought to volume in a volumetric flask using methylene chloride–methanol (2:1).

*Hydrogenation.* The extracted lipid solution was hydrogenated in the same manner as the standard lipid mixture.

### *Thin-layer chromatography*

*Solvent systems, developing chambers and plates.* Solvent I: diethyl ether–glacial acetic acid–petroleum ether (20:2:78); solvent II: chloroform–methanol–glacial acetic acid–water (65:20:6:4). 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 TLC plate. Just prior to use, chromatoplates (20 × 20 cm) with a 0.25 mm layer of Silica Gel G (Merck, Darmstadt, G.F.R.)* were placed in a developing chamber containing Solvent I for pre-washing. This step was necessary to move organic contaminants to the uppermost edge of the chromatoplates. After allowing the plate to

* Merck plates were preferred because the Silica Gel G layer does not slough off when sprayed to saturation with Rhodamine 6G.

dry, a light pencil line was drawn across the absorbent side of the plate 4.5 cm from the bottom to serve as the spotting line.

On the reverse side of the plate (glass side) a score mark was made with a glass cutter 5.0 cm from the bottom of the plate and parallel to the pencil line on the adsorbent side. The plate was then activated for 30 min at 110°.

*Application of sample*

All solutions to be spotted were evaporated to dryness in a centrifuge tube, resuspended in 50 $\mu$l of methylene chloride–methanol (2:1) and spotted. The tube was rinsed with an additional 50 $\mu$l of solvent which was also spotted in each case.

Samples were spotted 2.2 cm apart along the sample line using a 50 $\mu$l Hamilton syringe with a repeating dispenser (Hamilton Pb-600). A warm air blower was positioned under the plate to facilitate drying of the sample spotted on the plate. Spot size was not allowed to exceed 4 mm. Eight spots, 1, 2, 3, 5, 15, 25, and 35 $\mu$g standard lipid mixture, and the sample to be assayed, were applied to the plate.

*Development of chromatograms*

After spotting, the plate was developed in Solvent I for approximately 45 min until the solvent front was 5.0 cm from the top of the plate to separate simple lipids. The plate was then removed from the chamber, dried and broken along the scored line. The plate section containing the origin was then rotated 180 degrees and developed in Solvent II in a direction opposite to that in Solvent I. The solvent was allowed to rise until the solvent front was just at the top edge of the plate to separate complex lipids from non-lipid material which remained at the origin.

*Spraying.* After the plates were dried with warm air from a blower, the two sections were placed in a chromatographic plate spray shield (Supelco Inc., Bellefonte, Pa.). They were then sprayed to saturation four successive times with Rhodamine 6G (ref. 9) and dried between each spraying with warm air from a blower.

*Fluorometric scanning.* Fluorometric measurements were performed with a Turner Model 111 filter fluorometer and a Camag-Turner Model 2 automatic TLC scanner[3].

A low pressure, mercury, far-UV lamp with maximum emission at 254 m$\mu$ was installed in combination with the primary filter, Corning 7-54 (Turner 110-811) (transmittance from 220–400 m$\mu$). A Turner 110-815 UV absorbing filter (transmittance at 254–300 m$\mu$) was used in addition to the primary filter. The secondary filter most commonly used was a Turner 110-824 (color spec. No. 23A; max. transmittance at 570 m$\mu$) in combination with a Turner No. 110-823L range extension filter (transmission 10%). A sensitivity adjustment of 3 × and excitation slit width of 3 mm were optimal in most cases.

Emitted fluorescence was plotted on a Westronics Strip Chart Recorder (Model No. D113) with a chart speed of 24 in./h.

The scanning direction was perpendicular to chromatographic resolution in order to scan all spots of the same class on the same recorder tracing.

*Integration and calculation.* Fluorescence response peak areas for concentrations of samples and standards were determined by triangulation or planimetry and expressed in units of square millimeters.

Quantitation of the sample lipid classes was effected by comparing the peak

area for each class with the standard curve for each lipid class derived from various quantities of standard spotted on the plate.

## RESULTS AND DISCUSSION

### *Role of unsaturation as a quenching factor*

Both unsaturated standard lipids and biological extracts could not be induced to fluoresce completely with Rhodamine 6G in the UV-range and, therefore, could not be quantitated without prior hydrogenation. Unsaturated lipid samples having a large free fatty acid fraction displayed a characteristic purple-blue ring at the periphery of this spot. Upon hydrogenation, this quenching ring disappeared (Fig. 1). Although this quenching ring was not so apparent visually in those lipid classes containing esterified unsaturated fatty acids (triglyceride, cholesteryl ester, and phospha-

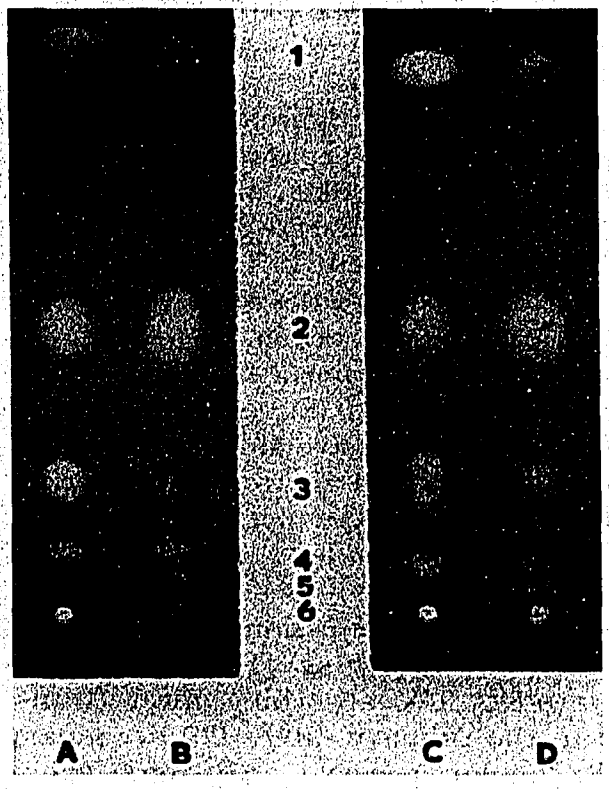


Fig. 1. Thin-layer chromatography of lipid classes in Solvent I before (B) and after (D) hydrogenation. Note presence of quenching ring in free fatty acid fraction prior to hydrogenation (arrow). A and C are saturated standard lipids. (1 = cholesteryl ester; 2 = triglyceride; 3 = free fatty acid; 4 = sterol; 5 = unidentified; 6 = origin, including phospholipid).

tidyl choline), quantitation of these classes was also impossible prior to hydrogenation. After hydrogenation, no difficulty was experienced in quantitation, and comparisons of gas–liquid chromatographic (GLC) analyses before and after hydrogenation showed that all unsaturated fatty acids had been saturated.

The mechanism by which unsaturation interferes with fluorescence from Rhodamine 6G is not known. It is possible that double bonds offer some steric hindrance to maximal association of Rhodamine dye with the lipid since, as the number of

TABLE I

RANGES FOR QUANTITATION OF LIPID CLASSES

| *Lipid* | *Class*[a] | *Limits of lower range (μg)*[b] | *Upper range (μg)* | *Coefficient of determination*[c] |
|---|---|---|---|---|
| 1,2,-Dipalmitoyl-*sn*-glycero-3-phosphoryl-choline | Phosphatidyl choline | 0.4–3.0 | 3.0–130.0 | 0.973 |
| Cholesterol | Sterol | 1.0–3.0 | 3.0– 70.0 | 0.986 |
| Stearic acid | Free fatty acid | 0.6–3.0 | 3.0– 70.0 | 0.980 |
| Glyceryl trioleate | Triglyceride | 0.6–3.0 | 3.0– 75.0 | 0.981 |
| Cholesteryl laurate | Cholesteryl ester | 0.6–3.0 | 3.0– 70.0 | 0.970 |

[a] Preliminary analyses have shown quantitation of other complex lipids such as phosphatidyl ethanolamine and sphingomyelin and hydrocarbons such as squalene to be feasible by the method.

[b] Although the standard curve passes through the origin on extrapolation, quantities below those stated cannot be detected.

[c] Values for coefficient of determination[13] represent the means from analyses of 23 standard curves. Due to limitations in the number of different concentrations of standard lipid that could be spotted on a single plate, these mean values were derived for various ranges. In no case did any individual value fall below 0.950.

double bonds increased, the purple quenching ring increased progressively in size. A saturated fatty acid such as stearic acid had no quench ring while mono-, di-, tri-, and tetraenoic acids (oleic, linoleic, linolenic, and arachidonic acids) had progressive larger ones. Although not a similar effect, NUTTER AND PRIVETT[6] demonstrated that unsaturation interfered with quantitation of lipid classes by charring and densitometry.

*Range of method*

Estimates of useful ranges of the method appear in Table I. The differences in the limits of the upper and lower ranges for various classes are a function of degree to which the lipid molecules can be induced to fluoresce in the UV-range with Rhodamine 6G (cholesterol was the least efficient in this respect), and the spot size which is influenced by migration distance and solubility in solvent. Any lipid classes with a spot size larger than 15 mm (the width of the window slit of the TLC scanner) had lower upper limits. This was a limiting factor in the free fatty acid, triglyceride, and cholesteryl ester classes.

*Accuracy and precision*

Accuracy of the method was determined by repeated analyses of aliquots of standard lipid and by comparison of fluorometric TLC and GLC quantitation of two aliquots of the free fatty acid fraction from chick liver homogenate. The accuracy was obtained by applying least squares analyses to sets of data points from twenty-three standard curves for each class over various ranges of concentrations. The predicted values as determined by least squares analyses were compared to actual values to obtain the residual errors for each lipid class. Accuracy of the method was then established by determining confidence limits[12] for the residual errors of each standard

TABLE II

MICROGRAM DEVIATIONS FROM MEANS OF REPLICATE ANALYSES OF PAIRED ALIQUOTS

| *Tissue* | *Lipid class* | | | | |
|---|---|---|---|---|---|
| | *Phosphatidyl choline* | *Cholesterol* | *Free fatty acid* | *Triglyceride* | *Cholesteryl ester* |
| Mouse fibroblasts | 0.0 (75.0)[a] | 0.5 (22.5) | 0.1 (10.5) | 0.2 (9.0) | 0.3 (6.3) |
| Chick fibroblasts | 0.0 (1.0) | 0.0 (3.4) | —[b] | — | — |
| Chick kidney epithelial cells | 0.8 (25.4) | 1.0 (19.0) | — | — | — |
| | — | 0.2 (2.8) | — | 0.5 (3.2) | — |
| Duck kidney epithelial cells | — | — | 0.0 (3.2) | 0.3 (3.6) | 0.2 (1.8) |
| Pigeon aorta cells | 0.3 (4.9) | 0.0 (3.0) | 0.2 (2.9) | 0.2 (3.8) | 0.1 (2.4) |
| | 0.1 (6.5) | — | 0.1 (4.4) | — | 0.1 (2.5) |
| Chick liver homogenate | — | 0.2 (25.2) | 0.5 (11.3) | — | 0.3 (6.9) |
| | — | — | 0.1 (4.3) | — | — |
| Mean of microgram deviations | 0.2 | 0.3 | 0.2 | 0.3 | 0.2 |

[a]Values represent differences of replicate analyses from the mean values which are given in parenthesis.

[b] Blank areas in the table for particular classes of some biological samples are due to different ratios of one class to another in various cells and tissues. In some cases minor components fell below the lower limit of detection, and large samples necessary to detect the minor classes caused major classes in these systems to be elevated above the upper limits of quantitation.

lipid class. Accuracy of the method was $\pm$ 0.2 $\mu$g for each lipid class at the 99.8% confidence level.

Fluorometric quantitation of the free fatty acid fraction of the tissue homogenate (25.4 $\mu$g) was within 4.9% of the GLC value (26.7 $\mu$g).

Replicate analyses of twenty-five paired aliquots representing five different biological systems appear in Table II. Confidence limits were established for the differences of replicate analyses from the mean values as an estimate of precision of the method[13]. The precision of the method between two replicate analyses was $\pm$ 0.2 $\mu$g from the mean value at the 99.5% confidence level. As was reported by ZÜRCHER *et al.*[10] and PATAKI[11], accuracy and precision of any direct quantitative method are entirely dependent on standardizing such factors as: application of substance; chromatography; developing distance; drying; scanning of the chromatogram; and spraying to saturation for uniform background. Such factors were strictly controlled in this method.

*Recovery data*

Recoveries of different quantities of standards of each of the five lipid classes added to lipid extracts from six different biological systems are shown in Table III. The per cent recoveries for this method are similar to those reported by BACHORIK AND ROGERS[14] for the direct fluorometric assay of conjugated bile acids.

The results summarized in this paper demonstrate the applicability of *in situ*

TABLE III

RECOVERIES OF STANDARD LIPIDS ADDED TO VARIOUS QUANTITIES OF BIOLOGICAL LIPID EXTRACTS

| *Lipid class* | *No. of analyses* | *Amount of standard lipid added (μg)* | *Amount of biological lipid (μg)*[a] | *Mean percentage recovery of standard lipid* | *Range of percentage recoveries of standard lipid* |
|---|---|---|---|---|---|
| Phosphatidyl choline | 12 | 1.0–30.0 | 3.0–26.0 | 103.5 | 92.5–113.0 |
| Sterol | 22 | 1.0–36.0 | 3.0–56.0 | 100.5 | 86.0–112.0 |
| Free fatty acid | 23 | 1.0–36.0 | 0.5–12.0 | 98.8 | 85.0–116.0 |
| Triglyceride | 15 | 1.0–30.0 | 3.0–13.0 | 96.3 | 82.0–113.3 |
| Cholesteryl ester | 14 | 1.0–30.0 | 1.0– 7.0 | 101.4 | 89.0–112.3 |

[a] Lipid extracts from all sources used to determine precision were employed.

fluorometry to lipid class analyses. In addition to the five classes described herein, preliminary work suggests a much wider applicability of the method to any lipid class that can be induced to fluoresce with Rhodamine 6G in the UV-range. Reported analyses of phospholipids by TLC and densitometry[6] conflict with the findings of BIEZINSKI *et al.*[5] who were unable to quantitate these lipids. However, the method described here can quantitate at least one class of phospholipid.

A recent report by ADAMS AND SALLEE[15] describes the relative quantitation of six neutral lipid and nine phospholipid classes by TLC and densitometry. Ranges of their method are similar to these described herein, but no data are presented to show adaptability of the procedure to absolute quantitation.

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