Journal of Chromatography, 82 (1973) 95–100
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 6672

A NEW PROCEDURE FOR INDUCING FLUORESCENCE IN QUANTITATIVE THIN-LAYER CHROMATOGRAPHY FOR ORGANIC COMPOUNDS

BRUCE G. SMITH

Department of Chemical Research Analytical, Special Separation Methods, G. D. Searle & Co., Skokie, Ill. 60076 (U.S.A.)

SUMMARY

A method is presented for introducing fluorescent chromophores into a variety of organic molecules on a thin-layer chromatogram for *in situ* analysis. The procedure involves impregnation of the adsorbent material with ammonium sulfate, developing the unknowns with known standards, exposing the developed plate to *tert.*-butyl hypochlorite and heating the chromatogram. Fatty acids, triglycerides, an amino acid, sugars, steroids, and prostaglandins have been analyzed in microgram quantities using this method.

EXPERIMENTAL

Apparatus

Fluorescent measurements were carried out with a Zeiss chromatogram spectrophotometer using the (Pr-M)[1] set-up with a mercury lamp light source in combination with a 365-nm activation filter. Scanning with the instrument was carried out perpendicular to the direction of chromatography using a slit aperture (2 mm × 12 mm). A Sargent-Welch recorder, Model SR, with a 1- to 100-mV span, was used to record results.

Areas of resulting peaks were calculated by triangulation (area = $^1/_2\ BH$). It is desirable for the same operator to calculate the same series of samples and standard curves.

Reagents

The chromatographically pure compounds used in this study include oleic acid, androstane-3,17-dione, xanthanoic acid, cholesterol *n*-propionate, N-methyl phenylalanine, D-glucose and triolein.

All solvents used to dissolve the samples or perform the chromatography were of analytical grade.

A Heraeus Type 350 oven was used to heat the chromatograms. The oven was placed in a hood and equipped with a vacuum source (water aspirator) for removal of vapors. A stainless-steel plate box supplied by Camag Inc., Model 23-250, was placed in the oven after the plastic cover had been removed. Application of the

solutions to the chromatographic plate was accomplished by the use of 5-μl capillaries, Dade P 4518-05.

The Silica Gel G adsorbent was obtained from Merck AG, Darmstadt. The adsorbent was washed with hot ethyl acetate and dried at 100° overnight and then stored in glass bottles. Ammonium sulfate was purchased from J. T. Baker, Catalog No. 0792.

Procedure

Plate preparation. In the preparation of the chromatographic plate, 1.5 g of ammonium sulfate are dissolved in 60 ml of distilled water and added to 30 g of Silica Gel G. After shaking the slurry for 15 sec, it is spread on clean chromatographic plates, 250 μ in thickness. The plates are allowed to dry for 16 h at room temperature and then stored in sealed containers for future use.

Production of fluorescence —Method 1. After application of samples and development in a normal saturated chamber, the plates are dried for 30 min in a nitrogen environment. Drying is accomplished by placing the plate in an empty chamber and inserting a rubber hose with a moderate nitrogen flow. The plate is put into a chamber (kept in hood) saturated with *tert.*-butyl hypochlorite vapors for 15 min (a 25-ml erlenmeyer flask half full of *tert.*-butyl hypochlorite kept in the bottom of the sealed chamber works nicely). After removal, the plate is inserted into a plate box, which had been put in the oven already and had been heated for 15 min at 150–180°. The particular temperature is dependent upon the compound to be analyzed (more specific temperatures will be given with each compound).

The plate is removed and allowed to cool for 10 min. It can then be scanned using a excitation wavelength of 365 nm and the optimum fluorescent emission determined and observed. The latter varies with the compound under analysis.

Production of fluorescence —Method 2. After development and drying, the plate is placed into the top slot of a plate box already in the oven for 15 min with the adsorbent surface down. To a separate coated chromatographic plate 8 × 8 in.

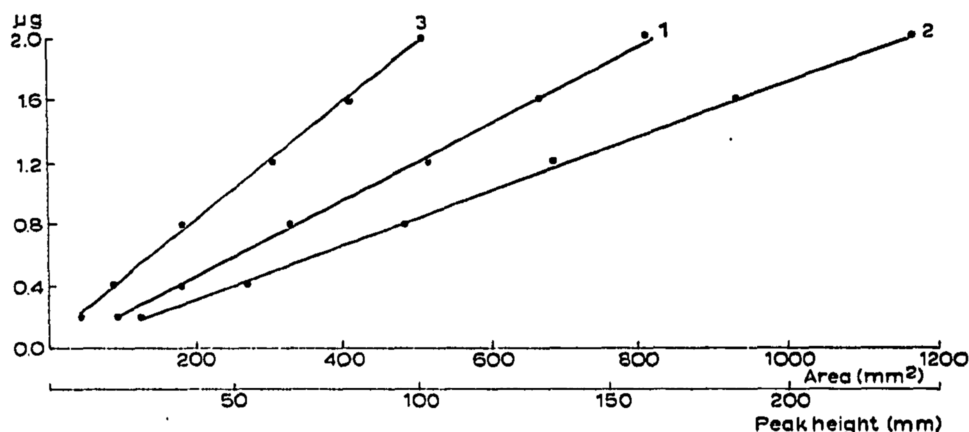

Fig. 1. Calibration curve for different amounts (0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 μg) of cholesterol *n*-propionate on ammonium sulfate–Silica Gel G. Solvent: 100% benzene, R_F 0.50. Emission measured at 600 nm; scan 30 mm/min. Fluorescence produced by Method 2 at 160°. 1 = Peak height; 2 = area; 3 = peak height of same plate scanned three days later.

in size, 2–3 ml of *tert.*-butyl hypochlorite are applied with a pipette in a random fashion and the plate is inserted into the plate box with its surface facing that of the plate to be quantitated. Fifteen minutes later the plate to be quantitated is removed and allowed to cool for 10 min before being scanned.

RESULTS AND DISCUSSION

In order to prove the reproducibility of direct fluorometry using this procedure, three spots, each containing 1.5 μg of cholesterol *n*-propionate, were applied to a Silica Gel G plate containing ammonium sulfate and developed with 100% ben-

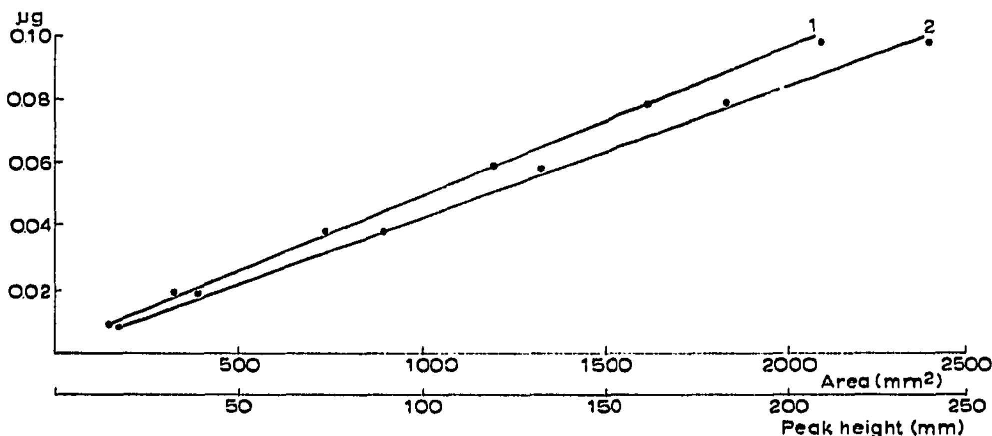

Fig. 2. Calibration curve for different amounts (0.01, 0.02, 0.04, 0.06, 0.08, and 0.10 μg) of xanthanoic acid (9-xanthene-carboxylic acid) on ammonium sulfate–Silica Gel G. Solvent: ethyl acetate–glacial acetic acid–benzene (20:1:79), R_F 0.35. Emission measured at 440 nm; scan 15 mm/min. Fluorescence produced by Method 2 at 170°. 1 = Peak height; 2 = area.

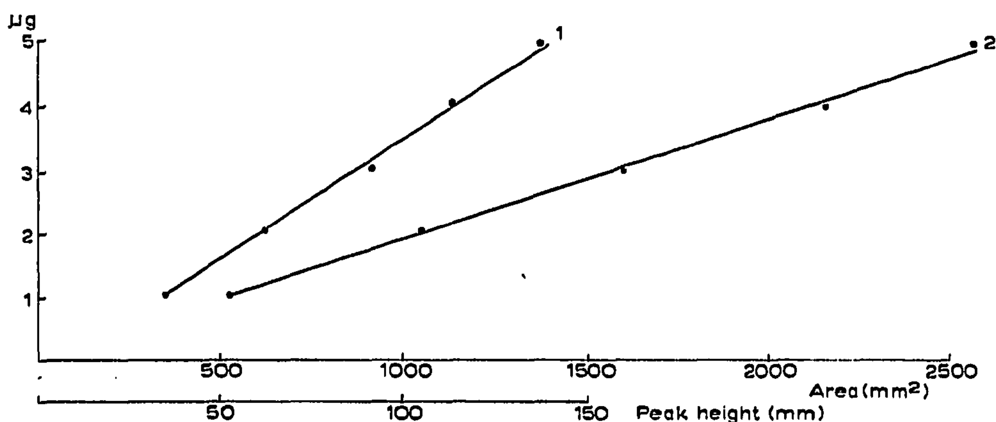

Fig. 3. Calibration curve for different amounts of androstane-3,17-dione (1.05, 2.06, 3.06, 4.07, and 5.07 μg) on ammonium sulfate–Silica Gel G. Solvent: ethyl acetate–benzene (30:70), R_F 0.52. Emission measured at 600 nm; scan 6 mm/min. Fluorescence produced by Method 1 at 175°. 1 = Peak height; 2 = area.

zene. After drying, the plate was subjected to Method 2 at 160°. Each spot (R_F 0.49) was scanned three times using a scan speed of 30 mm/min and the emission measured at 600 nm. This procedure gave a relative standard deviation ($n = 9$) of 2.7% for the area. One of the above spots was scanned five times to check the instrumental reproducibility and the effect of repeated scans on the ability of that chromophore to fluoresce. The relative standard deviation ($n = 5$) for the area was 1.5% and for the peak height 0.88%.

Three 1.5-μg portions of cholesterol *n*-propionate were applied to a Silica Gel G plate without ammonium sulfate, developed with 100% benzene, dried under nitrogen, sprayed with sulfuric acid-water (50:50) and heated at 110° for 15 min. Each spot (R_F 0.56) was scanned three times using a scan speed of 30 mm/min and the emission measured at 600 nm.

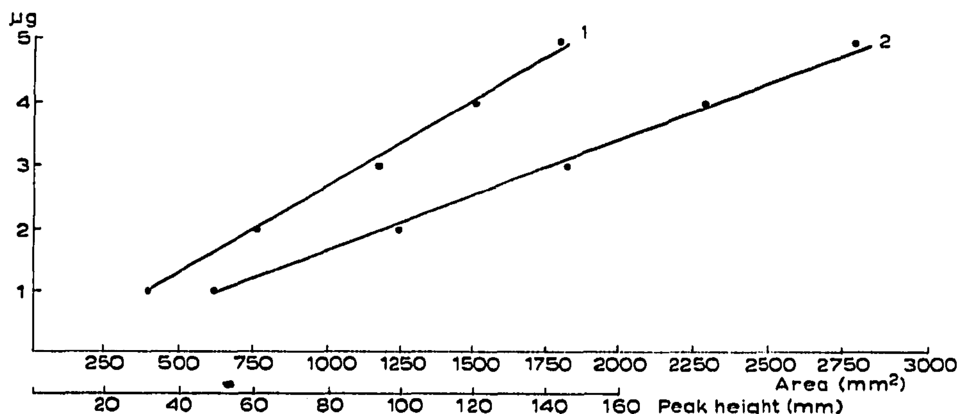

Fig. 4. Calibration curve for different amounts (1, 2, 3, 4, and 5 μg) of oleic acid on ammonium sulfate–Silica Gel G. Solvent: ethyl acetate–glacial acetic acid–*n*-hexane (25:1:74), R_F 0.41. Emission measured at 580 nm; scan 6 mm/min. Fluorescence produced by Method 1 at 160°. 1 = Peak height; 2 = area.

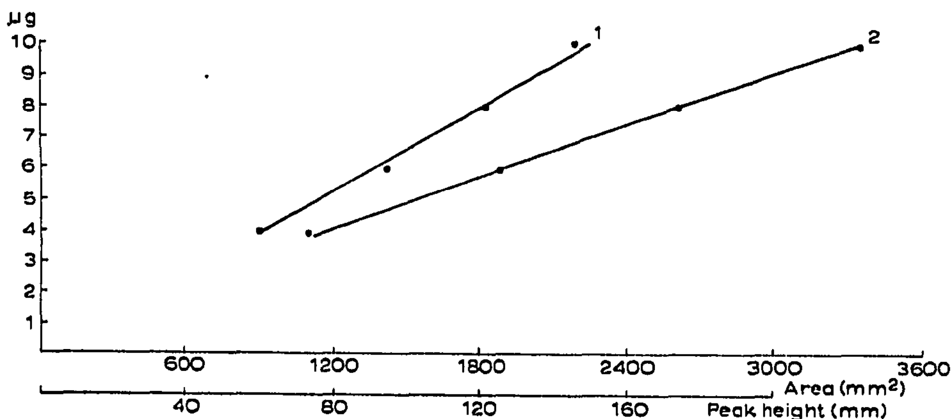

Fig. 5. Calibration curve for serial amounts (4, 6, 8, and 10 μg) of triolein on ammonium sulfate–Silica Gel G. Solvent: ethyl acetate–*n*-hexane–glacial acetic acid (7:92:1), R_F 0.46. Emission measured at 580 nm; scan 6 mm/min. Fluorescence produced by Method 1 at 180°. 1 = Peak height; 2 = area.

The relative standard deviation ($n = 9$) for the area was 11.3%. Scanning one of the spots five times gave a relative standard deviation for the area of 2.1% and for the peak height of 1.4%.

Quantitation of compounds which have reacted with sulfuric acid on a thin-layer plate to give a colored spot or fluorescent product has met with difficulty and has been overcome only by completely charring the compound to give a stable density spot with no fluorescence[2]. It can then be quantitated by spectrodensitometry.

Silica gel modified with ammonium bisulfate in thin- layer chromatography has produced fluorescence in steroids but little or no fluorescence with lipids[3]. The combination of ammonium sulfate and *tert.*-butyl hypochlorite produces a stable fluorescent product on a thin-layer chromatogram for a wide variety of organic compounds, which can give the user a broad-spectrum approach.

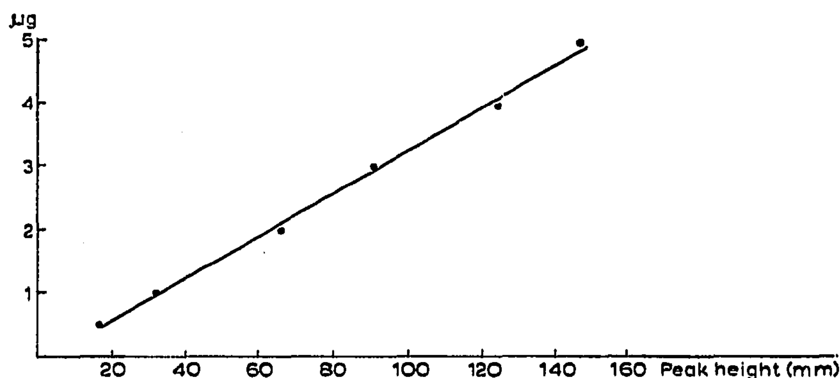

Fig. 6. Calibration curve for different amounts (0.5, 1, 2, 3, 4, and 5 µg) of D-glucose on ammonium sulfate–Silica Gel G. Solvent: ethanol–glacial acetic acid–distilled water (90:5:5), R_F 0.53. Emission measured at 440 nm; scan 150 mm/min. Fluorescence produced by Method 2 at 170°.

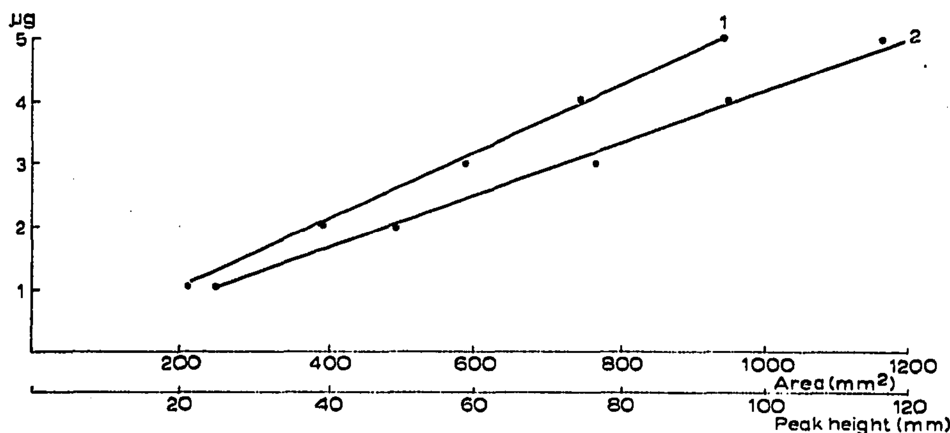

Fig. 7. Calibration curve for different amounts (1, 2, 3, 4, and 5 µg) of N-methyl phenylalanine on ammonium sulfate–Silica Gel G. Solvent: chloroform–methanol–distilled water–glacial acetic acid (64:30:4:2), R_F 0.36. Emission measured at 580 nm; scan 15 mm/min. Fluorescence produced by Method 1 at 175°. 1 = Area; 2 = peak height.

Method 1 was used to introduce fluorescence into the following compounds: androstane-3,17-dione, oleic acid, triolein and N-methyl phenylalanine. Results are represented in Fig. 3, 4, 5, and 7. Cholesterol *n*-propionate, xanthanoic acid and D-glucose were subjected to method 2 and results illustrated in Figs. 1, 2, and 6.

CONCLUSION

The fluorogenic and chromogenic reaction of organic compounds with *tert.*-butyl hypochlorite in combination with ammonium sulfate is unknown. It appears as though the *tert.*-butyl hypochlorite serves a dual purpose, *viz.* first to oxidize the organic compounds, and secondly to modify the ammonium sulfate to produce a more reactive reagent. Either ammonium sulfate or *tert.*-butyl hypochlorite subjected to this procedure without the use of the other reagent gives little or no fluorescence when tested with the present compounds and sixteen additional organic compounds.

The present procedure of fluorescence production is suitable for quantitative analysis in that the ammonium sulfate and *tert.*-butyl hypochlorite are applied to the chromatographic plate in a uniform manner. One reagent is dispersed in the adsorbent matrix through solution and the other applied uniformly in a vapor phase.

ACKNOWLEDGEMENT

I wish to thank Ms. Cynthia Liss for her technical assistance.

REFERENCES

1 E. Stahl and H. Jork, *Zeiss Inform.*, 68 (1968) 52.
2 J. Touchstone, T. Murawec, M. Kasparow and W. Wortmann, *J. Chromatogr. Sci.*, 10 (1972) 490.
3 J. Touchstone, T. Murawec, M. Kasparow and W. Wortmann, *J. Chromatogr.*, 66 (1972) 172.