Separation and detection of carboxylic acids by thin-layer chromatography

The non-volatile carboxylic acids occurring in living cells have been fractionated by several excellent paper chromatographic methods (review, *e.g.* in ref. 1). More recently thin-layer chromatography, which has a higher sensitivity and is faster, has been used for the same compounds. The stationary phase has been either silica gel[2–7], cellulose[5,7–10], or polyethylene glycol[11], and the mobile phase either acid[3–11] or alkaline[2,5,7,8,10].

The spots have usually been made visible by spraying the dried chromatograms with an appropriate indicator. The smallest amounts to be detected were 8.5 nmoles malonic acid (bromocresol green)[2] and 2.6–5.2 nmoles citric acid (8-hydroxyquinoline as fluorescence indicator)[9]. Most investigators, however, use at least 10 times this amount for thin-layer chromatography. The aniline–ribose spray used by HIGGINS AND VON BRAND[7] was less sensitive than the indicators. BACHUR[10] recently published another detection method based upon the ability of acids to inhibit the browning of pyridine treated cellulose in ultraviolet light. This method was sensitive for acids at a level of 2.5 nmoles, but compounds absorbing at 250 mμ would be detected as well.

The sensitivity of the detection method is limited by the difference in pH between the spots and the background of the plate, and by the choice of indicator. It might be difficult to increase the pH of the background because of the slow evaporation of the acid contained in the chromatographic solvent and because some stationary phases (especially cellulose powder) yield rather acid suspensions in water (pH about 4). GOEBELL AND KLINGENBERG[8] adjusted the pH of the suspension of cellulose powder to 11.5 with NaOH before preparation of the thin-layer plates and thereby avoided the use of ammonia vapor for neutralizing the developed plates[4,9].

The present note describes a modification of the thin-layer technique for cellulose plates which permits the use of the many solvent systems developed for paper chromatography. In addition a new and convenient indicator system with very high sensitivity is described.

Experimental

The plates were about 0.1 mm thick and prepared with the Desaga equipment (Desaga, Heidelberg) according to the specifications for the cellulose powder (MN 300, Macherey, Nagel & Co., Düren) (*cf.* ref. 12). The suspension of cellulose powder was adjusted to pH 9.0 with 0.1 *N* NaOH (about 1 ml per 90 ml suspension) before application to the plates. The start spots were placed about 2 cm from the edge of the plate and the front of the solvent moved 120 mm above the spots.

Most of the solvents used for paper chromatography consist of two phases, the organic phase is used for developing the chromatogram and the inorganic phase for saturating the tank atmosphere[1]. This was also done in the Desaga tank, the sides of which were covered with filter paper in the usual way. The inorganic phase of the solvent was used to wet this filter paper and the organic phase was contained in a stainless steel trough (203 mm long, 15 mm high, 22 mm wide) which rested on the bottom of the tank. A piece of stainless steel sheet was also placed here. This was bent and cut to hold the plate free of the inorganic phase during the equilibration period prior to the chromatography.

2′,7′-Dichlorofluorescein (Merck, Darmstadt) was used as indicator. In order to

avoid spraying with the indicator, it was dissolved in the organic phase of the solvent at a concentration of about 4 mg %. About 20 min drying of the plates with forced hot air was sufficient for the detection of most of the spots in ultraviolet light, but the contrast was improved by passive drying overnight, presumably because the liberation of acid from the plate was limited by diffusion in the cellulose layer.

The plates were photographed on 35 mm film in the light from two UVS-12 lamps (254 mμ) (Ultra-Violet Products, Inc., Calif.). The camera was placed about 50 cm from the plate and the lens was covered with an ordinary ultraviolet filter and a Wratten filter No. 12. The film (Adox KB 14, Adox Fotowerke GmbH, Frankfurt/Main) was developed (with developer Adox E 10) and rinsed in a wetting agent after washing in order to avoid drying marks on the negatives. The prints were made on Brovira BEH 1 paper (Agfa-Gevaert, Leverkusen) and developed with Kodak D 163 developer diluted with 1 vol. water (Kodak Ltd., London).

Results and discussion

Fig. 1 shows the use of a paper chromatographic solvent for thin-layer chromatography. Fumarate, succinate and malate were as well separated by the present method as by the original paper chromatographic method[13]. This was also the case with other solvents originally developed for paper chromatography[1]. As these plates were rather thin they could not carry more than 30–40 nmolos of fumarate or succinate with the solvent indicated. It was found necessary to equilibrate and develop the plates in a tank containing both the inorganic and the organic phase of the solvent. If only the organic phase was present, tailing and low R_F values resulted and the solvent acid would be very difficult to remove from the plates after chromatography. The time needed for the equilibration should be found by trial. With these plates 20 min might be sufficient.

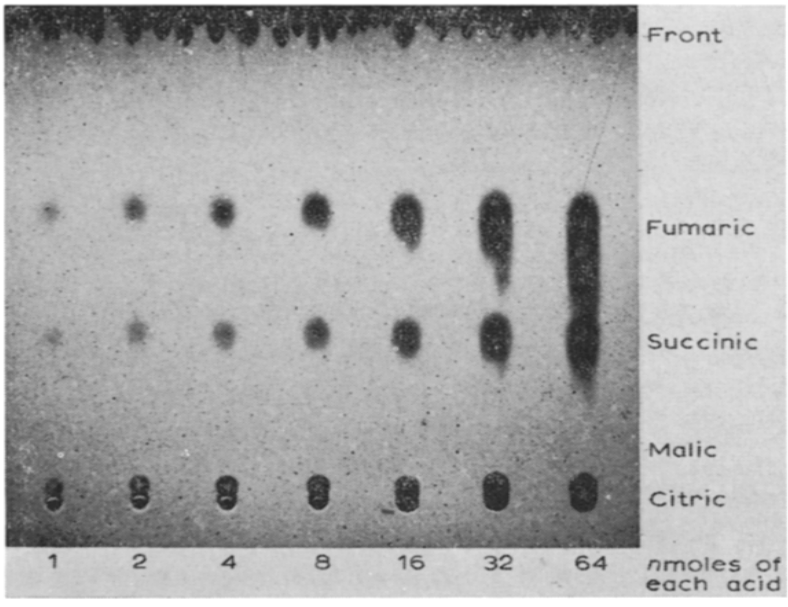

Fig. 1. An example of the adaptation of a paper chromatographic method to thin-layer chromatography. Solvent: chloroform–*tert.* amyl alcohol–100% formic acid–water (136:24:27:83)[13]. Plate equilibrated 1 h in the tank before development. Development time: 35 min. For further details, see text. The front contained impurities eluted from the stationary phase. (Exp. C 0052).

The R_F values are slightly dependent on the pH of the cellulose suspension, the thickness of the plates and the time of equilibration, but were in fairly good agreement with those published for paper chromatography[1]. The order of elution of the acids was the same with all solvents tried as it was for paper chromatography. An unknown acid might be identified preliminarily by the empirical system introduced by RADECKE[14].

Dichlorofluorescein was found to be an ideal indicator for the purpose. It permitted very easy documentation of the chromatograms by photography and it would be distributed evenly on the plates by most solvents. The sensitivity of the chromatographic method was at least 1 nmole succinate or fumarate (see Fig. 1). The use of plates made from cellulose suspensions of pH 7.0 or lower concentration of indicator does not increase the sensitivity. Substances absorbing at 250 mμ could be detected on the weakly fluorescing acid plates and the interference of such compounds with the detection of the acids can be avoided by photographing the plates in light at 366 mμ.

Preliminary experiments showed that fluorimetric scanning of the plates could be used for determination of the content of acid in the spots. The variation in the fluorescence of the background of the plates caused by the highly fluorescent grains (see Fig. 1) would amount to 5 % of the total fluorescence corresponding to about 1 nmole acid. There appeared, however, to be serious limitations to the applicability of this scanning method to extracts of biological materials, because the concentration distribution within the spots of these extracts was different from that of the standard spots.

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