notes on methodology

Complete separation of lipid classes on a single thin-layer plate

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SUMMARY A double-development procedure employing first a polar and then a nonpolar solvent system is described for the complete separation by thin-layer chromatography of the main lipid classes encountered in natural lipids. For better quantification, long plates (34 cm) are employed. Diglycerides were separated from cholesterol, 1,2- from 1,3-diglycerides, and monoglycerides from phospholipids.

KEY WORDS double-development · thin-layer chromatography · quantification · lipid classes

A SINGLE SOLVENT system capable of resolving a mixture of cholesterol ester, triglyceride, diglyceride, free fatty acid, free cholesterol, monoglyceride, and phospholipid has not been reported. Nonpolar solvents, though capable of separating hydrocarbons, cholesterol esters, and alkyl esters as classes, leave more polar lipids largely unresolved at or near the origin. More polar lipids are generally separated by solvent systems of petroleum hydrocarbon and diethyl ether in various ratios, together with 1–2% of glacial acetic acid (1). In the less polar range of this system (e.g., diethyl ether–hexane–acetic acid 25:75:1), cholesterol ester, triglyceride, fatty acid methyl ester, free fatty acid, and diglyceride are well resolved, but monoglyceride and phospholipid remained unresolved at the origin. Increasing the proportion of diethyl ether in the solvent mixture displaces monoglyceride from phospholipid slightly, but at the expense of cholesterol ester/triglyceride resolution. The system, moreover, suffers from the serious disadvantage that throughout the polarity range free cholesterol migrates with either 1,2- or 1,3-diglyceride. We describe here a one-dimensional, double-development system which gives complete separation of the lipid classes most frequently encountered in biological systems, when long (34 cm) plates are used.

The separated lipids have been determined by the dichromate-reducing method described by Amenta (2). A single reagent is employed and elution is unnecessary.

Materials and Methods. All chromatographic solvents were redistilled before use. The lipid standards used (cholesteryl oleate, tripalmitin, 1,3-dipalmitin, palmitic acid, cholesterol, monopalmitin, and lecithin) were purified before use and their purity was checked by TLC. Cholesterol after initial purification contained a very small amount of free fatty acid (apparent in Fig. 1*a* and 1*c*) and was rechromatographed for incorporation in the standard and synthetic mixtures. Standards and lipids for estimation were made up in chloroform–methanol 1:1 (v/v) for application to the plate. The thin layers (250 μ) of Silica Gel G (E. Merck, A.G., Darmstadt, Germany) were prepared on 34 × 20 cm glass plates, and the adsorbent was activated for 40 min at 105°C before use. The glass plates were cut from 3 mm standard plate glass; the developing tanks used were specimen jars (5 × 8½ × 14½ inches) fitted with ground-glass lids.

The plates were marked out into 3 cm lanes (Fig. 2). The standard lipid solution was applied to one lane, another lane was left blank, and the unknown lipid solutions were applied to two other lanes. The solutions were applied to give 50–250 μg of each lipid class on the plate, and were pipetted onto the origin as a series of discrete spots across the appropriate lane. Reference spots of each lipid class were applied in the two outer lanes. The plates were developed in saturated tanks as follows.

Solvent system 1: Diethyl ether–benzene–ethanol–acetic acid 40:50:2:0.2. The solvent front was allowed to run a distance of 25 cm from the origin. Development time was approximately 60 min. The plates were removed, air-dried for a few minutes, and heated briefly to remove traces of acetic acid. They were then transferred to another tank containing the second solvent.

Solvent system 2: Diethyl ether–hexane 6:94. This solvent was run to within 1 cm of the top of the plate. The time taken for development in this system was 100–120 min. The plates were then air-dried again and given a final drying at 60°C for 30 min. The development times given are for normal operating temperatures of about 22°C.

The outside reference lanes were examined under UV light after spraying them with a 1% aqueous solution of Ultraphor (Badische Anilin und Soda Fabrik A.G., Ludwigshafen-am-Rhein, Germany), and zones corresponding to lipid spots were marked in the unexposed lanes. These zones, together with a corresponding area from the blank strip, were scraped from the plate on to squares of cellophane paper, and transferred from the latter to 10-ml stoppered tubes for estimation.

The reagent and general procedure of the colorimetric estimation were as described by Amenta (2). We found 2 ml of reagent, and dilution of 1 ml of the developed solutions in 10 ml of water for the colorimetric reading, to be most suitable for the range of 50–250 μg of lipid. Absorbances were determined at 350 mμ on a Unicam SP 500 spectrophotometer against a distilled water blank.

Abbreviation: TLC, thin-layer chromatography.

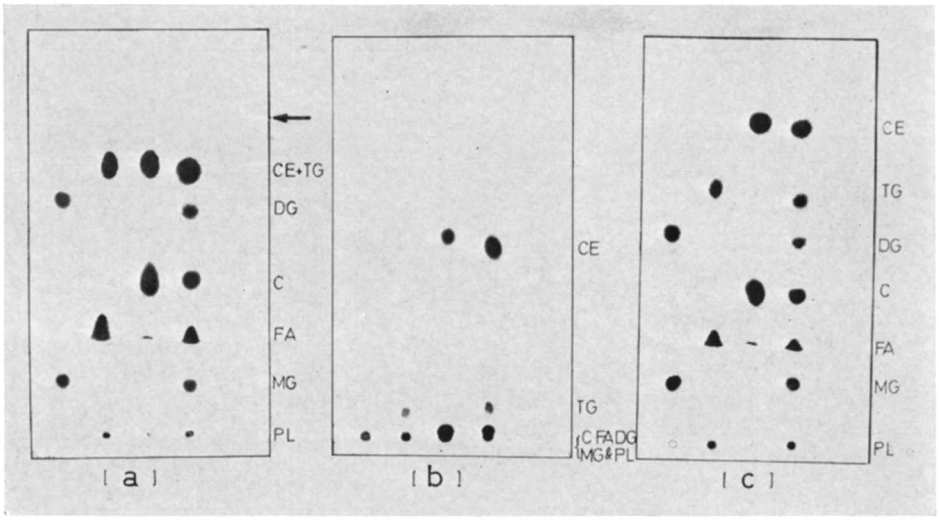

FIG. 1. Chromatoplates illustrating lipid class separation in two solvent systems, individually (*a* and *b*) and in successive combination (*c*).
(*a*) Solvent system 1, diethyl ether–benzene–ethanol–acetic acid 40:50:2:0.2.
(*b*) Solvent system 2, diethyl ether–hexane 6:94.
(*c*) Solvent system 1 followed by second development in solvent system 2.
CE, cholesterol ester; TG, triglyceride; DG, diglyceride; C, free cholesterol; FA, free fatty acid; MG, monoglyceride; and PL, phospholipid. Arrow indicates position of first solvent front.
The four mixtures applied across each plate, from left to right, were (i) monopalmitin and 1,3-dipalmitin, (ii) lecithin, palmitic acid, and tripalmitin, (iii) cholesterol and cholesteryl oleate, and (iv) a mixture of (i), (ii), and (iii).
Load: approx. 50 μg per spot.
Detection by charring after spraying with saturated solution of $K_2Cr_2O_7$ in 80% (by wt) H_2SO_4.

Results and Discussion. The separation achieved in solvent system 1 is shown in Fig. 1*a*. Cholesterol is clearly separated from diglyceride and monoglyceride from phospholipid, which remains at the origin; cholesterol ester and triglyceride migrate together near the solvent front. Solvent system 2 resolves this combination while scarcely affecting the other lipid classes (Fig. 1*b*). Hydrocarbons, when present, migrate with the solvent front in the second solvent system and as a result are separated from cholesterol esters, though hydrocarbon/cholesterol ester resolution is slightly inferior to cholesterol ester/triglyceride resolution. Development in the reverse order of solvent systems gave a slightly inferior separation of cholesterol ester from triglyceride and a less discrete fatty acid zone.

A typical chromatogram, run for the analysis of intestinal and plasma lipids of a pig, is shown in Fig. 2. The lipid classes separate as narrow bands and are almost equidistantly spaced; location of the lipid bands that would normally not be sprayed with detecting reagent is consequently unequivocal. It is felt that this justifies the lengthy development times required for the long plates. The total development time can be considerably reduced by running the plates at elevated temperatures or by employing the more normal 20 × 20 cm plates, but resolution is impaired. The 20 × 20 cm plates are very useful, however, for qualitative purposes with these solvent systems.

The linearity of response to dichromate reduction was verified over the working range 50–250 μg, for each of the standard lipids run through the complete chromatographic procedure, in agreement with Amenta (2). Amounts of lipid below (down to 15 μg) or above this range can be estimated by adjusting the amount of dichromate reagent added (2); the proportions of reagent used in the method described here were chosen to cover a working range suitable for general application.

A further check was made of the effect of unsaturation on the extent of dichromate reduction. With palmitic, oleic, and linoleic acids, a small but consistent decrease in reduction of dichromate with degree of unsaturation was observed. The decrease was of the order saturated: monoene: diene 1:0.98:0.91, these figures being the means of ten determinations. When highly unsaturated lipids are being examined, either a standard should be chosen whose degree of unsaturation is similar to that of the lipid being estimated or the sample should be completely hydrogenated prior to analysis and estimated against a saturated standard.

The accuracy and reproducibility of the technique was checked using synthetic mixtures. The results of a series of determinations on two different synthetic mix-

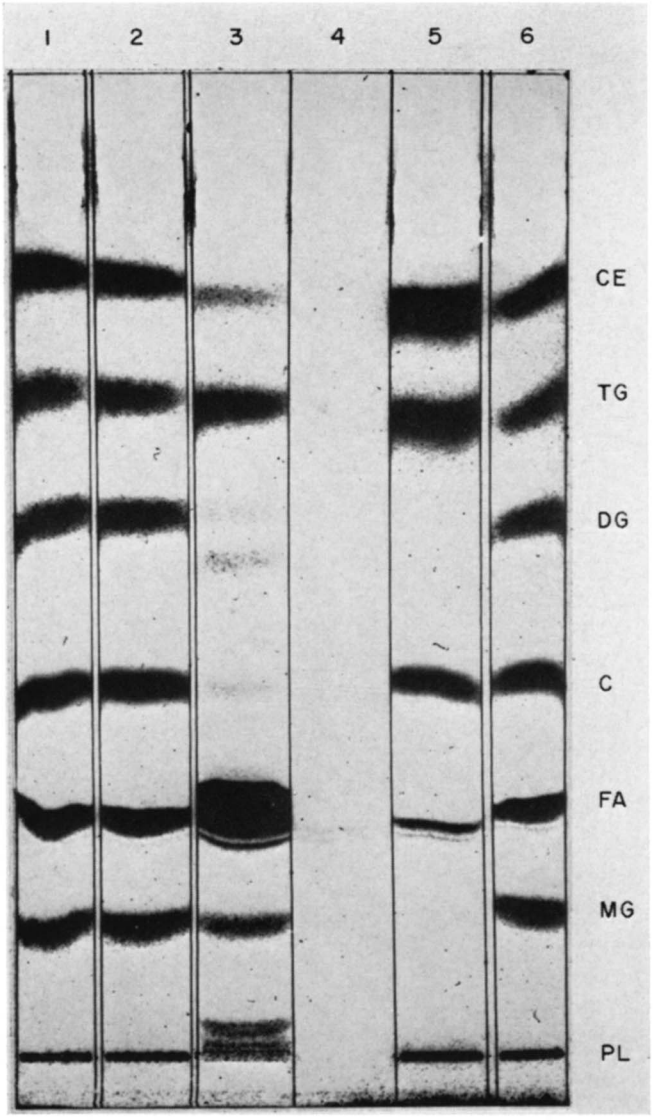

FIG. 2. Typical layout of analytical chromatoplate, charred as described in Fig. 1 to illustrate separation within lanes. Normally, lanes 1 and 6 only would be sprayed, as described in the text.

1 and 6, reference strips; 2, standard lipid class mixture (100 μg each lipid class); 3, pig intestinal lipids after fat feeding (400 μg applied); 4, blank lane; 5, pig plasma lipids (500 μg applied). Abbreviations as for Fig. 1.

1,2-diglyceride (the predominant isomer in pig intestinal content following the action of pancreatic lipase on triglyceride) is seen to be resolved from the faster moving 1,3-isomer (lane 3).

tures (with and without phospholipid) are shown in Table 1. The analyses on mixture 1 were carried out using solvent system 2 followed by solvent system 1; the normal order was employed for mixture 2.

The "absolute error" (3) recorded for the analyses of mixture 2 (about ±0.4%) compared with the analyses of mixture 1 (about ± 1.0%) reflects the improved chromatographic resolution observed when solvent system 1 is followed by solvent system 2, as opposed to the reverse

TABLE 1 TCL ANALYSES OF STANDARD LIPID CLASS MIXTURES

Synthetic Mixture No.	Lipid Class	Per Cent Composition		No. of Determinations
		Known	Found*	
1	Cholesterol ester	16.9	17.1 ± 0.9	6
	Triglyceride	17.4	18.4 ± 2.4	
	Diglyceride	20.0	17.5 ± 1.6	
	Cholesterol	13.5	13.3 ± 1.8	
	Free fatty acid	15.6	16.6 ± 0.9	
	Monoglyceride	16.5	17.1 ± 1.0	
	Phospholipid	0	0	
2	Cholesterol ester	11.4	11.4 ± 1.6	10
	Triglyceride	11.7	11.8 ± 1.3	
	Diglyceride	18.6	18.0 ± 2.4	
	Cholesterol	9.1	9.7 ± 1.6	
	Free fatty acid	17.6	16.9 ± 1.3	
	Monoglyceride	21.0	21.3 ± 2.0	
	Phospholipid	10.8	10.8 ± 1.6	

* Mean ± SD.

order. The standard deviation about the mean was quite consistent throughout.

The absolute recoveries, from application to the plate to scraping off the silicic acid areas, were checked with palmitic acid-1-^{14}C and glyceryl tripalmitate-1-^{14}C. The method of Snyder and Stephens (4) was used for scintillation counting of the silicic acid areas. The mean recoveries (four determinations) were 95.6 and 93.6% for the labeled palmitic acid and tripalmitin respectively. Application of the sample to the plate from chloroform–methanol 1:1 was more reproducible and quantitative than application from diethyl ether solution, presumably because of evaporation and consequent loss of lipid on the pipette tip when ether is used. As expected, the scraping-off operation accounted for most of the small loss recorded. This virtually quantitative recovery of label has been utilized in metabolic studies with labeled lipids to determine the specific activity of lipid classes; duplicate samples, one for mass analysis by the dichromate method and the other for counting as described above, were chromatographed on the same plate.

When this procedure is applied to extracts of naturally occurring lipids, caution should be used in attributing the dichromate reduction of material scraped from the origin entirely to phospholipid, since any very polar material in the extract will remain in this region. Marinetti (5) subjected silicic acid impregnated papers to a very short preliminary development with chloroform–methanol 1:1 (containing 2% water) in order to cause the polar lipids to migrate away from other material at the origin. In those cases where only a total phospholipid value is required, it may be preferable to carry out a phospholipid-phosphorus estimation (6) on the material at the origin in place of the dichromate reaction. Where a quantitative analysis of individual phospholipids is re-

quired, it is suggested that the lipid extract is first run in a system suitable for phospholipid separation. Individual phospholipid fractions can then be scraped from the plate and quantified by the dichromate reduction method. Neutral lipids, which will be located near the solvent front in such systems, can then be eluted as a group, and rechromatographed and estimated by the procedure as described here.

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