Two-way separation of carboxylic acids by thin layer electrophoresis and chromatography

In the course of investigations carried out in this laboratory, a method was required for the analysis of a mixture of carboxylic acids. Although some work has been reported on thin layer chromatographic separations using silica gel[1,2] and cellulose[3,4] as the stationary phases, with either an acid[1-4] or an alkaline[3] mobile phase, there does not appear to be any reference to a two-dimensional system including an electrophoretic separation. This paper describes a method which allows the simultaneous separation of at least 15 carboxylic acids, in a two-dimensional system within 5 h. The separation is started by electrophoresis in formic acid buffer, in the first dimension, followed by chromatography in isoamyl alcohol saturated with formic acid in the second.

Reagents

Cellulose powder MN 300 Macherey, Nagel & Co was used. Formic acid and isoamyl alcohol were of analytical grade from British Drug Houses; 2,7-dichlorofluorescein was from Merck, Darmstadt. The carboxylic acids were of analytical grade either from British Drug Houses or from Merck.

Apparatus

The cellulose layers were prepared with Desaga equipment (Desaga, Heidelberg) on 20 × 20 cm glass plates. Electrophoresis was carried out with a Desaga electrophoresis chamber with an aluminium alloy cooling plate, cooled by tap water, and a 1000 V Pleuger power supply CVC D, Belgium, with a built-in 60 mA ammeter. Desaga tanks were used for chromatography.

Experimental

The thin-layer plates were prepared with the Desaga equipment, using 17.5 g cellulose powder per 100 ml water. The plates were left to dry at room temperature for 24 h, and before use, they were prepared as shown in Fig. 1. The carboxylic acid mixture was applied with a Carlsberg pipette for volumes up to 5 μl. Larger samples were applied with an automatically driven Hamilton syringe, under a stream of cold air. The plates were cooled to about 4° and sprayed with a cold 0.2 *M* formic acid buffer, pH 2.5 (ammonia was used to raise the pH of the pure formic acid solution before adjusting to final volume). To avoid diffusion of the sample, a small (5 × 5 mm) piece of parafilm was placed over the application point during the spraying. The plates should be sprayed carefully in order that they do not become too wet, and spraying should be stopped when the surface appears shiny, indicating that the layer is moisture saturated[6]. Excess buffer on the glass edges was removed, and the plate was placed in the Desaga electrophoresis chamber. Two Whatman No. 1 paper wicks (10 × 20 cm) were used to connect the plate with the buffer troughs. In most runs a potential of 1000 V (50 V/cm) was applied for 30 min. Under these conditions the current only increased from an initial value of 20 mA to a final one of 25 mA.

After the electrophoresis the plates were removed and dried in a stream of cold air for about 30 min. During this time the buffer evaporated. Before chromatography, the edges of the plates (5 mm) which had been overlapped by paper wicks

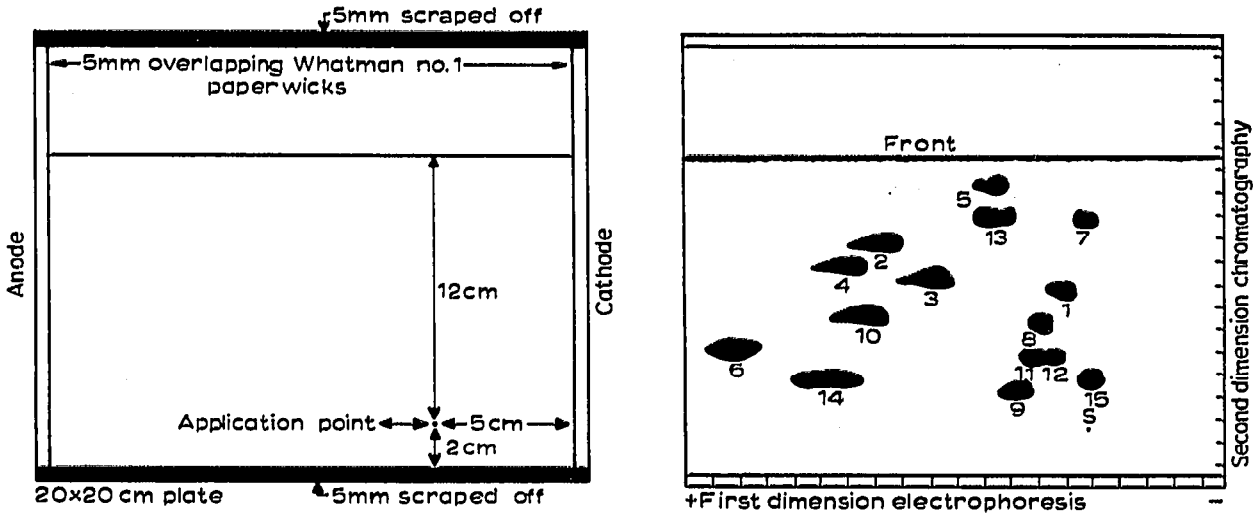

Fig. 1. Schematic diagram of a thin-layer plate showing the treatment of the plates prior to electrophoresis and chromatography. The line drawn at 12 cm was used to visualise the solvent front position during the chromatographic step.

Fig. 2. Thin-layer chromatogram of a two-dimensional separation of carboxylic acids. Cellulose layer 375 μ. An aqueous solution containing 10 μg of each acid was applied to the starting point (S). 1 = Glycolic; 2 = fluoroacetic; 3 = malonic; 4 = pyruvic; 5 = fumaric; 6 = maleic; 7 = succinic; 8 = malic; 9 = tartaric; 10 = α-ketoglutaric; 11 = citric; 12 = isocitric; 13 = *trans*-aconitic; 14 = *cis*-aconitic; 15 = ascorbic acid. For details see Experimental.

were scraped off. Chromatography was carried out in the second dimension with isoamyl alcohol–5*M* formic acid (2:1). A filter paper strip (15 × 20 cm), wetted in the lower acid layer, was used to line the tank in such a way that it did not touch the solvent lying in the bottom. A tank thus prepared could be used for at least three days. In order to obtain sharp spots it was found necessary to equilibrate the plates for about 30 min. The chromatographic separation was carried out using the upper phase of the above mixture as solvent, with 2,7-dichlorofluorescein (5 mg per 100 ml upper phase) dissolved in it[5]. This addition to the solvent allowed the detection of the carboxylic acids on the plates without spraying. The chromatography was stopped when the solvent front had reached 12 cm from the starting point. This took about 2½ h. After chromatography the plates were dried under a stream of cold air for about 30 min until all the solvent had been removed. This was necessary in order to increase the pH difference between the spots and the plate. On drying the carboxylic acid spots could be detected by observation under ultraviolet light (254 nm). Fig. 2 shows a typical chromatogram with the separated acids. For documentation the plates were photographed (Fig. 3) in ultraviolet light (254 nm) with a yellow filter (Kodak wratten 12) covering the camera lens.

Results and discussion

The method described allows the separation of a mixture of at least 15 carboxylic acids as shown in Fig. 2. With pure samples it was possible to start with the chromatographic separation, however with impure samples it was found convenient to start with electrophoresis as this step not only desalted the sample, but also separated the carboxylic acids from amino acids that could be present in the sample. Furthermore the use of a volatile buffer allows chromatography in any solvent system.

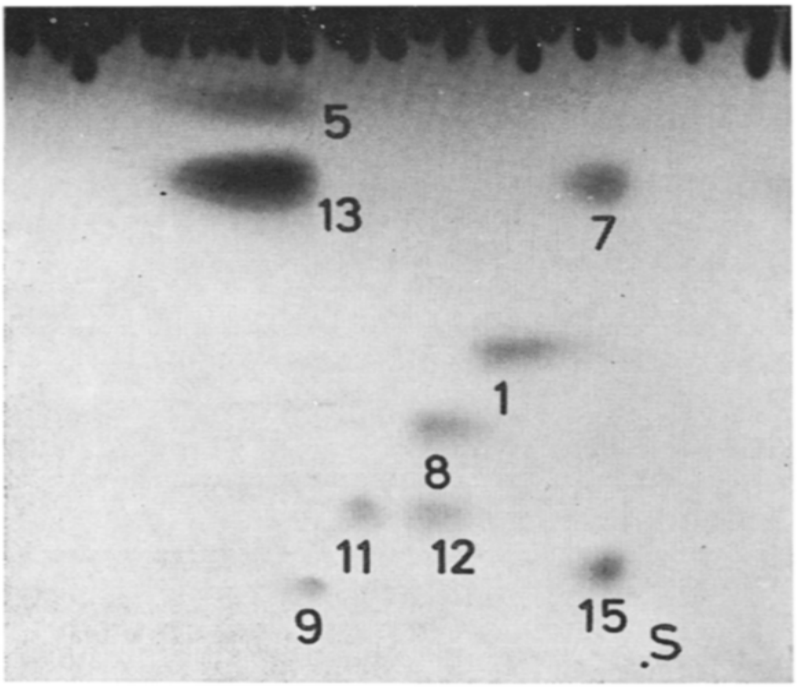

Fig. 3. Ultraviolet photograph of thin-layer chromatogram of a mixture of carboxylic acids, indicated by the same numbers as in Fig. 2. The electrophoresis has been carried out for 60 min to separate citric (11) and isocitric acid (12).

Some of the factors which affected the separation were:

Nature of the supporting material. Cellulose was preferred to silica gel, as with the latter too much heat was evolved on electrophoresis.

Plate thickness. It was found that 375 μ was the optimal thickness. Thinner plates could also be used with a lower capacity and reproducibility. On the other hand plates as thick as 500 μ could be used but the heat evolution was such that the power supply was easily overloaded.

pH. Similar separation patterns to the one presented were obtained with a lower pH, but the distances between the different spots were smaller. A higher pH than 2.5 resulted in increased heat evolution with no improvement in the resolution.

Buffer concentration. Lower concentrations than 0.2 *M* gave poorer separation, while higher concentrations resulted in increased heat evolution.

During the electrophoresis the acids migrated towards the anode, however owing to the endosmotic flow some acids appeared to remain stationary at the origin or even moved slightly towards the cathode. A longer run (60 min) resulted in the loss of maleic acid. On the other hand this permitted a separation of citric and isocitric acid (see Fig. 3). It should be mentioned that oxalic acid could not be detected by this method, as it formed very diffuse spots. It is important to take photographs within a few hours of development, since the spots weaken with time. BACHUR[8] has published a detection method based on the ability of acids to inhibit the browning of pyridine treated cellulose under ultraviolet light; this method could also be used.

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The detection of benzyloxycarbonyl-protected amino acid and peptide derivatives on thin-layer chromatograms

The application of thin-layer chromatography to peptide chemistry has been the subject of a recent monograph[1].

The classical benzyloxycarbonyl group, introduced by BERGMANN AND ZERVAS[2], is still the most common amino-protecting group used in the synthesis of peptides. We have for some time been investigating the possibility of detecting benzyloxycarbonyl-protected amino acid and peptide derivatives on chromatographic plates using ninhydrin. Our original plan was either to add a deblocking agent to the ninhydrin solution or to follow spraying with a deblocking reagent by treatment with ninhydrin. After inspection of the methods already developed for the removal of benzyloxycarbonyl groups in preparative work, it seemed possible to us that an acid, such as trifluoroacetic acid (WEYGAND AND STEGLICH[3]), would be worth testing. We chose for this purpose the less volatile trichloroacetic acid. In this way, by either using a ninhydrin solution in *n*-butanol containing 10% trichloroacetic acid, followed by heating to 100°, or by spraying with a 10% solution of trichloroacetic acid in glacial acetic acid, followed by heating to 100° and then spraying with ninhydrin, satisfactory spots could be obtained from some benzyloxycarbonyl compounds. However, a cleaner procedure, not involving the unpleasant trichloroacetic acid, seemed desirable.

Very recently WOLMAN AND KLAUSNER[4] published a procedure for the detection of *tert.*-butyloxycarbonyl derivatives on chromatograms, based on the sensitivity of these compounds to heat. Thus, after heating thin-layer chromatograms to 125–130° for 25 min, ninhydrin-positive spots were obtained.

We have now found that benzyloxycarbonyl compounds, too, are sensitive to heat. A somewhat higher temperature is needed for most benzyloxycarbonyl compounds than for *tert.*-butyloxycarbonyl compounds, although some can be detected