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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF NEUTRAL LIPIDS USING SEMI-SPECIFIC COLORIMETRIC AND TITRIMETRIC TECHNIQUES*

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SUMMARY

Quantitative thin-layer chromatography of standard and tissue neutral lipids was accomplished by eluting the separated lipids from the silica gel and utilizing semi-specific colorimetric and titrimetric methods. Reproducibility was good and recoveries of the various neutral lipids were between 94–101 %. Silica gel blank readings were reduced to low values by special washing of the silica gel.

INTRODUCTION

Quantitative thin-layer chromatography of neutral lipids has received considerable attention because of the speed and ease with which small samples can be separated[1–12]. Most of these quantitative methods are based on densitometric or gravimetric techniques[1–8]. Although these are relatively simple and accurate methods, they depend on both the complete conversion of lipid spots to elemental carbon and the precise separation of various lipid groups in order to avoid measuring two or more lipid substances in a given spot.

Additional quantitative methods have been proposed in which the separated lipids are first eluted from the absorbent and subsequently measured by spectroscopic means[9–12]. The use of individual chemical reactions for each group of neutral lipids should make their quantification more specific[7,10,11]. Most reports utilizing elution and spectroscopic techniques have not been published primarily to describe methodology. They therefore have only been concerned with quantifying a single specific neutral lipid[13–15]. Even in the two investigations concerned with quantitative methodology, not all the neutral lipids were studied and the recovery of some lipids was not complete[10,11]. In addition, no information was given regarding the range of various amounts of each neutral lipid that could be quantified. Further, no studies have systematically attempted to compare the thin-layer quantitative methods with silicic acid column chromatography techniques.

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The present study delineates a method utilizing previously described semi-specific colorimetric and titrimetric determinations of free and esterified cholesterol, free fatty acids, and triglycerides after they have been separated and eluted from thin-layer chromatoplates. Recoveries of 94–101 % were achieved over a range of 25–200 γ of each neutral lipid. These quantitative analyses by thin-layer chromatography were found to agree closely with silicic acid column chromatography techniques.

MATERIALS AND METHODS

Reagents

The absorbent for thin-layer chromatography was Silica Gel H[a]. Silicic acid (100 mesh) for column chromatography was obtained from Mallinckrodt Chemical Company[b]. The solvents were reagent grade and all except glacial acetic acid and 95 % ethanol were redistilled in glass prior to use[c].

Standards and tissue lipids

Neutral lipid standards were used individually and in various combinations to evaluate their recovery from the chromatoplates. Cholesterol esters from Applied Science Labs, Inc.[d] included cholesteryl myristate, palmitate, stearate, oleate and linoleate. Triolein and tripalmitate were purchased from Eastman Organic Chemicals[e] and palmitic, oleic, linoleic and stearic acids were obtained from Mann Research Labs, Inc.[f]. All standards were found to be at least 98 % pure by thin-layer or gas–liquid chromatography.

A mixture of reference neutral lipids[g] (Model Mixture No. 1) was used to identify the positions of the aforementioned neutral lipid standards or mixed tissue lipids with each chromatographic run. The mixture contained approximately equal weights of cholesterol, cholesterol oleate, triolein, oleic acid and methyl oleate. With the solvent system employed, methyl oleate and triolein were not separated (see Fig. 1).

Mixed tissue lipids were extracted from experimentally produced rabbit skin xanthomas with chloroform–methanol (2 : 1, v/v), as previously described[16]. Non-lipid impurities were removed by the method of FOLCH, LEES AND SLOANE-STANLEY[17]. The neutral lipids obtained were quantified by the thin-layer chromatography methods described and the results compared with an established quantitative technique utilizing silicic acid column chromatography[18].

Preparative washing of Silica Gel H

250 g of Silica Gel H was placed in a porcelain Buchner filter funnel (Coors No. 4) fitted with two disks of Whatman No. 2 filter paper. The funnel was placed on a 4 l filter flask and the following wash solutions were slowly poured over the absorbent

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[a] Brinkmann Instruments, Inc., Burlingame, Calif.
[b] Mallinckrodt Chemical Company, New York City, N.Y.
[c] Baker & Adamson, General Chemical Division, Morristown, N.J., Merck & Co., Inc., Rahway, N.J.
[d] Applied Science Labs., Inc., State College, Pa.
[e] Eastman Organic Chemicals, Rochester, N.Y.
[f] Mann Research Labs, Inc., New York City, N.Y.
[g] Hormel Foundation, Austin, Minn.

with suction applied: 0.5 l hexane–diethyl ether–glacial acetic acid (90:30:2, v/v/v); 1 l methanol–chloroform–formic acid (2:1:1, v/v/v); 0.5 l diethyl ether; 1 l methanol–chloroform–formic acid (2:1:1, v/v/v); and 2 l of glass-distilled water. Suction was continued until water no longer dripped through the funnel. The washed silica gel was then spread in a pyrex tray lined and covered with aluminum foil and dried at 110° for 48 h. Any large silica gel lumps were broken up after the first 24 h of drying.

Preparation of plates

36 g of washed silica gel was placed in a Waring Blendor (Model No. PB-5A with high and low speed controls) containing 64 ml of glass-distilled water. The resultant slurry was mixed at low speed for 3 min. Mixing for longer periods of time or at higher speed resulted in slurries with thin consistency which prevented optimal spreading on the chromatoplates.

This slurry was usually sufficient to coat five 20 × 20 cm pyrex plates with a 0.25 mm layer of silica gel using the Desaga adjustable spreader. The plates were first air dried and then activated for 1 h at 110° just prior to use.

Chromatographic procedures

Neutral lipid standards or the experimental xanthoma lipids were applied in chloroform solution with a microliter syringe* as narrow streaks, $^3/_4$ in. from the

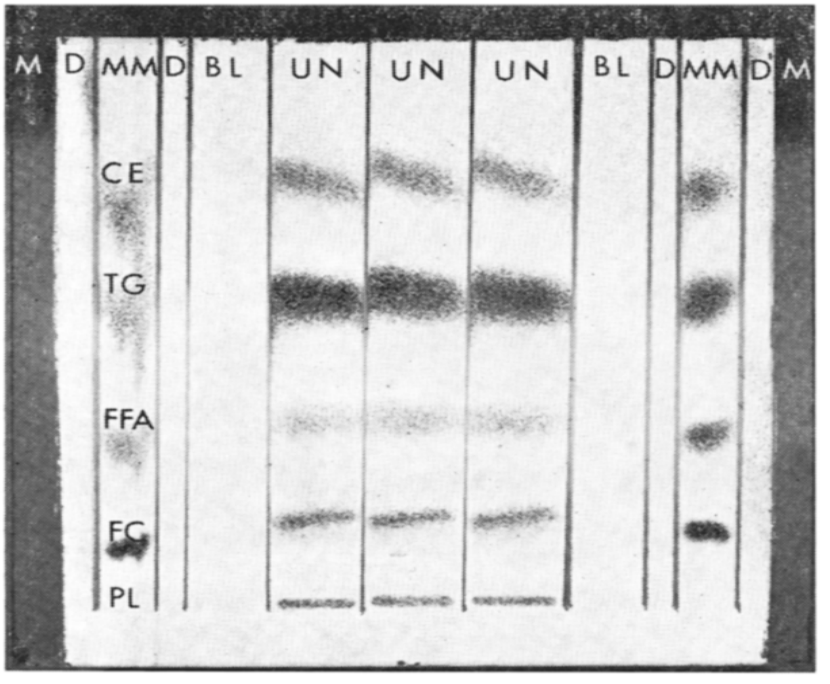

Fig. 1. A picture of the chromatographic separation of experimental rabbit xanthoma (three middle lanes marked "UN") and reference model mixtures (outer $^1/_2$-in. wide lanes marked "MM") shown to display the arrangement of the various lanes discussed in the text. Solvent used was hexane–ether–acetic acid (90:30:2). Detection for this picture was with sulfuric acid spray and charring. Lanes "BL" are blanks, and "D" are divider spaces. "M" is the margin of silica gel scraped from the sides and top of the chromatoplate to allow the teflon spacer of the saturation chamber to fit tightly against it. The streaked samples of rabbit xanthoma and the model mixtures are separated into the major neutral lipids: CE = cholesterol ester; TG = triglyceride; FFA = free fatty acids; and FC = free cholesterol. Phospholipids, PL, are seen in the xanthomas at theorigin.

* Hamilton Co., Inc., Whittier, Calif.

bottom of the plate, on three 1-in. wide lanes in the center of the chromatoplate (Fig. 1, lanes marked "UN"). On either side of the three middle lanes, 1-in. wide lanes served as blanks (Fig. 1, "BL"). Outside the blanks were strips of $^1/_4$, $^1/_2$ and $^1/_4$ in. in width. To the $^1/_2$-in. lanes a sample of the reference neutral lipid model mixture was applied (Fig. 1, "MM") to assist in identifying the positions of the various neutral lipids separated in the central lanes. The $^1/_4$-in. lanes on each side of the model mixture reference lanes were divider spaces (Fig. 1, "D").

The plates were developed in a saturation chamber described by PARKER AND PETERSON[19]. A $^1/_2$-in. wide, 3-sided teflon spacer was placed between the chromatoplate and a similarly sized glass cover plate lined with a piece of filter paper. The whole unit was held together with metal clips. To allow the teflon spacer to fit snugly against the chromatoplate, a $^1/_2$-in. wide margin of silica gel was scraped from the sides and top (Fig. 1, "M").

The saturation chamber was placed in a glass covered tank (27 × 29 × 10 cm) containing 183 ml of hexane–diethyl ether–glacial acetic acid (90:30:2, v/v/v). The solvent was allowed to rise to the top of the adsorbent with an average running time of 30 min.

Detection of chromatographed lipid spots

Since iodine vapors and other indicators such as Rhodamine G and fluorescein interfered with the triglyceride and fatty acid determinations they were not used. Instead, the central lanes, blanks, and inner divider spaces were covered with three layers of paper towels leaving only the outer "MM" and divider spaces exposed. Weights were placed on the edges of the towels and small amounts of concentrated sulfuric acid were sprayed on the uncovered lanes. After removing the towels, the sprayed lanes containing the lipid model mixture were charred with a Fisher microburner to identify the various neutral lipids. Horizontal lines were then drawn across the three center lanes and blanks in positions corresponding to the upper and lower poles of the triglyceride and free fatty acid spots. These areas were scraped individually with a glass slide into appropriate containers after the sulfuric acid sprayed lanes on one side of the plate were carefully wiped off the glass. This prevented sulfuric acid contamination of the scraped silica gel spots of the central lanes from otherwise displaying spurious fatty acid titrations.

The partially scraped chromatoplate was then exposed to iodine vapors to locate the positions of the remaining free and esterified cholesterol spots and to check on the completeness with which the triglyceride and free fatty acid spots were removed. After the iodine had vaporized, the free and esterified cholesterol and their appropriate blanks were scraped into individual receptacles.

Analysis of individual neutral lipids

Cholesterol esters. These compounds (25–200 γ) with their corresponding silica gel blanks were scraped into 20 ml culture tubes (15 × 150 mm). One ml of 1.0 *N* (5.6 %) KOH in 95 % ethanol (made fresh just prior to use) was added to the tubes which were covered with glass marbles and incubated at 80–85° in a constant temperature water bath for 1 h. The water level in the bath was maintained at the same height as the level of the liquid in the tubes to allow adequate reflux. After cooling, 3 ml of hexane was added, followed by 1.5 ml of distilled water. The tubes were shaken

for 20 sec after capping with polyethylene stoppers (size 0). Appropriate samples (usually 1 or 2 ml) were taken from the hexane layer, dried completely under nitrogen, and quantified by the ferric chloride method of COURCHAINE *et al.*[20], using the directions for total cholesterol determinations. Free cholesterol standards (25–200 γ) were carried through the entire procedure. Results were expressed as the amount of cholesterol in the cholesterol esters.

Free cholesterol. Four ml of glacial acetic acid were added to the samples and blanks, after they were scraped into 20 ml culture tubes, as well as to reagent blanks and standards of free cholesterol (over a range of 25–200 γ). The tubes were mixed on a Vortex Mixer, centrifuged for 10 min at 300 $\times$ g and 3 ml of the acid were taken for quantification using the COURCHAINE[20] method for total cholesterol determination.

Free fatty acids. The gel containing these lipids and the corresponding blanks were scraped into 12 ml conical centrifuge tubes. The fatty acids were eluted from the gel with 4 ml ether–methanol (9:1, v/v), followed by two separate 3 ml portions of chloroform. After the addition of each eluent, the tubes were covered with polyethylene stoppers, shaken 20 sec, and centrifuged for 5 min at 300 $\times$ g. The supernatants were entirely removed with a volumetric pipette without disturbing the silica gel and combined in 15 ml conical centrifuge tubes (without graduations) and dried under nitrogen. After twice rinsing the sides of the tube with small volumes of hexane, the fatty acids were quantified by the titration method of DOLE[21]. Palmitic acid was used as a standard over the range of 25–200 γ.

Triglycerides. These compounds and their corresponding blanks were scraped into glass funnels specially made by fusing a fritted glass funnel (porosity M, 2 ml capacity, 10 mm diameter disk) to a Kimax funnel. The lipid was eluted into 20 ml culture tubes (15 $\times$ 150 mm) with two portions of chloroform, 2.5 ml, and then two additional portions of diethyl ether (2.0 ml). These eluents were pushed through the silica gel in the funnel by nitrogen delivered through a rubber stopper perforated in the center by glass tubing. The combined eluents were dried under nitrogen and the sides of the tubes were rinsed twice with small volumes of ether. All samples, reagent and silica gel blanks, and standards (25–125 γ) where hydrolyzed by adding 1 ml of 0.4 % KOH in 95 % ethanol (made just prior to use), covering the tubes with glass marbles and heating for 30 min at 70–80° in a constant temperature bath[22]. The water level in the bath was kept at the same height as the level of liquid in the tubes for adequate reflux. After hydrolysis, 0.5 ml of 0.2 N H_2SO_4 was added and the samples stirred on a Vortex Mixer and placed in a boiling water bath for 15 min to remove the alcohol. Glycerol was then analyzed[22,23].

The optical density or titrimetric determinations obtained from the appropriate silica gel blanks were always subtracted from the readings derived from each lipid spot.

RESULTS

Quantification of neutral lipid standards is displayed in Figs. 2–5. For each there is a linear relationship between amounts of lipid used and optical density (or in the case of free fatty acids, μmoles of NaOH). This applies when the standards are placed either directly in test tubes (solid line) or are chromatographed (dotted line) and then quantified. The exact optical density readings are given in the inset tables accompanying each figure.

The silica gel blanks referred to Figs. 2–5 represent the optical density or titrimetic readings greater than that of the reagent blank readings. The optical density of silica gel blanks for free and esterified cholesterol average 0.003 ± 0.002 (Figs. 2, 3) which is the equivalent of 2–3 γ of cholesterol, while the triglyceride blanks average 0.030 ± 0.007 (Fig. 4) which represents 4–5 γ of glycerol. Similarly, the free fatty acid silica gel blanks correspond to no more than 5 γ of free fatty acid.

Neutral lipids extracted from experimentally induced cutaneous xanthoma were quantified by thin-layer chromatography in order to test the method on a mixture of unknown tissue lipids. The results obtained were compared with analysis of the lipids from another portion of the same extract after separation by silicic acid

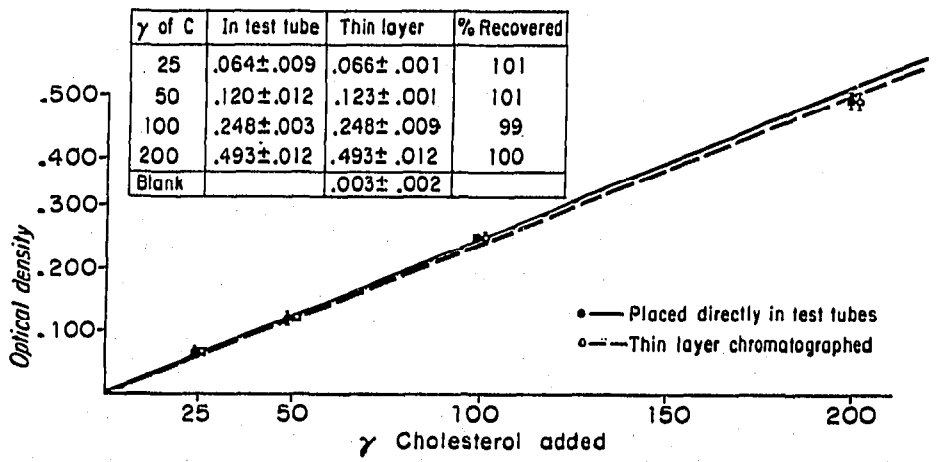

Fig. 2. When optical density readings shown in the table were plotted against increasing concentrations of free cholesterol standard, a straight line was obtained whether the colorimetric reaction was performed on the standard placed directly in test tubes (solid lines) or chromatographed (dashed line). The bars at each point represent one standard deviation and the points represent the mean of four runs. The two lines were almost superimposable reflecting the recoveries of 99–101% shown in Table I. The silica gel blank optical density reading (mean and standard deviation from 20 lanes) was small, but has been deducted from the chromatographed standards' readings before the values in the table and graph were used.

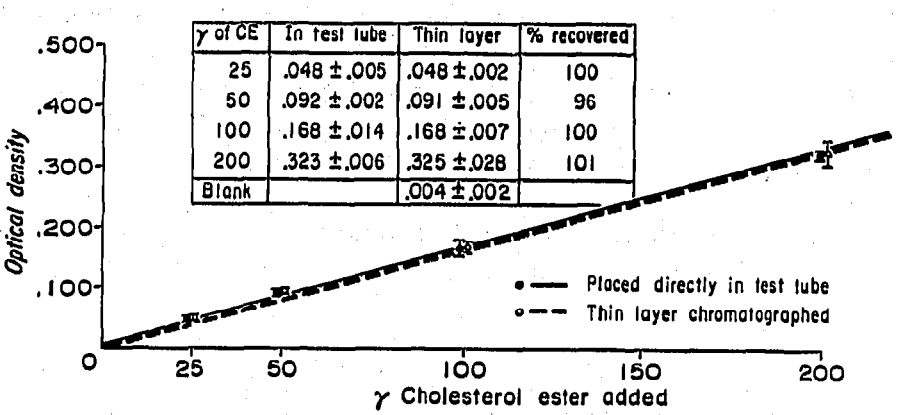

Fig. 3. A mixture of approximately equal amounts of cholesterol myristate, palmitate, stearate, oleate and linoleate was used to display satisfactory recovery of cholesterol esters. On the graph the points were the average of four runs and the bars represent one standard deviation. The linear relationship between concentration of the cholesterol ester standards added and optical density readings were plotted according to the figures given in the table. The silica gel blank optical density reading was small and the mean (20 determinations) has been deducted from the chromatographed standards' readings before inclusion in the table or graph.

column chromatography. Table I summarizes these results. As can be seen, there is close agreement between the two methods.

TABLE I

COMPARISON OF THE QUANTITATIVE THIN-LAYER CHROMATOGRAPHY METHOD TO THAT OF SILICIC ACID COLUMN CHROMATOGRAPHY[a]

Method of chromatography	*Free cholesterol (mg/ml of xanthoma lipid sample)*	*Cholesterol ester (mg/ml of xanthoma lipid sample) (as free cholesterol)*	*FFA (μg/ml of xanthoma lipid sample)*	*TG (mg/ml of xanthoma lipid sample) (as glycerol)*
Thin-layer	0.917 ± 0.030	0.539 ± 0.049	152.0 ± 12.4	15.19 ± 0.30
Column	0.886 ± 0.044	0.573 ± 0.011	161.0 ± 12.2	15.62 ± 0.26
% Recovery[b]	103%	94%	94%	97%

[a] Results of one experiment with each lipid determination run in quadruplicate.
[b] Amount of lipid recovered by thin-layer chromatography when compared with recovery of lipid from the same sample after silicic acid column chromatography.

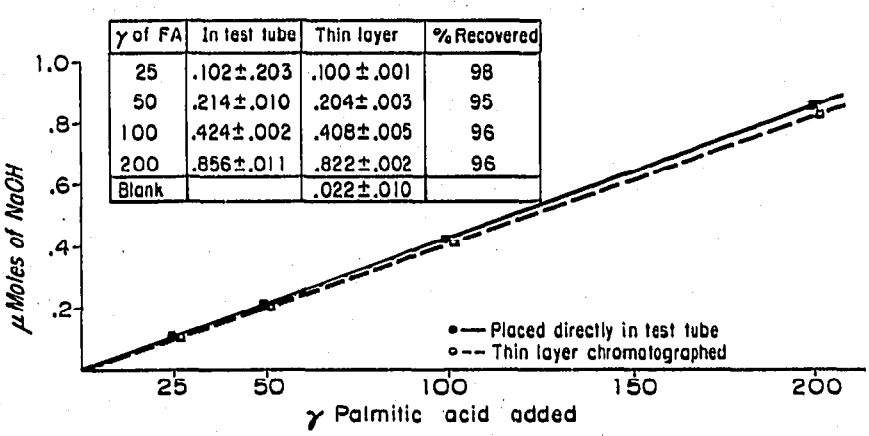

Fig. 4. Recovery of free palmitic acid from chromatoplates was depicted in this graph and table representing the average and one standard deviation of four runs. Between 96–98% recovery was achieved when 25–200 γ of free fatty acid was analyzed. The silica gel blank was the average of 20 determinations.

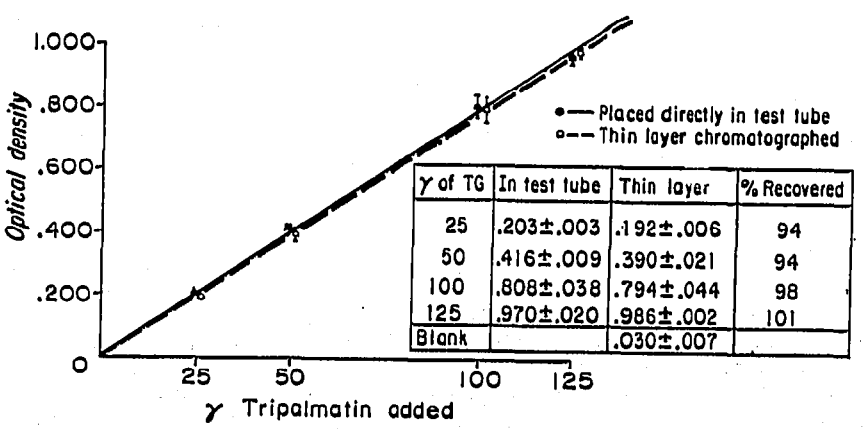

Fig. 5. Tripalmitin recovery from thin-layer plates was 94–101% when 25–125 γ of the standard was analyzed. The graph and table represent the mean and one standard deviation of six runs. The silica gel blank was an average of 20 determinations.

DISCUSSION

Quantitative thin-layer chromatography is particularly useful in dealing with small samples of lipid where laborious biochemical and chromatographic separatory methods are best avoided. The present method provides a means for precise quantification of 25–200 μg of each major group of neutral lipid. The results of this thin-layer method are comparable to those obtained by silicic acid column chromatography.

Elution of the separated neutral lipids avoids the need for special densitometry equipment. Subsequent quantification utilizing semi-specific colorimetric and titrimetric techniques assures measurement of a given lipid even when two or more lipids may reside in a given spot.

Complete elution of the various neutral lipids was achieved with a variety of solvents and methods. Free cholesterol was removed from the gel with glacial acetic acid, while esterified cholesterol was first hydrolyzed and the resulting free cholesterol was extracted with hexane. It was necessary to saponify the cholesterol esters prior to using COURCHAINE's method since cholesterol stearate was not completely soluble in the glacial acetic acid. Elution of free fatty acids required sequential shaking with mixtures of ether, methanol and chloroform. Triglycerides seemed particularly difficult to elute and required the use of simply constructed funnels to hold the silica gel samples while chloroform and ether were allowed to trickle through the funnel. Using this method, recoveries of 94–101 % were achieved. When similar eluents were shaken with the same triglyceride standards on silica gel in tubes, recoveries of only 85–92 % were achieved. These latter recovery figures are similar to those reported by GLOSTER AND FLETCHER[11].

Care must be given to the saponification procedures used for cholesterol esters and triglycerides. Complete hydrolysis depended on maintaining the original volume of the KOH–ethanol solution added to the tubes. If evaporation occurred, hydrolysis was incomplete and results were erratic. To prevent evaporation, long tubes were used (15 × 150 mm culture tubes) and the depth of the water bath was maintained at the same height as the top of the KOH–ethanol solution to allow maximal reflux within the containers.

This method has proved of value to date in accurately analyzing neutral lipids obtained from rabbit and human sera, xanthoma and blood vessels.

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