Journal of Chromatography, 79 (1973) 179–185

CHROM. 6589

APPLICATION OF DIRECT SPECTROPHOTOMETRY TO THE ANALYSIS OF CHROMATOGRAMS

III. A NEW MICRO THIN-LAYER CHROMATOPLATE AND ITS APPLICATION TO THE ANALYSIS OF TOCOPHEROLS

TAKAAKI ARATANI

Department of Chemistry, Faculty of General Education, Hiroshima University, Higashisenda, Hiroshima (Japan)

KIYO MITA

Department of Food and Nutrition, Faculty of Home Economics, Hiroshima Women's University, Ujina, Hiroshima (Japan)

AND

FUMIE MIZUI

Department of Food and Nutrition, Hiroshima Bunka Women's Junior College, Gion, Hiroshima (Japan).

(Received December 12th, 1972)

SUMMARY

A method for the more efficient utilization of thin-layer chromatoplates is described.

Dipping quartz microscope slides into 46 % hydrogen fluoride solution is used to etch plates on which very thin layers (in general above 20 μm thick) can then be spread, and the layers are thin enough to separate very small amounts of materials in a short time. Furthermore, thin layers of sorbent prepared in hollows etched on the quartz plates are protected from mechanical damage and are stable without the need for special storage conditions.

INTRODUCTION

Transmission spectrophotometric procedures have been widely used for the quantitation of small amounts of materials in biological systems. In particular, thin-layer chromatography (TLC) on microscope slides has become a well established technique in analytical chemistry[1–5]. Many kinds of ready-made or laboratory-made chromatoplates with sorbent layers are available and the preparation of micro thin-layer plates has been proposed by many workers.

ZWEIG[6] reviewed many different types of adsorbent layers and MACEK AND BEČVÁŘOVÁ[7] also reviewed papers, ready-to use plates and flexible sheets for TLC. SNYDER[8] summarized advanced concepts in TLC. PIEL[9] described the separation of dyes on a silica gel with a particle size less than 1 μm, but made no further advances.

MARIÁN[10] proposed the preparation of sheets with very thin layers with a silica gel membrane of a few microns thickness, and EDGAR[11] suggested that micro thin-layer chromatoplates could be prepared by placing the adsorbent in a scratch (0.2 mm wide and 0.1 mm deep) made with a sharp steel file in a microscope slide. DE OSSÓ[12] proposed the use of very long development paths on pre-coated zigzag-shaped silica gel layers (25 μm thick) on the plates (20 × 20 cm), and KIRCHNER[13] described a new method of preparative TLC involving the use of thicker layers contained within a stainless-steel framework with thin stainless-steel wires to support the adsorbent layers (1/8–1/2 in. thick). BROICH *et al.*[14] showed that chromatography on extra-thin films of silica gel provides increased sensitivity in the detection of drugs. SVETASHEV AND VASKOVSKY[15] recently described the TLC of lipids on a silica gel with a particle size of 2–7 μm.

In order to evaluate our procedure as a potential quantitative method for microanalysis the shifts in the absorption maxima of *dl*-α-tocopherol with varying concentrations and time stability of the spectra were investigated. In part II[16], we showed that a linear decrease in the thickness of the sorbent layer is useful in decreasing the shift of the absorption maximum. In this paper, we describe the detailed preparation of very thin layers which are protected from mechanical damage on quartz plates using silica gel, and sensitive and rapid direct transmission spectrophotometric procedures for the determination of tocopherols.

EXPERIMENTAL

Preparation of the micro thin-layer chromatographic plates

Quartz microscope slides (76 × 26 × 0.7 mm) were cleaned thoroughly with a detergent solution, rinsed with water and dried. Two cleaned quartz slides were held together firmly with clips and the four sides and the edges (3 mm width) of both surfaces were coated with solid paraffin. The pair of coated quartz slides was then detached from the clips and dipped into 46% hydrogen fluoride solution at 30° for different periods of time. After boiling the slides repeatedly in distilled water, they were rinsed with xylene, methanol and distilled water and then allowed to dry at 110°. The relationship between the time of dipping in the hydrogen fluoride solution and the depth of the hollow etched in the quartz slides is shown in Fig. 1.

A slurry of Silica Gel G (Merck, Kieselgel G; above 325 mesh) sorbent was spread into the hollow on the quartz slide with a glass roller and the excess of the slurry was scraped off with a flat spatula so as to make the surface of the sorbent in the hollow level with the surface of the quartz slide. Superfluous sorbent was wiped off the chromatoplates, which were dried at 110° for 1 h and stored in a desiccator over dry silica gel until required for use.

Materials

Authentic *dl*-α-tocopherol and tocopherols from soyabean oil were used as samples. A stock benzene solution of tocopherols suitable for micro-TLC was prepared so as to contain 6.1 μg/μl and stock ethanolic solutions of *dl*-α-tocopherol suitable for obtaining the calibration curve contained 1–20 μg per spot. All solvents were optically pure.

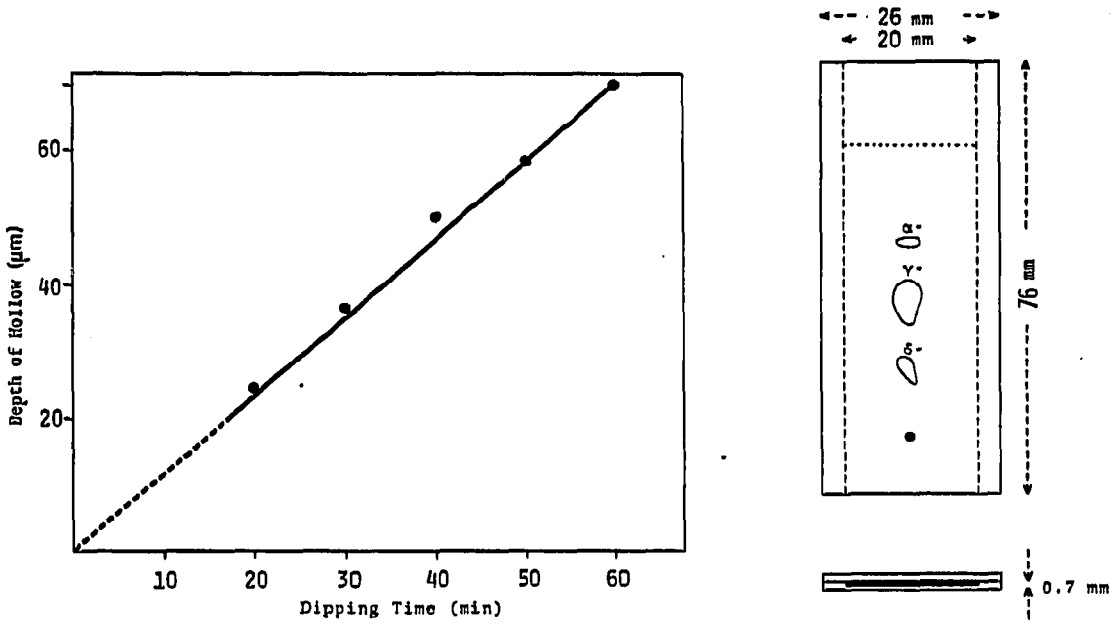

Fig. 1. Effect of time of dipping in 46% hydrogen fluoride solution on the depth of the hollow etched in the quartz plate at 30°.

Fig. 2. Separation of tocopherols using a very thin layer of silica gel. Development with *n*-hexane–chloroform (50:50). R_F values for tocopherols: α-, 0.74; γ-, 0.47; δ-, 0.23. Amount of sample, 3.1 μg. Particle size of silica gel, above 325 mesh; layer thickness, 53 μm.

Chromatography of tocopherols

A Hamilton microsyringe was used to apply the samples on to the chromatoplates. Chromatograms on the quartz plates were developed under nitrogen in a glass tank (85 × 85 × 33 mm) containing 10 ml of solvent (*n*-hexane–chloroform, 50:50), and then placed in a vacuum desiccator at room temperature in the dark. The chromatograms were sprayed with bathophenanthroline–$FeCl_3$ reagent[17]. Chromatograms of tocopherols (3.1 μg) in thin layers (53 μm thick) of silica gel (above 325 mesh) are shown in Fig. 2.

Preparation of the calibration curve of dl-α-tocopherol

The double-beam spectrophotometer used was a Shimadzu Model MPS-50 (Japan) recording instrument equipped with end-on photomultiplier tubes and a scanner having a roller to move the plate at the rate of 45 mm/min. A deuteron lamp was used.

The direct transmission spectrophotometric procedure involved covering the plate with a thin quartz plate of identical dimensions, fixing the edges together with masking tape and introducing the combined plates into the transmission attachment.

The centre of the spot was located by manual adjustment of the position of the plate until the maximum signal was registered by the photomultiplier microphotometer, and the plate was aligned so that the centre of the spot was scanned. Each spot was scanned in the direction of the solvent flow.

The calibration curve for *dl*-α-tocopherol obtained from the relationship between the amount of sample present (2.4, 5.6, 7.5, 9.8, 14.8 and 19.6 μg per spot) and the area under each absorption peak (291 nm) using the thin layer (70 μm thick) of silica gel (above 325 mesh) is shown in Fig. 3.

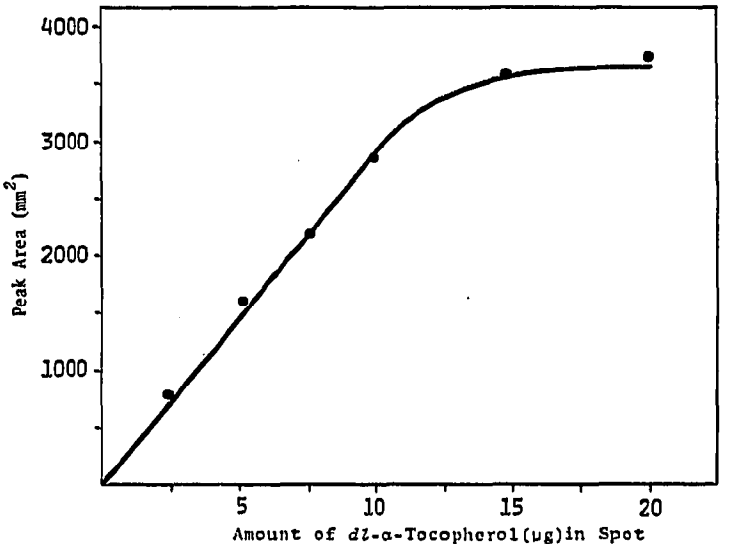

Fig. 3. Calibration curve for *dl*-α-tocopherol using a very thin layer of silica gel by transmittance at 291 nm. Particle size of silica gel, above 325 mesh; layer thickness, 70 μm.

RESULTS AND DISCUSSION

RADECKA AND WILSON[18] reported that attempts to standardize satisfactorily the conditions of chromatography from plate to plate were not successful, coefficients of variation for integral readings between plates being as high as 20%. It is important, therefore, that the preparation of the thin adsorbent layers should be carried out with great care so as to prevent the occurrence of defects, as also pointed out by GOLDMAN AND GOODALL[19].

In this investigation, chromatographic micro thin-layers of good quality were conveniently made in the hollow prepared on the plate by dipping an ordinary-quartz microscope slide into 46% hydrogen fluoride solution. Because optical inhomogeneities would have been produced on the surface of the plates if they were cleaned in concentrated chromic acid solution, the plates were rinsed in a suitable boiled detergent solution.

Fig. 1 shows that the depth of the hollow in the plate can be considerably influenced by the length of time the quartz plate is dipped in the hydrogen fluoride solution. The plate must therefore be prepared with extreme care and the hollow must be uniform throughout its depth. Furthermore, it is necessary to carefully clean the surface and underside of both the chromatographic plate and the cover plate so as to remove sorbent before scanning.

MADSEN AND LATZ[20] reported that the peak areas obtained by scanning in the direction of plate development are greater than those obtained by scanning perpen-

dicularly to the direction of plate development. This behaviour is observed because the chromatograms are elliptical, with the major axis in the direction of plate development. The chromatograms were therefore scanned in the direction of plate development.

Shift of the absorption maximum of dl-α-tocopherol

PORTER *et al.*[21] reported that the absorption maxima in the ultraviolet spectra of tocopherols spotted on silica gel were shifted 10 nm towards the ultraviolet. SHELLARD AND ALAM[22] used light petroleum and liquid paraffin in order to reduce the scattering of the incident light by the particles of the thin-layer sorbents, but it was impossible to prevent it completely. In the present studies, the absorption maximum of *dl*-α-tocopherol in the sorbent layer (70 μm thick) of silica gel (above 325 mesh) on the quartz plate was found to fluctuate by a maximum of only 1 nm towards the ultraviolet compared with that (292 nm) in the ethanolic solution, and it is therefore obvious that this technique is suitable for identification purposes.

Calibration curve for dl-α-tocopherol

Fig. 3 shows that when more than 10 μg of *dl*-α-tocopherol per spot are used, the relationship between the amount of sample and the area under the absorption peak (291 nm) is no longer linear. Thus, as little as *ca.* 1 μg of *dl*-α-tocopherol could be measured by this technique.

The reproducibility of the peak area for each spot (2.4, 5.0, 7.5 and 9.8 μg per spot) was determined by scanning five times, and the standard deviation was 2 % for the recorded peak area of each sample.

The linearity of the calibration curve is dependent on the correct choice of the scanning wavelength, so that provided care is taken to scan the chromatogram at the absorption maximum, the quantitative analysis of an unknown sample is capable of giving accurate results when using the calibration curve. Furthermore, the method of sample application affects the linearity of the calibration curve, and highly concentrated areas of the applied spot should be avoided as the sample tends to flow, resulting in uneven spreading of the sample.

SAMUELS AND FISHER[23] indicated that a diffuse spot can show a greater total colour in the colour development of a chromatogram than does the same amount of material in a compact spot. Serious non-uniformity of the thin layer on the plate, therefore, results in non-linearity of the calibration curve. It must be pointed out that although the use of a double-beam instrument overcomes some of the problems due to non-uniformity of the sorbent layer thickness, background scatter, sample application and other factors, the greatest sensitivity occurs at the wavelength corresponding to the absorption maximum of each compound.

Chromatography of tocopherols from soyabean oil

In general, useful separations of tocopherols by TLC have been achieved by using the following systems: on alumina with benzene[24] or benzene–diethyl ether (50:50)[25]; on silica gel with chloroform[24,26] or benzene[27]; on alumina–zinc carbonate (3:1) with chloroform[28]; on Aluminium Oxide G–zinc carbonate (3:1) with chloroform[29]; on Kieselgel G–zinc carbonate (2:1) with benzene–cyclohexane (30:70)[29]; and on silica gel with chloroform and then diisopropyl ether–light petroleum (20:80)[30].

Fig. 2 shows that the best separation of tocopherols (3.1 μg) from soyabean oil was achieved in about 13 min by using a very thin layer (53 μm thick) of silica gel (above 325 mesh) with *n*-hexane–chloroform (50:50). Under these conditions, R_F values for α-, γ- and δ-tocopherols were 0.74, 0.47 and 0.23, respectively, and these values were confirmed by comparison of their UV spectra and thin-layer chromatograms with those of authentic samples.

High resolution with very thin layers in TLC can be achieved by the application of a very small amount of sample. Therefore, strict attention in the preparation of plates with very thin layers must be paid, especially to the accuracy in any quantitative microanalysis.

CONCLUSIONS

Major problems still to be investigated in TLC include increased resolution, shorter separation times and improved quantitation and reproducibility, as also pointed out by SNYDER[8]. Furthermore, because the reduced shift of the absorption maximum is a very important factor in chromatographic microanalysis, it is necessary to use very thin layers in the application of direct spectrophotometry to TLC.

In this paper, a new method for quantitative detection with very thin layers in TLC using a sorbent layer prepared in a hollow on a thin quartz plate has been described. The chromatography of tocopherols (3.1 μg) on very thin layers (53 μm thick) of silica gel (above 325 mesh) provides rapid and high resolution, increased sensitivity and a decreased shift of the absorption maximum. Furthermore, the very thin layers are protected from mechanical damage by covering the plate with a thin quartz slide of identical dimensions.

REFERENCES

1 H. GÄNSHIRT, in E. STAHL (Editor), *Thin-Layer Chromatography: A Laboratory Handbook*, Springer-Verlag, New York, 2nd ed., 1969, p. 133.
2 U. A. TH. BRINKMAN, G. DE VRIES AND E. VAN DALEN, *J. Chromatogr.*, 25 (1966) 447.
3 F. W. H. M. MERKUS, *J. Chromatogr.*, 41 (1969) 497.
4 U. A. TH. BRINKMAN AND G. KINGMAN, *Z. Anal. Chem.*, 249 (1970) 16.
5 G. DE VRIES AND U. A. TH. BRINKMAN, *J. Chromatogr.*, 64 (1972) 374.
6 G. ZWEIG, *Anal. Chem.*, 42 (1970) 349R; 44 (1972) 51R.
7 K. MACEK AND H. BEČVÁŘOVÁ, *Chromatogr. Rev.*, 15 (1971) 1.
8 L. R. SNYDER, *J. Chromatogr.*, 63 (1971) 7.
9 E. V. PIEL, *Anal. Chem.*, 38 (1966) 670.
10 M. MARIÁN, *J. Chromatogr.*, 42 (1969) 117.
11 D. EDGAR, *J. Chromatogr.*, 43 (1969) 271.
12 F. J. DE OSSÓ, *J. Chromatogr.*, 58 (1971) 294.
13 J. G. KIRCHNER, *J. Chromatogr.*, 63 (1971) 45.
14 J. R. BROICH, D. B. HOFFMAN, S. ANDRYAUSKAS, L. GALANTE AND C. J. UMBERGER, *J. Chromatogr.*, 60 (1971) 95.
15 V. I. SVETASHEV AND V. E. VASKOVSKY, *J. Chromatogr.*, 67 (1972) 376.
16 T. ARATANI AND F. MIZUI, *J. Chromatogr.*, 79 (1973) 173.
17 C. C. TSEN, *Anal. Chem.*, 33 (1961) 849.
18 C. RADECKA AND W. L. WILSON, *J. Chromatogr.*, 57 (1971) 297.
19 J. GOLDMAN AND R. R. GOODALL, *J. Chromatogr.*, 47 (1970) 386.
20 B. C. MADSEN AND H. W. LATZ, *J. Chromatogr.*, 50 (1970) 288.
21 W. L. PORTER, A. S. HENICK, J. I. JEFFERS AND L. A. LEVASSEUR, *Lipids*, 6 (1971) 9.
22 E. J. SHELLARD AND M. Z. ALAM, *J. Chromatogr.*, 32 (1968) 472 and 489; 33 (1968) 347; 35 (1968) 72.
23 S. SAMUELS AND C. FISHER, *J. Chromatogr.*, 71 (1972) 291.

24 A. SEHER, *Mikrochim. Acta*, (1961) 308.
25 D. C. HERTING AND E. E. DRURY, *J. Chromatogr.*, 30 (1967) 502.
26 P. A. STURM, R. M. PARKHURST AND W. A. SKINNER, *Anal. Chem.*, 38 (1966) 1244.
27 R. A. DILLEY AND F. L. CRANE, *Anal. Biochem.*, 5 (1963) 531.
28 H. SCHMANDKE, *J. Chromatogr.*, 14 (1964) 123.
29 P. G. ROUGHAN, *J. Chromatogr.*, 29 (1967) 293.
30 K. J. WHITTLE AND J. F. PENNOCK, *Analyst (London)*, 92 (1967) 423.