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A METHOD FOR MICRODETERMINATION OF MAJOR NEUTRAL LIPIDS AND AN APPLICATION OF THE PROCEDURE TO TISSUE LIPIDS

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Abstract: Cholesterol, cholesteryl esters, triglycerides and fatty acids as major neutral lipids and phospholipids were examined in quantitative analysis. The method consisted of three steps: (1) separation of lipids by one-dimensional thin-layer chromatography on silica gel plates; (2) elution of neutral lipids from scraped silica gel with chloroform-methanol (4 : 1); and (3) colorimetric determination of individual neutral lipids in eluates and phospholipids in silica gel. The conditions were modified for chromotropic acid reaction for determining triglycerides. Laurell's method for determining fatty acids was also modified to apply to quantitative thin-layer chromatography. The accuracy of the modified methods was well-defined as the absorbance values were on a linear curve. A quantitative study was made of the recovery of triglycerides and fatty acids after chromatography. Combining these modified methods and colorimetry for determination of cholesterol, cholesteryl esters and phospholipids, the author established a micromethod for determining the major neutral lipids and phospholipids by thin-layer chromatography. Lipids from HeLa, S-3 cells were analyzed to examine the applicability of this method to tissues. The results indicated that the new method permitted a reliable estimation of the major neutral lipids and phospholipids from small amounts of tissues.

Thin-layer chromatography (TLC) is often used to determine neutral lipids. Photodensitometry (1, 2), lipid oxidation in acidic dichromate solution (3, 4) or hydroxaminic acid colorimetric test for ester groups (5, 6) are simple and applicable to small amounts of materials, but these procedures are not specific for each lipid class.

Skipski *et al.* (7) made an extensive study of the separation and quantification of neutral lipids. These investigators devised a one-dimensional, two-step development system for an accurate separation of minor lipids, as well as major ones. Combining the two-step system and quantitative methods including colorimetry, titration and infrared spectrophotometry (8), Skipski *et al.* (9) extended the range of lipid classesde terminable by the specific method.

This method was successfully applied to obtain precise data on lipid composition of rat liver plasma membranes (10). Although a quantitative TLC method for neutral lipids was established (9, 10), this method had various shortcomings. The main shortcoming was the inability to measure small quantities of lipids (at least 10 mg of lipids were needed for determination). Another disadvantage was that it required time-consuming and complicated steps of analysis.

It appeared worthwhile to develop a specific method which is simple and capable of determining neutral lipids on relatively small amounts of sample material, especially in the analysis of lipids of tissue culture cells, plasma membranes or other organelles. The author has already reported on a microdetermination method by TLC of cholesterol, its esters and phospholipids (11). Among other major neutral lipids are triglycerides and fatty acids. As a quantitative method for triglycerides, Van Handel introduced the chromotropic acid method (12, 13). Laurell and Tibbling (14) determined free fatty acids in serum by the fatty acid-cupric soap method. The present paper describes a new method for determining triglycerides and fatty acids by TLC. Furthermore, examinations were conducted on establishing of a method for microdetermination of major neutral lipids. An application of the method to lipids from HeLa, S-3 cells is also described.

MATERIALS AND METHODS

Reagents: All solvents used were of reagent grade. They were purchased from Wako Pure Chemical Industry, Ltd. and Katayama Chemical Industries, Co., Ltd. Silica gel G containing 13% $CaSO_4$ (E. Merck AG, Darmstadt) was used as adsorbent throughout the study. Chromotropic acid disodium salt and 2-(2-thiazolylazo)-p-cresol were of reagent grade and were obtained from Dojin Laboratories. All reference lipids were of reagent grade. Cholesterol from Wako Pure Chemical Industry, Ltd. was further purified by recrystallization from hot ethanol. Triolein from P. L. Biochemicals, Inc. and oleic acid from Applied Science Laboratory, Inc. were found to be pure by TLC.

Preparation of tissue lipids: HeLa, S-3 cells were grown in monolayer culture in Eagle's minimum essential medium supplemented with 5% calf serum. Cells weighing about 300 mg (wet wt.) were harvested at the confluent stage and washed with phosphate buffered saline soluiton by centrifugation. Lipid was extracted by stirring in 10 ml of chloroform-methanol (2:1, v/v) at room temperature. The crude lipid extracts were washed with 20 vol. of 0.05% NaCl as described by Folch *et al.* (15).

Thin-layer chromatography: Thin-layer chromatoplates were prepared as described previously (11). After activation, they were prewashed with chloroform-methanol (2:1, v/v) by allowing the solvent to run overnight or longer

and were reactivated prior to use. Quantitative applications of lipid samples to chromatoplates were carried out by transferring the preweighed samples with a capillary. The plates were developed with the solvent system composed of petroleum ether (30-60°C b.p.), diethyl ether and acetic acid in a volume ratio of 82 : 18 : 2. Spots on chromatograms were visualized with iodine vapor. Elution of neutral lipids from silica gel was performed as follows. The tubes containing silica gel were rotated mechanically, followed by centrifugation at 3000 rev./min (Hitachi centrifuge 03P). Silica gel containing phospholipids was scraped into a Kjeldahl flask and was calcified. The details of all these procedures were described in the previous report (11).

Colorimetric determination of triglycerides: Triglycerides were determined by the modified method of Van Handel and Zilversmit (12). The principle of the method is the same as that of the original method, which utilizes the color development between chromotropic acid and formaldehyde produced by periodate oxidation of glycerin.

Reagents. (A) Alcoholic potassium hydroxide solution; 2.5g/dl KOH-ethanol (1 : 19, v/v) was prepared immediately before use. (B) 0.025 M $NaIO_4$. (C) 5g/dl $NaHSO_3$; the solution was stored in a cool, dark place and used within three days. (D) Chromotropic acid reagent; 0.2g of chromotropic acid (disodium salt) was dissolved in 50 ml of distilled water and 60 ml of reagent grade sulfuric acid (Wako Pure Chemical Industry, Ltd.) was added. To prevent a temperature rise in the mixture, sulfuric acid was added slowly, while stirring the mixture in an ice bath. The solution was stored in a brown bottle at 0-4°C. It was prepared fresh every one to two weeks.

Procedure. One ml of alcoholic potassium hydroxide solution was added to glass-stoppered tubes containing dried triglycerides. The tubes were well shaken and incubated in a water bath at 60°C for 15 min. After cooling to room temperature, 0.2 ml of 1 N H_2SO_4 was added and mixed well. The remaining water and ethanol were evaporated off on a rotary evaporator. The evaporating procedure was ceased immediately upon complete evaporation, since prolongation led to remarkable losses of glycerin. One ml of distilled water was added and the oxidation of glycerin to formaldehyde was initiated by the addition of one drop of $NaIO_4$ solution. After incubation in the dark for exactly 10 min at room temperature, the reaction was stopped by addition of one drop of $NaHSO_3$ solution. The tubes were left for 5 min, and 4 ml of chromotropic acid reagent was added. The tubes were stoppered and allowed to stand in boiling water bath for 30 min, protected from direct exposure to light. Absorbance was read against a blank at 570 nm.

Colorimetric determination of fatty acids: The method of Laurell modified by Maehata and Naka (16) was used for determination of fatty acids with slight modifications to accomodate quantitative TLC. The original method is based on fatty acid conversion to copper soaps, followed by colorimetric determination of the metals.

Reagents. (A) Solvent; Chloroform-heptane (4 : 3, v/v). (B) Cu-TEA solu-

tion; 10 ml of 1 M Cu $(NO_3)_2 \cdot 3H_2O$ were added to 5 ml of triethanolamine, and the mixture was immediately shaken vigorously to avoid turbidity. The solution was diluted to 100 ml with saturated NaCl solution. The pH was adjusted to 8.2-8.3 with 1 N NaOH. The solution was stored in the dark. (C) TAC solution; 10 mg of 2-(2-thiazolylazo)-p-cresol was dissolved in 100 ml of ethanol. Precipitates were removed by filtration. The solution was stored in a brown glass-stoppered reagent bottle. It was stable for at least a month.

Procedure. Three ml of the solvent and 2.0 ml of Cu-TEA solution were added to glass-stoppered test tubes containing dried fatty acids. The tubes were immediately capped tightly with glass bulbs, shaken mechanically for 30 min and centrifuged at 3000 rev./min for 10 min (Hitachi centrifuge 03P). Two ml of upper phase were transferred to another tube containing TAC solution. After a few minutes, absorbance was read against a blank at 610 nm.

Other colorimetric determinations: Cholesterol was determined by the method of Zak *et al.* (17) with the color reagent of Rosenthal *et al.* (18). Cholesteryl esters were subjected to hydrolysis by ethanol-KOH and were determined in the same manner as cholesterol. The details of the method were described in the previous report (11). Phospholipids were determined by the method of Allen modified by Kates *et al.* (19). Lipid phosphorus was multiplied by 25.

All glassware was placed in chromic acid mixture and then in conc. HCl and rinsed well with distilled water.

RESULTS

Chromatographic separation: Fig. 1 shows the chromatographic separation of reference lipids and the total lipid extracts from HeLa, S-3 cells. The chromatography shows good resolutions of cholesteryl esters, triglycerides, fatty acids, cholesterol and phospholipids. All these lipid classes were not sufficiently separated that they could be individually collected. Monoglycerides and phospholipids were not distinctly separated. Cholesteryl esters were considered to contain hydrocarbons according to the system of Malins and Mangold (20) and Skipski *et al.* (7). The position of diglycerides was not determined on chromatogram, but the lower two spots observed in the application of triolein were derived from hydrolysis of triolein during storage, indicating that diglycerides were above or very close to cholesterol. However, hydrocarbons, diglycerides and monoglycerides were not included in this chemical analysis, and the colorimetry used was specific for cholesterol or phospholipids. Therefore, it was concluded that good separation for determining the above lipids could be achieved by the development system used in the present study.

Calibration curves of triglycerides: Calibration curves of triglycerides are given in Fig. 2. Linearity was obtained from 0 to 0.15 mg of triolein. In

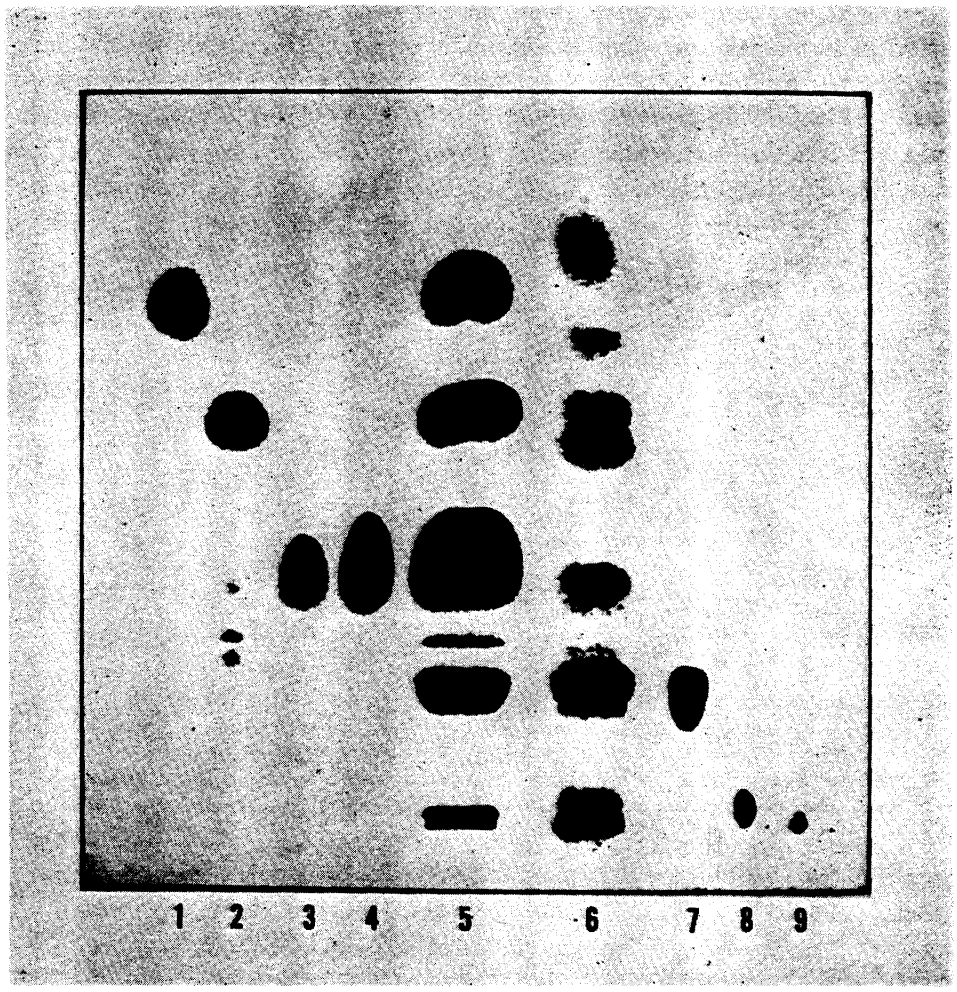

Fig. 1. Separation of neutral lipids by thin-layer chromatography performed on reference compounds and total lipid extract from HeLa, S-3 cells. 1, cholesteryl stearate; 2, triolein; 3, palmitic acid; 4, oleic acid; 5, mixture of 1-4 and 7-9; 6, total lipid extract from HeLa, S-3 cells; 7, cholesterol; 8, monoolein; 9, phosphatidylcholine. A total of 1 mg of lipid from cells was applied. The developing solvent was a mixture of petroleum ether (b. p. 30-60°C)-diethyl ether-acetic acid, 82 : 18 : 2 (v/v/v) (11). Detection of spots was by charring with sulfuric acid spray.

addition, the calibration curve of triolein determined after TLC was in good agreement with that directly determined. To diminish errors due to distillation of ethanol from glycerin, the reaction mixture was evaporated to dryness on a rotary evaporator. As Van Handel and Zilversmit (12) indicated, direct evaporation caused remarkable losses of glycerin. The addition of 0.2 ml of

1 N H_2SO_4 to the mixture prevented the loss of glycerin. Table 1 shows the effect of evaporation time on the amount of glycerin present in the residue. After the addition of 0.2 ml of 1 N H_2SO_4, loss of glycerin due to evaporation was negligible. The addition of 0.2 ml of water, on the contrary, resulted in a remarkable loss in the yield of glycerin. The concentration of $NaIO_4$ and the suitability of $NaHSO_3$ as an inhibitor of $NaIO_4$ were already

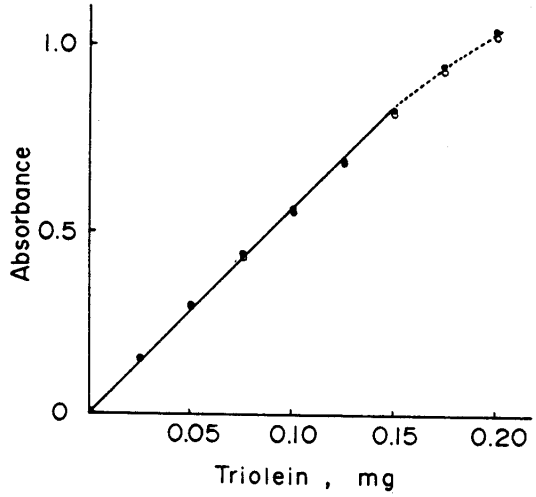

Fig. 2. Calibration curve for triolein. ●——●, triolein determined before chromatography; ○——○, triolein determined after chromatography. Absorbance measurement was carried out as described under Materials and Methods.

TABLE 1 THE EFFECT OF EVAPORATING TIME ON AMOUNTS OF GLYCERIN IN THE RESIDUE

Evaporation time (min)	Absorbance at 570 nm					
	Exp. 1			Exp. 2		
	1	2	Mean	1	2	Mean
7.0	0.543	0.554	0.549	0.522	0.527	0.525
8.5	0.562	0.581	0.572	0.530	0.534	0.532
10.0	0.548	0.539	0.544	0.561	0.553	0.557
12.5	0.576	0.574	0.575	0.535	0.563	0.549
15.0	0.564	0.537	0.551	0.518	0.562	0.540
30.0				0.560	0.528	0.544
Mean			0.558±0.017			0.541±0.016

0.1 mg of triolein was used. After saponification of triolein, evaporations were at 20°C. Absorbance values represent amounts of glycerin in the residues. Absorbance measurement was carried out as described under Materials and Methods. Two experiments were carried out on duplicate samples.

discussed by other investigators (21, 22).

Calibration curves of fatty acids: Fig. 3 shows the calibration curve of oleic acid. In the range of 0-0.04 mg of oleic acid, linearity was clear. Moreover, the calibration curve of oleic acid which was applied to TLC with

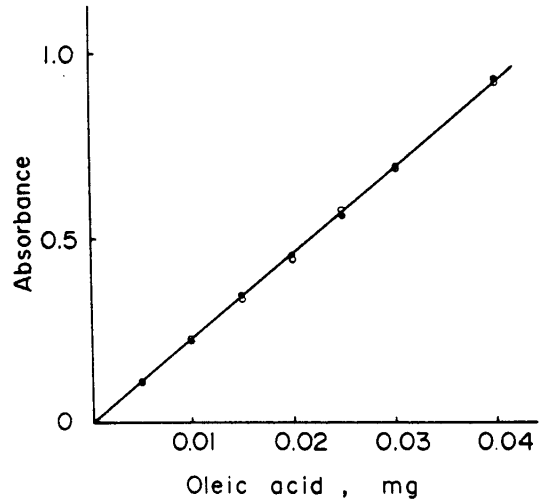

Fig. 3. Calibration curve for oleic acid. ●——●, oleic acid determined before chromatography; ○——○, oleic acid determined after chromatography. Absorbance was measured as described under Materials and Methods.

subsequent elution also showed a linear relationship and was identical with that obtained from direct determinations. A vortex-mixer or hand shaking was often used to mix Cu-TEA solution with the solvent. However, under these conditions occasional failure to achieve complete mixing resulted in low values. Therefore, mechanical shaking (Mono-shin, Taiyo Kagaku Kogyo, Co., Ltd.) was employed. The effect of time on the formation of Cu-fatty acid complex was studied (Table 2) and 30 min was chosen for the shaking time. The error range in the calibration curve was within 5%. The calibration curve prepared with palmitic acid did not differ significantly from that with oleic acid of equimolarity with palmitic acid, whether applied to TLC or not.

TABLE 2 THE EFFECT OF TIME ON THE FORMATION OF Cu-FATTY ACID COMPLEX FOR COLORIMETRIC DETERMINATION OF FATTY ACIDS

Time (min)	Absorbance	
	0.01 mg	0.02 mg
10	0.194	0.456
20	0.234	0.466
30	0.240	0.485
45	0.242	0.473
60	0.231	0.469

0.01 and 0.02 mg of oleic acid were used. Mechanical shakings were performed at room temperature.

Recoveries of triolein and oleic acid: 0.1 mg of triolein and 0.02 mg of oleic acid were applied to TLC. After developing on chromatoplates, triolein and oleic acid were eluted from silica gel. Examination was carried out on eight different applications in a single experiment. Table 3 shows the

TABLE 3 RECOVERY OF TRIOLEIN AND OLEIC ACID IN ELUATES FROM SILICA GEL AFTER TLC

	0.1 mg of triolein		0.02 mg of oleic acid	
	Reference	After TLC	Reference	After TLC
Absorbance	0.535	0.547	0.459	0.465
	0.526	0.555	0.473	0.474
	0.567	0.540	0.485	0.470
	0.571	0.523	0.480	0.466
	0.533	0.541	0.456	0.471
	0.576	0.576	0.469	0.474
	0.534	0.548	0.484	0.485
	0.555	0.552	0.477	0.452
Average	0.550	0.548	0.473	0.470
Average percent recovery		99.6±4.9%		99.4±4.4%

absorbance values of the applied and non-applied lipids determined colorimetrically. The recovery of both lipids applied to TLC was evaluated by comparison of their absorbances with those of non-application. The average percent recovery of 0.1 mg of triolein and 0.02 mg of oleic acid was 99.6± 4.9% and 99.4±4.4%, respectively. The error range did not indicate inherent errors of colorimetry but variations in the degree of elution, because the non-applied lipids also gave approximately the same error range. It may, therefore, be assumed that both lipids were eluted completely.

TABLE 4 THE CONTENT OF NEUTRAL LIPIDS AND PHOSPHOLIPIDS OF HeLa, S-3 CELLS

Lipids	Amount in 1 mg of total lipid extract (μg)				
	1	2	3	4	Mean
Cholesteryl esters*	13.9	13.4	13.4	13.9	13.7 ±0.3
Triglycerides*	73.7	75.6	73.7	74.7	74.4 ±1.2
Fatty acids*	5.76	6.04	5.52	5.92	5.81±0.29
Cholesterol	99.0	98.3	101	95.7	98.5 ±2.8
Phospholipids (P×25)	598	610	613	603	606 ±8

* Calculated as cholesteryl oleate, triolein and oleic acid, respectively.

Analysis of lipids from HeLa, S-3 cells: The applicability of this method to microdetermination of tissue lipids was examined using lipids extracted from HeLa, S-3 cells. 4.1 mg of total lipids were obtained from cells weighing 300 mg (wet wt.). One mg of total lipids was applied quantitatively to TLC. The content of cholesterol, cholesteryl esters, triglycerides, fatty acids and phospholipids was obtained from four analyses. It is apparent from Table 4 that the results were in close agreement. The inherent error by this method was within 5%.

DISCUSSION

The original method for determining triglycerides was modified to obtain more accurate estimations. Van Handel removed the remaining ethanol by placing the tubes in gentle boiling water bath after adding 0.5 ml of 0.2 N H_2SO_4 to saponified samples. This procedure gives rise to large errors due to differences in the removal of ethanol and water from individual tubes. Accordingly, the author added 0.2 ml of 1 N H_2SO_4 and evaporated ethanol and water to dryness on a rotary evaporator. Although the addition of sulfuric acid produced aggregates, which sometimes led to boiling during evaporating, there was no significant error which could be attributed to boiling. The presence of excess water did not affect the absorbance values, while the residual ethanol affected these values. Complete dryness is not necessary, if ethanol odor disappears. The presence of trace amounts of silica gel in eluates had no effect on the final results of chemical analysis, as regards cholesterol and its esters. As to triglycerides and fatty acids, however, materials from blank areas were slightly colored, and they therefore should be measured using the solution derived from the adjacent unstained areas as a blank. Van Handel and Zilversmit (12) reported that the calibration curve was linear up to absorbance of 1.9 at 570 nm. On the other hand, it was found with the present method that Beer's law was strictly applicable through 0.15 mg of triolein (which corresponded to absorbance of 0.825), but above this value curvilinearity was observed. Such departure from linearity can be explained in the following way. Since periodate oxidation was carried out at low concentration of $NaIO_4$ and at low temperature (10°C), oxidation of higher concentrations of glycerin was incomplete.

The original method for determining fatty acids was also modified to apply to quantitative TLC. Since fatty acids in tissue lipids were usually much lower than the other major neutral lipids, the sensitivity of the method is of first importance. Laurell's method is sensitive enough to allow an estimation of oleic acid in the range from 0 to 0.035 mg, and the molar extinction coefficient is about 5.2×10^4 l/mole·cm. The author employed

Laurell's method to determine fatty acids by quantitative TLC. Consequently, color developing in eluates from TLC was unstable, compared to that in control solutions whose application to TLC was omitted. The color intensity increased in a few minutes, and thereafter decreased rapidly and fell to one-third of the control value after 30 min. From the adsorption capacity of gel (23), it can be presumed that complex formation between Cu ion and diphenylcarbazide was inhibited by adsorption of Cu ion onto silica gel in eluates. On the speculation that a color reagent of stronger chelating capacity than diphenylcarbazide does not hinder the forming of Cu-complex, TAC was used as a color reagent. The resulting color remained stable for a few hours both in applied and non-applied lipid. Thus, the obtained molar extinction coefficient was about 2.5×10^4 l/mole·cm. The sensitivity of diphenylcarbazide is higher than TAC, but the latter gave better results in quantitative TLC. As Laurell noted, it is desirable to use siliconized pipettes when the upper phase is transferred after mechanical shaking. Since the evaporation of the solvent causes large and serious errors, it is necessary to stopper the tubes tightly during incubation. With attention to these details, 0 to 0.04 mg of fatty acids can usually be estimated within an error of a few percentages.

Judged from analysis of lipids from HeLa, S-3 cells, the present method makes it possible to assay microquantities of neutral lipids and phospholipids. The characteristic of this method is that colorimetry is combined with TLC in all the lipids tested. Therefore, high specificity can be obtained despite analysis of lipids in the range of 1 to 2 mg. Quantitative application is critical for microassay of lipids and is dependent upon accurate gravimetry. The microdetermination method of neutral lipids cannot be scale down to analyze less than 1 mg, because of the limitations of gravimetry as well as colorimetry. Compared with the method of Skipski *et al.* (9), the present method has several advantages. They are summarized in Table 5. The

TABLE 5 COMPARISONS OF THE METHOD OF SKIPSKI *et al.* (9) AND THE PRESENT METHOD

	Skipski's method	Author's method
Amounts of lipid used	10–30 mg	1–2 mg
Development system	Two-step	One-step
Detection of chromatogram	Spraying with Rhodamine 6G and ultraviolet light	Iodine vapor
Elution from silica gel	Chloroform-methanol (4 : 1) and ether*; centrifugation and filtration through sintered-glass funnels	Chloroform-methanol (4 : 1); centrifugation
Chemical analysis	Colorimetry, titration and infrared spectrophotometry	Colorimetry

* Chloroform-methanol and ether were used properly according to classes of lipids

present method uses only colorimetry for chemical analysis and the elution procedure is simplified.

The author is now investigating methods to simplify the elution step. From preliminary experiments with the recovery of cholesterol and cholesteryl stearate after TLC, almost complete elution was obtained by a simpler procedure. The elution was carried out by mechanical rotation twice at room temperature for 10 min and shaking for a few seconds. By means of this procedure $96.5 \pm 2.2\%$ of cholesteryl stearate and $97.6 \pm 2.2\%$ of cholerterol were recovered.

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