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Note

Sensitive and highly reproducible quantitative fluorescent thin-layer chromatographic visualization technique for lecithin and sphingomyelin

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Although numerous thin-layer chromatographic (TLC) procedures have been developed for the separation, visualization, and quantitation of lecithin (L) and sphingomyelin (S), relatively few techniques have employed sensitive fluorescent dyes for visualization[1–7]. Fluorescent dyes reported in the chemical literature include: Rhodamine 6G (ref. 1), Rhodamine B (ref. 2), 2′,7′-dichlorofluorescein (DCF)[3–5], 1-anilino-8-napthalene sulfonate[6] and 8-anilino-1-naphthalene sulfonate (8-ANS)[7]. These dyes are most often applied by spray techniques which are adequate for sensitive visualization. However, spray techniques contribute to uneven stain distribution as well as chromatogram-to-chromatogram variation in stain density. For accurate quantitation purposes by densitometry or reflectance spectrofluorometry of the TLC plates, the aforementioned variations may result in a low signal-to-noise ratio of varying background fluorescence intensity. To a large extent, these variations have been eliminated by employing a timed dipping technique[5]. Although the dip technique has been quite successful, aging problems have been reported for DCF.

The present investigation reports a new visualization technique which incorporates the fluorescent dye directly into the solvent system. Experimental data are presented for the applicability of this technique for the visualization and quantitation of microgram quantities of L and S.

EXPERIMENTAL

ITLC-type SG chromatography sheets were obtained from Gelman (Ann Arbor, MI, U.S.A.). The sheets were prepared and activated as previously described[5].

Lecithin (L-α-lecithin, from egg yolk, Type V-E) and sphingomyelin (from bovine brain, type I) from Sigma (St. Louis, MO, U.S.A.) were used to prepare the L/S ratio standards. A stock solution of S was prepared by adding 0.5 g of S to a 5-ml volumetric flask which was filled to volume with chloroform–methanol (9:1). A 1:1 L/S ratio standard was prepared by adding 50 μl of the stock sphingomyelin to 50 μl of lecithin [1 g per 10 ml of chloroform–methanol (9:1)] in a 5-ml volumetric flask which was filled to volume with chloroform–methanol (9:1). Subsequent ratios of 1.5:1, 2.0:1, 2.5:1, and 3.0:1 were similarly prepared by adding 75, 100, 125 and 150 μl of the commercial lecithin solution, respectively. Chromatography discs containing preapplied L/S ratio standards were prepared as previously described[5].

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A stock dye solution was prepared by adding 15 mg of 8-anilino-1-napthalene sulfonate (Mg salt) (Eastman Kodak, Rochester, NY, U.S.A.) to a 50-ml volumetric flask which was filled to volume with absolute ethanol. The chromatography solvent system, methylene chloride–stock dye solution–water (100:25:3), was prepared fresh weekly. The aqueous portion of the solvent system should be added last to prevent the formation of a cloudy solution. The solvent system is stable for more than 1 week if it is stored in a tightly stoppered brown bottle and shaken vigorously before use.

A 4-ml volume of well-mixed solvent system was transferred to a Seprachrom chromatography trough. The chamber was assembled and the solvent system was allowed to ascend for 3.0 min. The developed chromatogram was immediately removed and air dried in the dark for 20 min. Both L and S fluorophores were visible as bright green spots on a dark purple background when examined under long-wave UV light (375 nm). On occasion, chromatograms exhibiting tailing or elongation of the fluorophore spots were not scanned and the analysis was repeated.

The reflected fluorescence intensity of the chromatogram spots was measured with a Farrand Mark I spectrofluorometer which was equipped with a TLC scanning attachment and modified as previously described[5]. Measurements were made at an excitation wavelength of 365 nm (± 3 nm) and an emission wavelength of 465 nm (± 3 nm). The area of the recorded peaks was measured in triplicate with a Koizumi compensating polar planimeter.

A fluorescence stability study was performed as previously described[5]. The detection limits were established by serial dilution of the lipid solutions and TLC development until the lipid fluorophores could no longer be detected by the spectrofluorometer at a range setting of 1.0.

RESULTS AND DISCUSSION

The major advantage of incorporating the dye within the solvent system is that errors attributable to the spraying and dipping techniques are eliminated. Of secondary importance, the new visualization technique reduces the procedure time by eliminating the separate staining and subsequent drying steps. Preliminary investigation has shown that either DCF or 8-ANS could successfully be employed within the solvent system. Because of undesirable aging characteristics of DCF[5], the present study has evaluated 8-ANS. To facilitate dissolution of 8-ANS within the solvent system, the dye was dissolved in the absolute ethanol fraction. The optimal dye concentration was approximately 15 mg of 8-ANS per 50 ml of absolute ethanol. The water fraction of the solvent system should be added last to prevent the formation of a cloudy solution.

Separation distances using this technique are excellent: R_F values for L and S were 0.65 and 0.47, respectively. The detection limit of both the L and S fluorophores was below 0.1 μg.

The background fluorescence of the developed chromatograms was practically zero. Smooth baselines, without unsatisfactory irregularities, were observed upon scanning the chromatograms. A linear relationship was obtained for L/S ratios between 1 and 3 when the L/S area ratios were plotted *versus* the L/S weight ratios (Fig. 1). The linear regression parameters for these results are: correlation coefficient, 0.999; y-intercept, 0.048; slope, 0.72. Each data point represents an average of six test

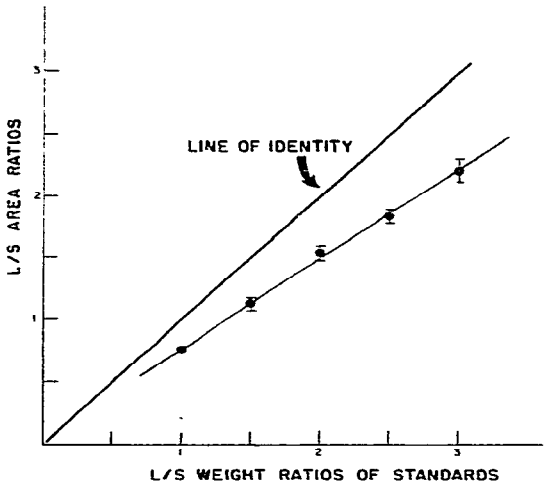

Fig. 1. Correlation of L/S area ratios *vs.* L/S weight ratios. The results depicted represent an average of six test analyses for each quantity studied. Refer to text for complete details.

results, with the exception of a single 1:1 L/S ratio test result which was eliminated by Dixon's criterion[8] for the elimination of outliers using a 95% confidence limit. The standard deviation for each data point is indicated on the graph. The average coefficient of variation was 3.5%.

Fluorescence stability studies were conducted over a 24-h period. Three 24-h, one 12-h, and two 1-h studies were performed and the average results of these studies are reported in Table I. In general, the L and S fluorophore intensity was observed to decrease slightly over 12 h and quite dramatically over 24 h, however, the L/S ratio was stable throughout. The results reported in Table I represent both wet and dry chromatogram measurements of the reflected fluorescence intensity. For accurate quantitation, chromatograms should be scanned either wet or dry, but not during the drying process.

TABLE I
FLUORESCENCE STABILITY OF 8-ANS STAINED L AND S
Relative fluorescence is reported in percentages of the fluorescence intensity of 6 μg of lecithin as measured at 0 h, after 5 min drying. Refer to text for complete details.

Time (h)	*Relative fluorescence*		*L/S ratio*
	L (6 μg)	*S (3 μg)*	
0.0	100.0	71.8	1.39
0.25	110.8	73.4	1.51
0.5	110.1	75.9	1.45
1	114.0	78.7	1.45
3	113.8	76.9	1.48
6	108.3	77.3	1.40
9	102.7	74.3	1.38
12	93.2	66.6	1.40
24	72.2	50.5	1.43

The fluorometric TLC procedure described herein is highly recommended for accurate and reproducible measurement of the L/S ratio. The advantages of the present method include: uniform staining, lower and more stable baselines, stable fluorescence over 12 h, and detection of μg quantities of L and S.

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