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QUANTITATIVE DENSITOMETRIC THIN-LAYER CHROMATOGRAPHY OF LIPIDS USING COPPER ACETATE REAGENT

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SUMMARY

A copper acetate spray reagent has been used for the quantitative densitometric thin-layer chromatography of glycolipids and phospholipids. The precision of the method ranges between zero and $\pm$ 4.5%. Glycolipids and phospholipids from human CNS myelin have been analyzed.

INTRODUCTION

The quantitative determination of phospholipids separated by thin-layer chromatography (TLC) on silica gel is frequently based on the determination of phosphorus in the separated fractions after elution and mineralization[1–3], or following direct ashing in the presence of the adsorbent[4–7]. Other investigators[8,9] have employed densitometric measurements of lipid spots visualized under standard conditions. Earlier quantitative TLC techniques for lipids have been extensively reviewed[10].

As analyses of brain lipids in this laboratory involved limited quantities of material available and the presence of trace amounts of certain components such as cholesterol ester in brain, a single, rapid, sensitive procedure for the quantitative determination of neutral lipids, glycolipids and phospholipids was desirable.

The sulfuric acid–potassium dichromate spray reagent[11] used in charring of lipid spots for densitometry requires a forced-draught oven to ensure complete decoloration of the background. In addition, oversprayed chromatograms tend to absorb moisture on standing following the charring procedure.

By incorporating and refining certain features of previously described techniques, the quantitative densitometric TLC of lipids was re-evaluated using a copper acetate spray reagent. This report describes its application to the analysis of neutral lipids, glycolipids and phospholipids. As slight variations in technique can reduce the accuracy of the method, detailed descriptions are presented.

MATERIALS AND METHODS

Redistilled ACS reagent grade solvents were degassed with nitrogen before use.

This contained less than 5 p.p.m. of oxygen (prepurified nitrogen, Matheson Colman Company, Cucamonga, Calif.). All solvent ratios were measured on a volume basis.

The following reference lipids were employed: Cholesterol, recrystallized from acetone, and mixtures of cholesterol esters, fatty acids, and triglycerides (Nutritional Biochemicals Corporation, Cleveland, Ohio) purified by preparative TLC on 500 mμ Silica Gel G plates. The individual lipid classes were extracted from the adsorbent with diethyl ether. Each lipid fraction gave a single spot after chromatographing on Silica Gel G plates. Individual neutral lipids had a final concentration of 2–4 μg per μl. Phospholipid and sphingolipid standards isolated from beef brain were used (Applied Science Laboratories, Inc., State College, Pa.) with the exception of the sodium salt of inositol glycerophosphatide, which was isolated from wheat sprouts and generously supplied by Dr. M. FAURE, Pasteur Institute, Paris. The concentrations of individual phospholipids and sphingolipids were in the order of 10 μg per μl.

Thin-layer chromatography

Silica Gel H (E. Merck, A.G., Darmstadt, Germany) mixed with 10% magnesium silicate (Allegheny Industrial Chemical Corp., Butler, N.J.) was employed as adsorbent. The magnesium silicate binder has been shown to eliminate almost completely the spreading of acidic lipid spots[12]. For routine examinations of neutral lipids only, Silica Gel G can be substituted as adsorbent.

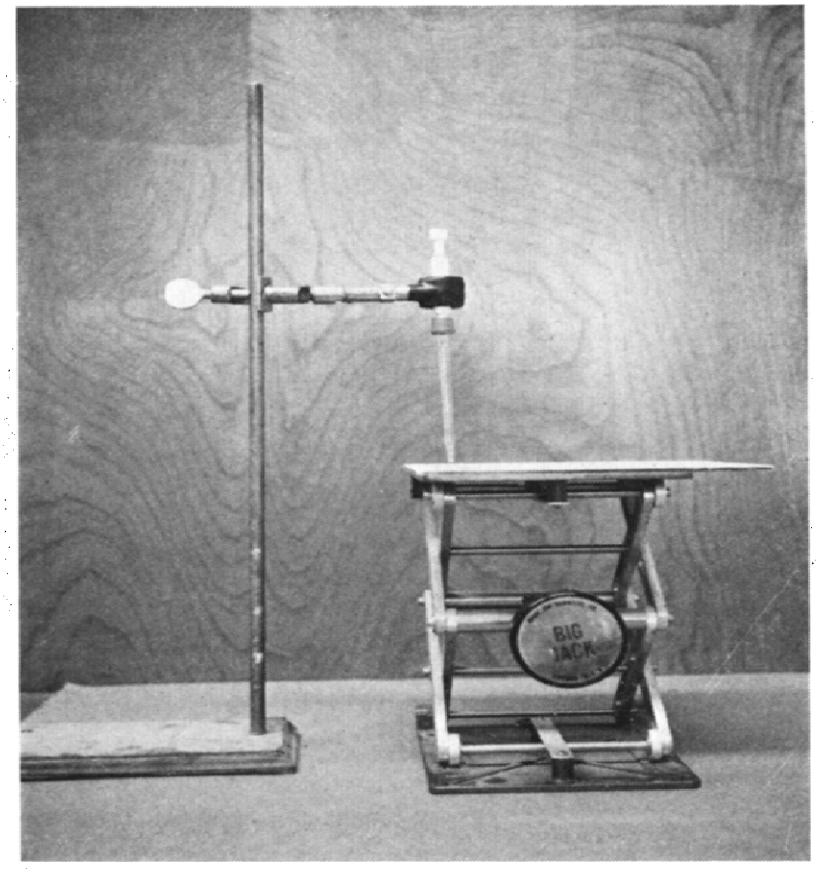


Fig. 1. Apparatus for application of sample to chromatoplate.

The slurry was prepared from 20 g of adsorbent in 60 ml of water by inverting the flask continuously for approximately 50 sec. Vigorous shaking was avoided to minimize the incorporation of air bubbles. After coating with silica layers 250 mμ thick using Desaga-Brinkmann equipment (Brinkmann Instruments Co., Great Neck, N.Y.) the plates (20 × 20 cm) were air-dried for 30 min, heated at 110° for 1 h, cooled and developed the full length of the plate in chloroform to remove organic contaminants[10]. The plates were activated at 100° for 1 h prior to use. The solvents (100 ml) used for reference compounds included chloroform, chloroform–methanol (4:1) and chloroform–methanol (2:1) for neutral lipids, sphingolipids, and phospholipids, respectively.

Each spot was applied 1.5 cm from the lower edge of the plate and 2 cm apart using a 10 μl Hamilton syringe for volumes up to 10 μl and a 50 μl Hamilton syringe for larger aliquots. The material was applied in successive, superimposed drops 5–6 mm in diameter and not more than 30 mm^2 under a continuous stream of nitrogen. The nitrogen stream was directed through a needle valve into a 25 cm length of Tygon tubing closed at the distal end. Syringe needles (Yale Sterile Disposable 25 g 3/8) were inserted into the tubing 2 cm apart with each hub directing a stream of nitrogen directly over a spotting site. Visible traces of solution remaining at the end of the syringe needle were removed by directing the needle parallel to the plate with the tapered tip just above the spotting site and the needle orifice on the under side. Care was taken throughout all applications to avoid scratching the silica gel layer. The amount of standard lipid applied ranged from 4 to 20 μg.

An alternate spotting method using lambda micropipets was also found to be satisfactory. A lambda pipet was connected to an ultramicro accropet (Van Waters & Rogers, Inc., Los Angeles, Calif.) rigidly attached to a ring stand. The accropet controlled the flow rate of the pipet. The thin-layer plate was placed on a bench lab jack and raised to within 1/8 in. of the tip of the pipet for dispensation of the sample. The surface of the silica gel was not disturbed by this procedure and the sample spot size was kept at a minimum (Fig. 1).

The chromatoplates were developed in unequilibrated tanks lined with Whatman No. 3 MM filter paper saturated with solvent mixture just before chromatography[12]. The solvents were allowed to ascend at room temperature to a distance of 12 cm from the origin for neutral lipids and 10 cm for sphingolipids and phospholipids except in the case of mixtures containing phosphatidyl choline and sphingomyelin, where a 15 cm solvent front gave improved resolution.

The solvent systems for neutral lipids included petroleum ether–diethyl ether–acetic acid (85:15:1)[8], or for the analysis of cholesterol and diglyceride mixtures, petroleum ether–ethyl ether (70:30) or chloroform. Phospholipid analyses in this laboratory included preparative TLC to separate the lipid classes for subsequent determination of fatty acid compositions. The overlapping of bands on unidimensional TLC was avoided by a preliminary separation of total lipid extracts into smaller lipid mixtures by DEAE-cellulose chromatography[12,13]. Phosphatidyl ethanolamine and phosphatidyl serine eluted as pure fractions, thus the amounts of these lipids in lipid mixtures were calculated from the dry weights of column eluates. Other phospholipids eluted as mixtures with sphingolipids and were determined by quantitative TLC[13]. Chloroform–methanol–water (65:25:4)[14] was used for the analysis of phosphatidyl choline, sphingomyelin and cerebroside, and chloroform–methanol–water

(65:35:5) modified slightly by ROUSER *et al.*[12] from the original system of WAGNER *et al.*[14] for cerebroside sulfate and inositol glycerophosphatide. In this solvent system the methanol contained 2% NH_4OH.

After chromatography, the plates were dried for 10 min in a fume hood and sprayed with a 3% cupric acetate solution in 8% aqueous H_3PO_4 (ref. 15) until uniformly transparent. Overspraying is not critical but a fine spray is essential to avoid pitting of the silica gel. The plates were laid directly on a pyroceram hotplate set at 180° for 25 min in a fume hood.

Optical densities of the charred spots were measured with a TLC densitometer including a Light Source Model 52-C with TLC stage equipment, Multiplier Photometer Model 501-A (Photovolt Corp., New York) and Model G-14 Strip-Chart Graphic Recorder (Varian Associates, Palo Alto, Calif.). A metal strip with slit 2 cm long and 0.5 mm wide was fitted to the adjustable arm of the light source unit and a similar strip was fitted over the photocell. The lengths of these slits were adjusted with black tape until just greater than the width of the spots of the particular lipid class to be measured on a single plate[10].

For the analysis of samples, a Hamilton syringe having a Chaney adaptor was used for the accurate repetitive measurement of fixed volumes of sample and standard. The volumes of sample used were those shown in preliminary runs to give spots of comparable density to the standard following charring. For the standard, 10 μl of solution containing in the order of 10 μg was used. The sample and standard were spotted alternately to give at least three identical aliquots of each per chromatoplate. The recorder was adjusted to give peak heights for the standards on a single plate approximately 80% of the calibrated width of the chart paper. Peak areas were measured by triangulation by multiplying the peak height by the width at half

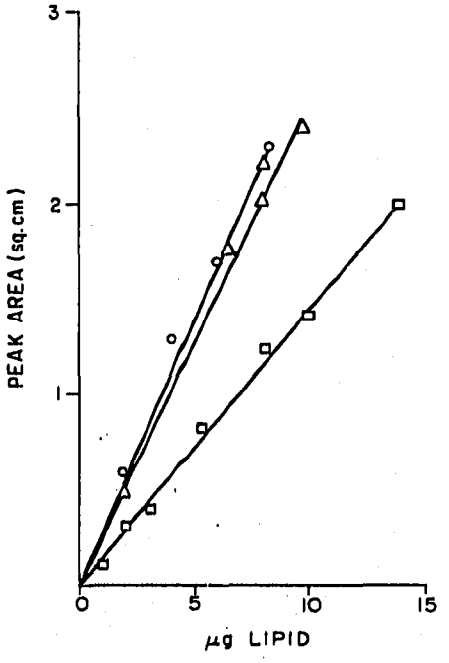


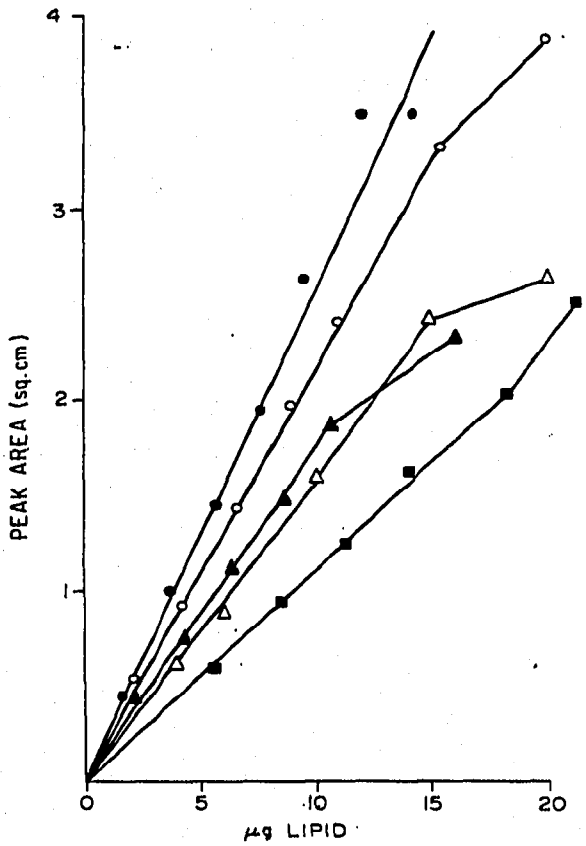
Fig. 2. Calibration curves showing relationship between area under the curve (cm^2) and concentration of neutral lipids. ○, triglyceride; △, free fatty acid; □, cholesterol.

height. The amount of lipid present was determined by comparison with the charred standard spots.

RESULTS AND DISCUSSION

The analysis of lipid spots charred with copper acetate reagent showed that a linear relationship resulted which passed through the origin when the densitometric peak area was plotted against the amount of lipid spotted over the range 2–15 μg (Figs. 2–4). For the analysis of samples, lipid standards in the range of 10–15 μg should be alternately spotted with volumes of sample previously shown to give spots of comparable density following charring. Quadruplicate or triplicate spotting of both standard and sample on the same plate is advisable to reduce errors including those involved in the measurements of peak widths during triangulation. The widths of the peaks at half height ranged from approximately 2.0 mm for most lipid samples and standards to approximately 3.5 mm for galactolipids. Table I shows the accuracy of the method for a typical series of analyses. The precision ranged between zero and $\pm$ 4.5%.

It is essential to run standards for each lipid to be analyzed on each chromato-



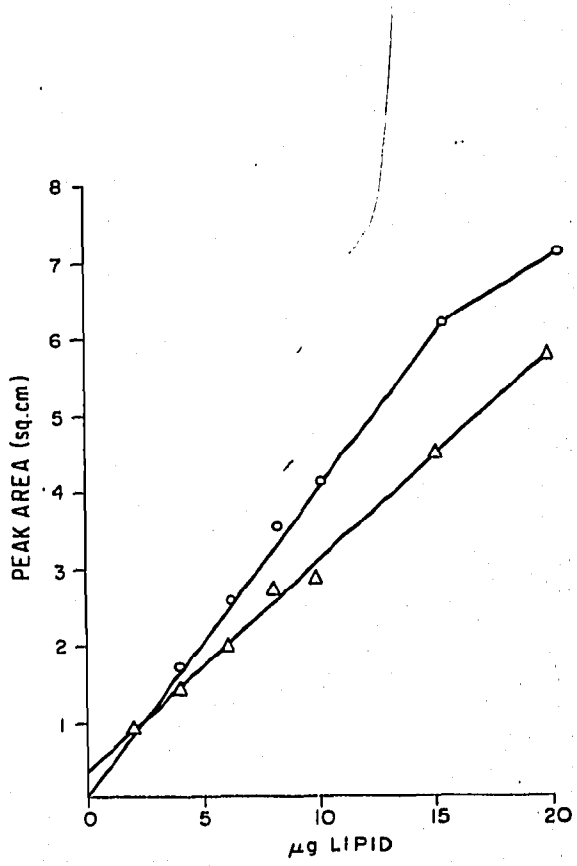


Fig. 3. Calibration curves showing relationship between area under the curve (cm^2) and concentration of phospholipids. ●, phosphatidyl ethanolamine; ○, phosphatidyl inositol; ▲, phosphatidyl serine; △, sphingomyelin; ■, phosphatidyl choline.

Fig. 4. Calibration curves showing relationship between area under the curve (cm^2) and concentration of glycolipids. ○, cerebroside; △, cerebroside sulfate.

TABLE I

PRECISION OF QUANTITATIVE THIN-LAYER CHROMATOGRAPHY USING COPPER ACETATE REAGENT

		Peak area (cm^2)				*Mean ± S.E.*
		1	*2*	*3*	*4*	
Choline glycerophosphatide	Standard	2.54	2.89	2.83	2.63	2.72 ± 0.10
	Sample	3.59	3.43	3.58	3.44	3.51 ± 0.00
Inositol glycerophosphatide	Standard	2.51	2.55	2.57		2.54 ± 0.00
	Sample	2.46	2.73	2.45		2.55 ± 0.10
Sphingomyelin	Standard	1.93	2.18	2.05	1.95	2.03 ± 0.00
	Sample	3.13	3.45	3.11	3.19	3.22 ± 0.10
Cerebroside	Standard	2.67	2.81	2.62		2.70 ± 0.00
	Sample	2.11	2.30	2.20		2.20 ± 0.10
Cerebroside sulfate	Standard	2.53	2.46	2.48	2.37	2.46 ± 0.00
	Sample	2.12	2.46	2.18	2.18	2.24 ± 0.10

plate, as equal amounts of different lipids give varying densitometric areas when chromatographed on the same plate. Different areas are also obtained when the same quantity of any individual lipid is chromatographed on consecutive plates.

In contrast to sulfuric acid–potassium dichromate reagent[11], overspraying of the chromatograms is not critical with copper acetate reagent, and there is no darkening of the background. Some fading of the charred spots, however, results when chromatograms are left overnight, thus optical density analyses should be carried out as soon as possible after charring and cooling the chromatoplates.

There is a delay in the rate of appearance of triglyceride spots compared with other lipid classes examined but the accuracy of the results is unaffected. Cholesterol and cholesteryl esters develop consecutively in color to red, deep blue and black during the heating procedure after spraying with copper acetate reagent. This provides a useful aid to the rapid identification of these lipid classes.

TABLE II

COMPARISON OF LIPID COMPOSITION OF HUMAN CNS MYELIN DETERMINED BY THIN-LAYER CHROMATOGRAPHY WITH RESULTS OF OTHER AUTHORS

	Present work		CUZNER *et al.*[a] *(ref. 16)*	O'BRIEN AND SAMPSON[b] *(ref. 17)*
	Case 1	*Case 2*		
Choline glycerophosphatide	8.8	9.0	9.4	10.6
Inositol glycerophosphatide	1.2	4.3	0.7	
Sphingomyelin	6.1	6.3	8.7	6.6
Cerebroside	17.9	13.2	20.0	20.5
Cerebroside sulfate	7.3	7.8	4.6	4.4

[a] Figures representing the average for two subjects were recalculated assuming the following molecular weights: phospholipid, 775, cerebroside, 804.9, cerebroside sulfate, 892 (O'BRIEN AND SAMPSON[17]), and galactolipids, 828.9 (EICHBERG *et al.*[18]).

[b] Figures for a 55-year-old subject.

The application of copper acetate reagent to the analysis of two cases of human CNS myelin phospholipids and galactolipids is shown in Table II[13]. The results are in general agreement with those obtained by other authors using TLC followed by colorimetric determinations on the separated, eluted fractions[16] or diethylamino-ethyl-cellulose, silicic acid and Florisil column chromatography[17].

ACKNOWLEDGEMENTS

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