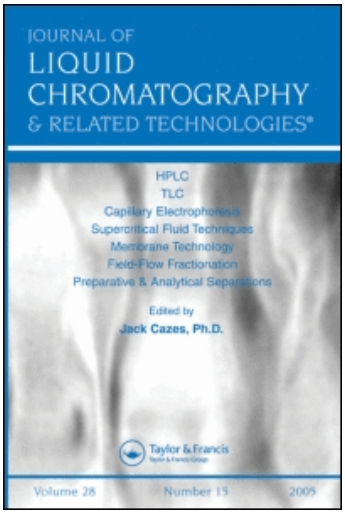

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Comparison of two Copper Reagents for Detection of Saturated and Unsaturated Neutral Lipids by Charring Densitometry

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COMPARISON OF TWO COPPER REAGENTS FOR DETECTION OF SATURATED AND UNSATURATED NEUTRAL LIPIDS BY CHARRING DENSITOMETRY

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ABSTRACT

The intensity of staining of each of four classes of neutral lipids (monoacylglycerol, diacylglycerol, triacylglycerol and fatty acid methyl ester) is shown to be dependent on the number or amount of carbon-carbon double bonds (C=C) when using 3% cupric acetate in 8% phosphoric acid. In contrast, staining with 10% cupric sulfate in 8% phosphoric acid is relatively independent of the number or amount of C=C.

INTRODUCTION

The quantitation of lipids by charring densitometry is a well established tool in use in thin-layer chromatography. One of the more widely used reagents is cupric acetate (3% in 8% phosphoric acid). Since its introduction by Fewster, Burns and Mead (1 (personal communication from R. Kopp)), there has been evidence which demonstrates that unsaturated lipids will char to a greater degree than saturated ones (2 - 4), but a quantitative study of a particular lipid class, by varying amounts of unsatur-

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ation, has not been previously done. Description of the extent of charring have either been evaluated by + or - (2,3) or a qualitative scale of 1 - 10 (4).

Our aim in this paper is to present a quantitative examination of the extents of charring, as monitored by densitometry, of lipid species with varying degrees or amounts of unsaturation. We have compared the intensity of staining, under identical charring conditions, of neutral lipids with cupric acetate (1) and cupric sulfate (2,3) reagents.

MATERIALS

Reference standards containing 25% each monoacylglycerol (MAG), diacylglycerol (1,2-DAG + 1,3-DAG), triacylglycerol (TAG) and fatty acid methyl ester of stearin, olein, linolein and linolenin were obtained from Nu Chek Prep (Elysian, MN). Fatty acid methyl esters quantitative mixtures (FAME) were obtained from both Nu Chek Prep and Alltech Associates, Inc. (Deerfield, IL). Whatman (Whatman Inc., Clifton, NJ) LK-5, 250u, 20x20 cm TLC plates, with a preadsorbent area, were scored to obtain 1 cm lanes, developed with chloroform-methanol 1:1 (v/v) and activated at 110°C for 60 min. All solvents were glass distilled or nanograde and chemicals were reagent grade.

METHODS

Thin-Layer Chromatography

Aliquots of quantitative mixtures, dissolved in chloroform

TABLE 1

FAME QUANTITATIVE MIXTURES

Mixture	Composition	mmoles C=C/gm
1	20% each of 16:0, 18:0, 20:0, 22:0 and 24:0	0.00
2	50% each of mixture 1 and mixture 3	1.45
3	20% each of 16:0, 16:1, 18:0, 18:1 and 18:2	2.91
4	1% 14:0, 4% 16:0, 3% 18:0, 45% 18:1, 15% 18:2, 3% 18:3, 3% 20:0, 3% 22:0, 20% 22:1 and 3% 24:0	3.51
5	20% each of 20:0, 20:1, 20:2, 20:3 and 20:4	6.24
6	25% each of 18:2, 18:3, 20:4 and 22:6	12.0

(neutral lipid mixture of MAG, DAG (1,2- + 1,3-), TAG and fatty acid methyl esters) or hexane (FAME), containing 0.25, 0.5, 1, 2 or 4 μg were streaked 1 cm below the silica gel-preadsorbent area boundary. The composition of the FAME quantitative mixtures and the calculated amounts of C=C (mmoles/gm = sum of (wt lipid/MW x no. C=C/wt total lipids)) are given in Table 1.

Every third lane was left empty as a reference during densitometry. Neutral lipids were resolved by development with chloroform/methanol (47:3) to 1.5 cm above the origin, dried *in vacuo* at room temperature for 20 min followed by development with hexane/diethyl ether/acetic acid (62:13:0.75) (5) to 2.5 cm from the

top of the plate. The FAME quantitative mixtures were spotted, dried in vacuo and developed with hexane/diethyl ether (47:3) (6). The minimal volume of sample streaked was 5 µl. Following development, plates were dried in vacuo at 100°C for 30 min. Nitrogen was admitted into the oven when releasing the vacuum and was used when spotting the samples.

Charring Densitometry

Plates were sprayed, until glistening wet (but not running with liquid), with either 3% cupric acetate in 8% phosphoric acid (1) or 10% cupric sulfate in 8% phosphoric acid (2,3). They were then air dried for 5 - 10 min, heated to 120°C for 2 min and charred at 170°C for 9 min (for neutral lipid mixtures) or for 5 and 5 or 9 min respectively (for FAME). These times were chosen so that the plate background remained white (LK-5 plates contain an organic binder which can darken if plates are heated too long or at temperatures > 170°C). Quantitation of the lipids was accomplished by densitometry using a fiber optic scanner (Kontes, Model 800, Vineland, NJ) equipped with a 440 ± 150 nm filter and an integrator (Hewlett Packard, Model 3390A, Avondale, PA) which gave numeric area responses. These were linear in the range tested and area/pg was determined by averaging the values calculated for each level of lipid species (DAG was the sum of the 1,2- and 1,3- species). The upper limit of linearity varied with the area of the lipid band. MAG bands were very tight (1.5 x 10 mm) and linearity was good only to 0.5 - 1 µg while fatty acid

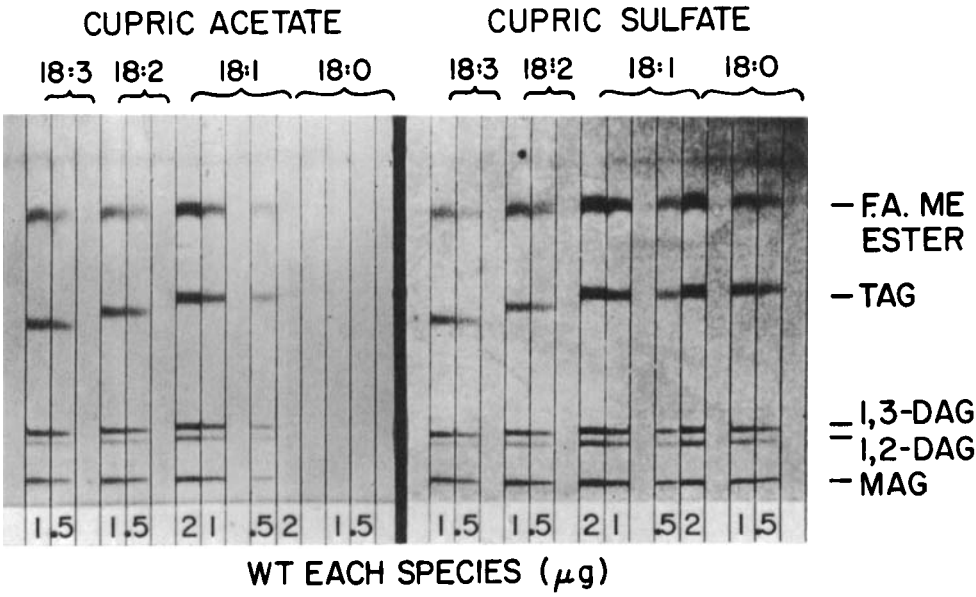

FIGURE 1. Two representative plates stained with cupric acetate and cupric sulfate reagents. Lipids were separated (5) and stained as described in the Methods (2 min at 120°C and 9 min at 170°C). F.A. ME ESTER = fatty acid methyl ester.

methyl esters were broader (5 x 10 - 7 x 10 mm) and linearity was maintained to 3 - 4 μg.

RESULTS AND DISCUSSION

A comparison of the staining intensity of a series of neutral lipids, each containing an 18 carbon acyl chain with 0, 1, 2 or 3 carbon-carbon double bonds (C=C), is shown in Figs. 1 and 2. All four saturated species were barely detectable (significant area levels were obtained at >1 μg) after charring with the cupric acetate reagent. Staining increased as the number of C=C

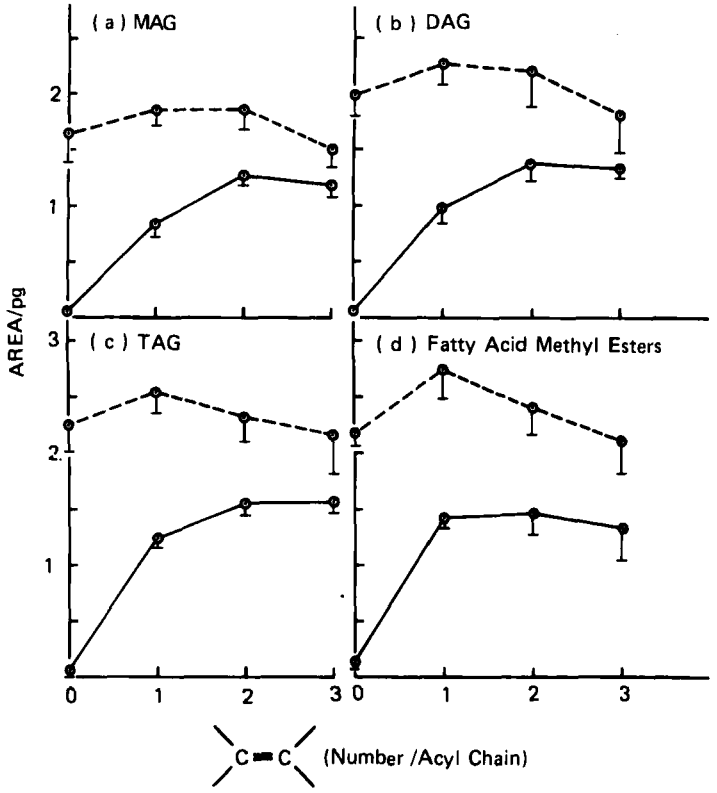

FIGURE 2. Staining of neutral lipids with cupric acetate and cupric sulfate reagents. The solid line is the amount of stain detected with cupric acetate reagent and the dashed line is the amount with cupric sulfate reagent. The values are averages of four plates, two each of separately weighed neutral lipid mixtures of MAG, DAG, TAG and fatty acid methyl ester. Sample standard deviations, with corrections for bias, are indicated (n = 4).

increased. For MAG, DAG and TAG maximal staining was acheived with the linoleate species whereas maximal staining of the fatty acid methyl esters was acheived with methyl oleate. In contrast, the cupric sulfate reagent was able to stain lipids strongly, fairly independent of the degree of unsaturation. However, there

appears to be a slight, but general, increase in staining of the mono and di unsaturated species as compared to the saturated and tri unsaturated species. Paired t tests indicate that these differences are generally significant ($p < 0.05$) when comparing 18:0 - 18:1, 18:1 - 18:3 and 18:2 - 18:3. There was no difference between 18:0 - 18:2 lipids ($p > 0.05$) (except for the fatty acid methyl esters ($p < 0.05$)). With the cupric sulfate reagent, the extent of staining of the different classes was: fatty acid methyl esters = TAG > DAG > MAG. With the cupric acetate reagent maximal staining was approximately equal for all four classes.

The FAME quantitative mixtures also showed increases in staining with increases in C=C using the cupric acetate reagent (Fig. 3a) and relatively small changes using the cupric sulfate reagent with 9 min of charring (Fig. 3b) However, if plates were charred for only 5 min, then there was usually a general decrease in the response using either reagent. In particular, with the cupric sulfate reagent the relative staining of the saturated fatty acid methyl esters decreased about two fold.

These results clearly demonstrate that the cupric acetate reagent will yield an intensity of staining which increases with increasing amounts of unsaturation (Figs. 2 and 3a). The cupric sulfate reagent apparently chars all lipids, within a particular class, to about the same extent providing that the plate is heated for a sufficient length of time.

Plates stained with the cupric sulfate reagent have been observed to vary in response, from plate to plate (Fig. 2 and

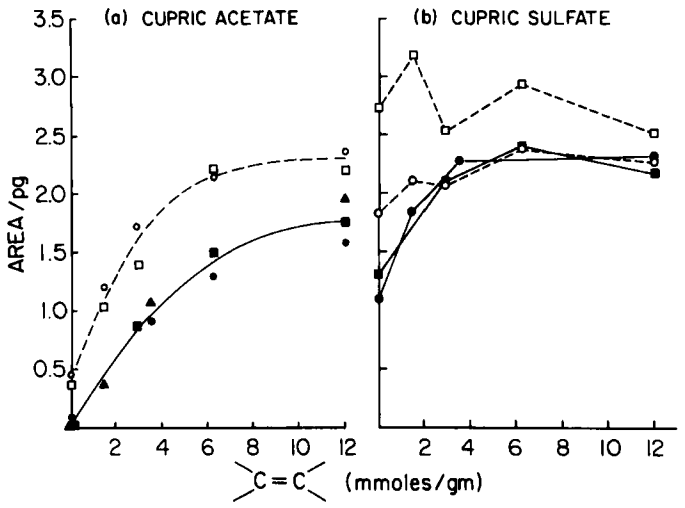

FIGURE 3. FAME stained with cupric acetate and cupric sulfate reagents. Samples were applied to plates, developed (6), stained and quantitated as described in the Methods. Plates were charred at 170° for 5 min (closed symbols, solid lines) or for 9 min (open symbols, dashed lines). Each symbol represents values from a single plate.

3b), but by including standards in each run we have found that they are internally consistent (7). TLC of naturally occurring phospholipids (phosphatidylinositol, phosphatidic acid, phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine), yielded average responses, on five separate plates, of 1.10 $\pm$ 0.09, 1.07 $\pm$ 0.07, 1.11 $\pm$ 0.14, 1.08 $\pm$ 0.07 and 1.06 $\pm$ 0.17 area x 10^6/nmole Pi and were linear up to 5 nmoles Pi (approximately 4 µg phospholipid). Sphingomyelin consistantly gave responses which were higher, about 1.7 area x 10^6/nmole Pi.

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