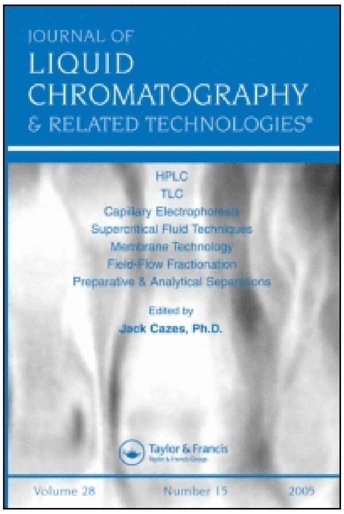

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An Improved Copper Reagent for Quantitative Densitometric Thin-Layer Chromatography of Lipids

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AN IMPROVED COPPER REAGENT FOR QUANTITATIVE DENSITOMETRIC THIN-LAYER CHROMATOGRAPHY OF LIPIDS

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ABSTRACT

Eleven cupric compounds were screened to determine whether a different cupric anion might give greater and more uniform charring responses with lipid classes on TLC plates than cupric acetate. A procedure was developed which used 10% $CuSO_4$ in 8% H_3PO_4 as a charring reagent. Improved reproducibility of charring was obtained by temperature-programmed heating of the thin layer plates from 30 to 180°C at 10 degrees per minute in the oven of a commercial gas-liquid chromatograph.

INTRODUCTION

Direct determination of lipid classes on thin layer plates requires 1) conversion of the separated colorless lipid spots into colored or fluorescent products and 2) quantitative determination by instrumental analysis. Reagents which react with specific groups such as phosphorus, glycerol or ester linkages present in the lipids may be used but are not applicable to all classes. Consequently, reagents which react generally with organic compounds have been used to visualize lipids.

Oxidative reagents such as sulfuric, perchloric, chromic and phosphoric acids have been widely employed for destructive charring to produce brown or black charred areas. Upon heating with the oxidizing agent, cleavage, oxidation and dehydration reactions occur in the carbon-hydrogen chain of the lipids to yield deposits of elemental carbon or something approaching elemental carbon (1). Two problems frequently encountered in charring lipid classes in situ on TLC plates are a) different lipid classes yield different responses since, structurally, they consist of widely diverse compounds, and b) unsaturated lipids yield greater responses than saturated lipids because the double bond provides a ready means of attack for the oxidative charring reagent.

Privett et al. (2) obtained approximately equal charring of lipid classes on silica gel with a sulfuric acid-dichromate reagent at 200°C. Downing (1) used 50% sulfuric acid and heated to 220°C to char lipids for quantitative densitometry. Many commercial hard layer silica gel plates contain an organic binder, added for mechanical stability. Application of these charring procedures to such plates produced background areas which were too dark to differentiate from charred lipid spot areas and to satisfactorily quantitate the lipids.

Fewster et al. (3) demonstrated that neutral lipids and phospholipids could be visualized and quantitated using a copper acetate-phosphoric acid reagent. After spraying the TLC plates, they were heated directly on a hot plate at 180°C for 25 minutes. In contrast to the sulfuric acid procedures (1, 2), the copper acetate reagent gave light colored backgrounds and resulted in greater sensitivity in densitometric scanning. However, large differences in response of the individual lipid classes were observed.

We therefore screened eleven copper compounds to determine whether different copper salts might give a more uniform response with the different lipid classes. In this paper, we report an improved copper reagent for visualization of lipids on TLC plates, suitable for quantitative densitometry.

MATERIALS AND METHODS

The TLC plates used were analytical 20 x 20 cm silica gel plates (250μ thick), either Whatman K5 precoated silica gel glass plates (Whatman Inc., Clifton, NJ) or Merck silica gel 60 plates (E. Merck, Darmstadt, Germany). Standard lipid mixtures were separated into eight lipid classes (from origin: phospholipids (PL), monoglycerides (MG), free fatty acids (FFA), cholesterol (C), 1,2-diglycerides (1,2-DG), 1,3-diglycerides (1,3-DG), triglycerides (TG), cholesterol esters (CE)) by a two-development, one dimensional thin layer chromatographic method developed in our laboratory (4). Phospholipids were separated into five classes (from origin: sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylethanolamine (PE)) by the procedure of Sherma and Touchstone (5).

The standard unsaturated lipid mixture (A) was composed of lipids containing equal amounts of C_{18} monounsaturated (oleic) and polyunsaturated (linoleic) fatty acids in the lipid moiety. Lipid standard B was a triglyceride mixture we have called saturated to correspond more closely to the composition of bovine milk fat lipids. It consisted of a mixture of 30% triolein, 30% tripalmitin, 15% tristearin, 15% trilaurin and 10% trimyristin. Lipid standard C was cholesterol. The phospholipid standards were purchased from Supelco Inc., Bellefonte, PA. All other lipid standards were purchased from Nu Chek Prep, Inc., Elysian, MN.

For visualization, the TLC plates were dipped for 3 seconds into the reagent contained in a Kontes Chromaflex dipping tank (5 x 170 x 475 mm, Kontes, Vineland, NJ). The eleven copper compounds were prepared as 3% solutions in 8% aqueous phosphoric acid for initial screening. After dipping, the TLC plate was drained for 2 minutes on a paper towel, the back of the glass plate was wiped, and the plate was heated. Sources of the cupric compounds were: borate, chloride, chromate, hydroxide, molybdate, permanganate, phosphate (ICN Pharmaceuticals Inc., Plainview, NY); carbonate, nitrate (Fisher Scientific Co., Fair Lawn, NJ); acetate (Matheson Coleman and Bell, East Rutherford, NJ); sulfate (Merck and Co., Rahway, NJ). TLC plates were heated either in a NAPCO Model 5850 vacuum oven (National Appliance Co., Portland, OR), or on the metal surface of a Thermolyne Type 1900 hot plate of an Analtech VPF Chamber (Analtech, Newark, DE), or in the oven of a Hewlett-Packard Model 7620A gas chromatograph (Palo Alto, CA).

Optical densities of the charred spots were measured at 350 nm with a Shimadzu CS-910 Dual-Wavelength TLC Scanner (Shimadzu Scientific Instruments, Inc., Columbia, MD) as described previously (6). In screening experiments, the intensity of charring was evaluated by visual assessment on a scale of 1 to 10, 1 being very weak and 10 being very strong.

RESULTS AND DISCUSSION

Eleven copper salts were screened to determine whether a different copper anion might be better than copper acetate for lipid charring. Table 1 compares the charring yield given by copper acetate with that given by borate, carbonate, chloride, chromate, hydroxide, molybdate, nitrate, permanganate, phosphate, and sulfate salts of copper when applied to an unsaturated lipid class mixture (A), a saturated triglyceride mix (B), and cholesterol (C). After applying 10 μg of A, B, and C on a micro Whatman K5 silica gel plate, the plate was dipped into a 3% solution of the copper salt in 8% H_3PO_4, and the lipids were charred at 130°C for 50 minutes. The color of the background was also evaluated since any optical densitometric procedure depends upon the difference between background absoprtion and the absorption of the sample. Ideally, the background should remain white and exhibit little absorption while the sample should absorb strongly.

TABLE 1
Comparison of Copper Salts for Lipid Charring

Cupric Compound	A Unsatd Lipid Class Mix	B Satd TG	C Chol	Background
Acetate	7	3	7	light
Borate	7	3	7	light-med
Carbonate	7	3	7	light
Chloride	7	3	7	light
Chromate	7	1	1	green
Hydroxide	7	3	7	light
Molybdate	7	3	7	dark blue
Nitrate	7	2	6	light
Permanganate	7	4	6	light-med
Phosphate	7	4	7	light-med
Sulfate	7	5	7	light-med

10 µg of standard lipids A, B and C were placed on micro Whatman K5 TLC plates. Lipid A: Mixture of 8 lipid classes, CE, TG, 1,3-DG, 1,2-DG, C, FFA, MG, PL. The fatty acid moiety consisted of equal amounts of 18:1 and 18:2 acids. Lipid B: Triglyceride mixture of 30% triolein, 30% tripalmitin, 15% tristearin, 15% trilaurin and 10% trimyristin. Lipid C: Cholesterol.

Plates were dipped in 3% solutions of the cupric compound in 8% H_3PO_4 and charred by heating for 50 min. at 130°C in a vacuum oven.

Intensity of charring was evaluated on a scale from 1 to 10 with 1 being very weak and 10 being very strong.

All copper salts, with the exception of chromate, were nearly equal in their ability to char the unsaturated lipid class mix, A, or cholesterol, C (Table 1). Less charring and more variation was observed in relative ability to char saturated triglycerides, B. Chromate and nitrate salts gave less response than cupric acetate. Permanganate, phosphate and sulfate salts gave more response than cupric acetate, and cupric sulfate was clearly the best charring reagent. Except for the chromate and molybdate salts which colored the background green and blue, the background areas of the TLC plate were light in color.

Several of the variables which affect the charring reaction (concentration of copper sulfate, concentration of phosphoric acid, charring temperature and time) were studied in an attempt to optimize the densitometric response. Plates containing standard lipids A, B and C, were charred by heating at 130°C for 45 min after dipping in solutions of 0, 1.5, 3, 5 and 10% copper sulfate in 8% H_3PO_4. Solutions of 5% and 10%

cupric sulfate gave greater charring than lower concentrations but the saturated mixture, B, still yielded less response than A or C. Increasing the temperature at which the plates were charred from 130°C to 180°C resulted in a 40-50% increase in charring. Charring for 10 minutes at 180°C with 10% $CuSO_4$ in 8% H_3PO_4 resulted in maximal intensities. The effect of varying phosphoric acid content was also studied using 2, 4, 8, 12, 20 and 40% concentrations, but solutions above 8% did not increase charring intensity of the unsaturated lipid standard mixture. The permanganate and phosphate salts had yielded almost as much charring as sulfate in the original screening of 3% copper salts at 130°C (Table 1). When these salts were tested at higher concentrations and at the higher 180°C temperature, neither was as good as 10% copper sulfate in 8% H_3PO_4 for 10 minutes at 180°C. Greater charring responses were obtained when lipids were dipped in the 10% $CuSO_4$-8% H_3PO_4 reagent than when they were sprayed.

Generally, charring of unsaturated triglycerides proceeded at rates ca 1.5 to 3X that of saturated triglycerides (Table 1). It seemed possible that equal charring of unsaturated and saturated triglycerides might be achieved if the lipids could be heated longer or at higher temperatures. The Whatman K5 silica gel plate has a recommended temperature limitation for charring of 130°C, probably because of the presence of the organic binder. Charring experiments were conducted with saturated and unsaturated triglycerides in which temperatures varied between 130 and 245°C with heating times between 1 and 30 min on a hot plate. As the temperature was increased to 230-245°C, the saturated lipids charred to almost the same extent as the unsaturated lipids, but the background became too dark for quantitative densitometry. Lipid charring on Whatman K5 plates was then compared with charring on Merck silica gel 60 plates. The Merck plates could be heated for periods as long as 38 minutes at temperatures up to 232°C and still be scanned. The backgrounds of Whatman K5 plates heated to 200°C for even 20 minutes were too dark to scan.

Heating glass thin layer plates on the metal surface of a hot plate did not yield consistently reproducible charring results. We attributed this to variable heating. The metal surface of the hot plate (often 200-350°C), affords complete contact between the glass plate and the metal heating surface, thereby providing very efficient heat transfer. However, it is very difficult with this system to provide a definite and reproducible heating period. It is very easy to overshoot heating period conditions both with regard to time and temperature because a) it is difficult to remove the plate from the heating surface exactly at the end of the heating period and

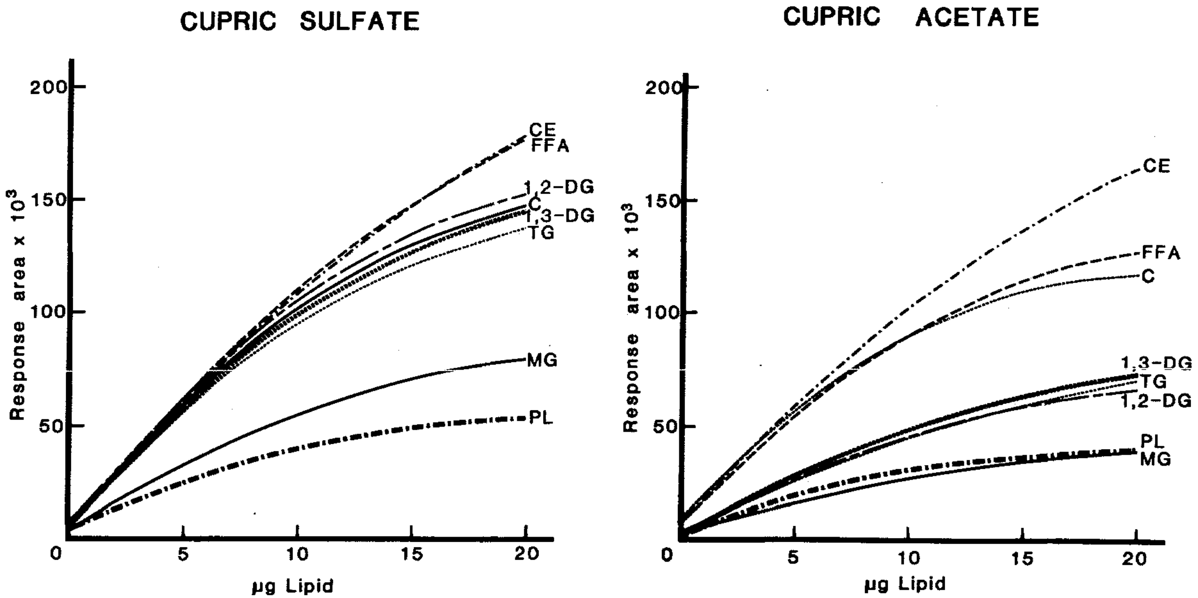

FIGURE 1. Densitometric response of neutral lipid classes on precoated Silica Gel 60 plates (Merck). Developed TLC plates were dipped for 3 seconds in 10% cupric sulfate in 8% aqueous H_3PO_4 (left) or in a saturated solution of cupric acetate (ca 7%) in 8% aqueous H_3PO_4 (right) and charred by temperature programmed heating in a GLC oven from 30-180°C at 10°/minute. Curves were drawn from 2nd order least squares regression equations based on 0, 0.2, 0.5, 1, 2, 5, 10, 15 and 20 μg of lipid.

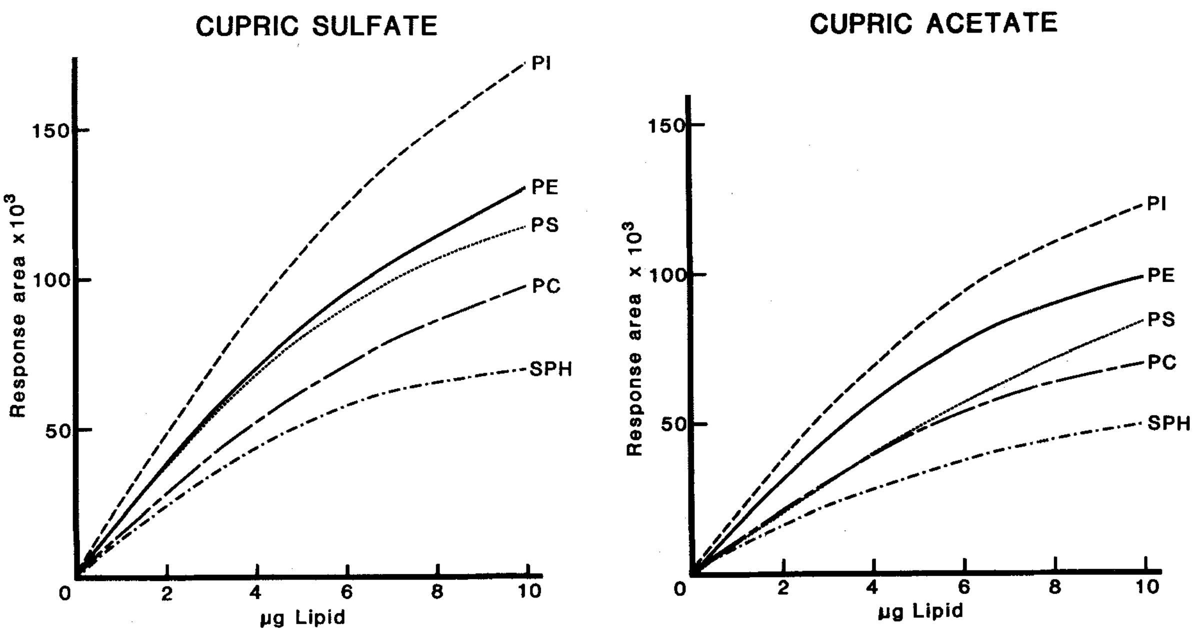

FIGURE 2. Densitometric response of phospholipid classes on precoated Silica Gel 60 plates (Merck). Developed TLC plates were dipped for 3 seconds in 10% cupric sulfate in 8% aqueous H_3PO_4 (left) or in a saturated solution of cupric acetate (ca 7%) in 8% aqueous H_3PO_4 (right) and charred by temperature programmed heating in a GLC oven from 30-180°C at 10°/minute. Curves were drawn from 2nd order least squares regression equations based on 0, 0.2, 0.5, 1, 2, 5, and 10 μg of lipid.

b) the glass plate continues heating due to absorbed heat even if removed from the metal surface.

Much more reproducible results were obtained by heating from 30°C to 180°C under closely controlled conditions. Downing (1, 7) has recommended heating on a cold aluminum slab to 220°C but we obtained variable results when charring was conducted in this manner. Very reproducible results were achieved by heating in the oven of a gas chromatograph using temperature programmed heating at 10°/minute. The TLC plate was placed on a 20 x 20 cm glass plate (0.25 cm thick) which rested on a wire screen in the oven of a Hewlett-Packard Model 7620A gas chromatograph. The well insulated GLC oven provided excellent heat uniformity. At the end of the heating period, the oven was programmed to cool automatically and quickly, thereby avoiding overheating problems.

Comparisons of the cupric sulfate and cupric acetate charring procedures for neutral lipid classes are shown in Figures 1. Greater charring was observed with the cupric sulfate procedure and all classes except monoglycerides and phospholipids yielded almost equal charring response. Whether the lower values with MG and PL were due to the nature of the compounds or to lower R_F values (less migration and higher density of spots) could not be ascertained. Charring responses for monoglycerides, diglycerides and triglycerides were approximately doubled by use of cupric sulfate. Greater charring responses were also observed with the phospholipids (Fig. 2). Cupric sulfate produced approximately 50% greater charring with all phospholipid classes.

The results obtained in this study demonstrate the usefulness of 10% $CuSO_4$ in 8% H_3PO_4 as a charring reagent for lipids on TLC plates. Use of the cupric sulfate reagent and temperature-programmed heating improved reproducibility and produced densitometric responses 2X those given by cupric acetate.

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