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Near-horizontal long-bed chromatography: a means of quantitatively separating compounds of close R_F value

Compounds having close R_F values in chromatography (within 0.02–0.10 R_F unit of each other) may often be distinguished qualitatively by a small difference in migration, but are too close together to be separated quantitatively by conventional paper and thin-layer chromatography (TLC). Although TLC presents a choice of many types of supporting medium and an infinite combination of solvent systems, it still offers but limited separability of some types of compounds (*e.g.*, isomers with close chemical properties) except through the use of very long bed lengths. The length of bed available to separate such compounds is, however, limited during ascending TLC by the decreased speed of solvent movement with height of climb. The opposite technique of descending TLC[1] has the disadvantage, for long strips, of requiring exceedingly delicate manipulation and is prone to siphoning of solvent. By running long TLC strips in a near-horizontal position, advantages of both ascending and descending chromatography, without their pitfalls, are available in a feasible, reasonably rugged TLC system. This paper illustrates the use of such near-horizontal TLC of a simple design.

Experimental

Glass strips, 710 mm long by 12 mm wide (cut from 28 in. flat-drawn window glass, 3/16 in. thick), were coated, by means of a Desaga spreader, with a 0.5 mm film of a slurry of Avicel Microcrystalline Cellulose (Brinkmann Instruments, Inc.)*,**. The slurry consisted of a mixture of 50 g Avicel powder, 1.5 g rice starch ground to a smooth water paste, 200 ml water, all blended in a Waring blendor and heated in a steam bath for one half hour with occasional stirring, and additional water added to make a flowing paste. (Note: after drying, the edges of the strips should be carefully freed of adhering powder so as to prevent creeping of solvent over the edge during chromatography.) Foil-backed adsorbents, commercially available in rolls, could be similarly used by cutting off strips of appropriate dimensions and bending these in a slightly trough-like contour to give them sufficient rigidity for handling.

The chromatographic chamber tube, 830 mm overall length by 16 mm I.D., with ground glass stoppers, is illustrated in Fig. 1. Solvent flow is from the left in the figure, in a slightly upward direction. The side arm (a) serves to accommodate a Whatman No. 3MM paper extension wick (b) (doubled if required) fastened by paper clip to the upper end of the TLC strip (c). The wick serves to extend the distance of solvent flow beyond the upper end of the strip and may be supplemented by a paper coil (D) when required for compounds of particularly low R_F value. A suitable support block (E) is illustrated, bored to hold, side by side, as many chamber tubes as desired.

* Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

** Microcrystalline celluloses Avicel TG-101 and Avicel PH-105 (American Viscose Division, FMC Corporation), with and without starch binder, were later used with similar results. The latter Avicel gave the fastest rates of movement of solvent and compounds, so that vanillic acid could be separated from isovanillic acid within an 8-h period. (Proportions: 60 g cellulose, 200 ml water.)

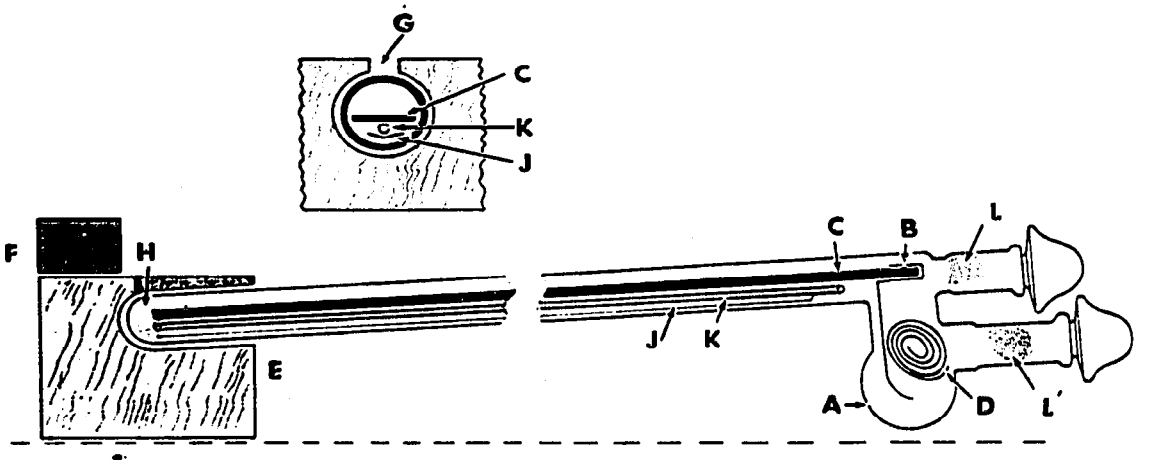

Fig. 1. Chromatographic chamber (section).

The block may be weighted down by a lead brick (F). The cross-sectional drawing at the top of Fig. 1 illustrates a 1 cm wide slot (G) in the top of the block above each chamber tube. This allows viewing of the TLC strip during chromatography. The angle of incline of the chamber tube should be about 1° from horizontal so that the developing solvent will accumulate at the bottom end. Each tube is prepared with a rounded wad of glass wool sewn into a loose ball inserted at the bottom end to act as solvent reservoir (H). A 12 mm wide strip of Whatman No. 1 paper of convenient length (*e.g.*, 57 mm) folded in half lengthwise and reopened (to give an open-V contour), is inserted touching the glass wool, to act as a saturating wick (J), and a length of 3 mm diameter glass rod (K) reaching almost to the side arm of the tube is placed over the paper strip to hold it in place and also act as a spacer supporting the TLC strip. Before inserting the TLC strip for chromatography, a volume of 2–3 ml of developing solvent sufficient to complete the chromatographic run is run down the saturating wick to the wad of glass wool.

The solution to be chromatographed is added as a small spot near the lower end of the TLC strip. The strip is inserted (long forceps) to make contact evenly with the solvent in the wad of glass wool. Insertion is made in such a manner that the paper extension wick (B) attached at the upper end of the TLC strip slides down into the side arm. A supplementary paper coil wick (D) may be added through the side arm stopper if needed. After insertion small wads of paper tissue (L,L') are placed in the stopper openings before closure and wetted with small amounts of developing solvent to improve atmospheric saturation in the chamber tube. (Care must be taken not to use an excess of solvent on the wads, or to allow solvent to touch the upper end of the TLC strip or its paper extensions.)

Vanillic acid (V), isovanillic acid (iV), ferulic acid (Fer), isoferulic acid (iFer), 3-O-methylgallic acid (3-MG), 4-O-methylgallic acid (4-MG); and protocatechuic acid (kindly supplied as crystalline compounds by Drs. J. CORSE AND L. JURD of this laboratory) were prepared as solutions in ethanol. Vanillic acid (^{14}C-methyl) (V-^{14}C) and isovanillic acid (^{14}C-methyl) (iV-^{14}C) were prepared by methylation of an aqueous solution of protocatechuic acid through the action of an O-methyltransferase system from pampas grass[2], using S-adenosylmethionine (^{14}C-methyl) (Calbiochem) as methyl donor.

The developing solvent used for chromatography was a mixture of toluene, acetic acid and water in the proportions 125:72:3. The chromatograms were run for 16 to 72 h in these experiments and could of course be run longer with additional amounts of extension wick if required for special separations. Alternatively, collection of eluate could be accomplished by allowing it to drip off the extension wick (B) into the empty side arm.

Radioactivity on TLC strips was measured by a Packard Radiochromatogram Scanner with its TLC platform modified to handle long strips.

Results

R_F values of 10–50 μg amounts of the pure test compounds are shown in Table I., V and iV as well as Fer and iFer could be separated in 32 h[**] (using an extension wick, B in Fig. 1), with results as shown in the table. The isomer pairs could be separated quantitatively, with clear zones between the isomers as indicated in the table. The

TABLE I

MIGRATIONS AND SEPARATIONS OF CHROMATOGRAPHED COMPOUNDS

Compound	R_F[a]	*Spot migration (cm) in 32 h*[b,c]	*Clear zone distance (cm) between isomers*	*Spot migration (cm) in 72 h*[d]	*Clear zone distance (cm) between isomers*[d]
V	0.64	53.2	1.5 ± 0.2	—	
iV	0.57	46.3		—	
Fer	0.62	55.0	0.65 ± 0.15	—	—
iFer	0.57	51.0		—	
3-MG	0.26	22.4	0.45 ± 0.05	38.9	11.0 ± 0.2
4-MG	0.22	19.4		27.2	

[a] Solvent front at 60–65 cm (16 h).
[b] Measurement made to arithmetic center of spot, judged visually by UV fluorescence.
[c] Mean value of two runs.
[d] Mean value of four runs ± maximum deviation.

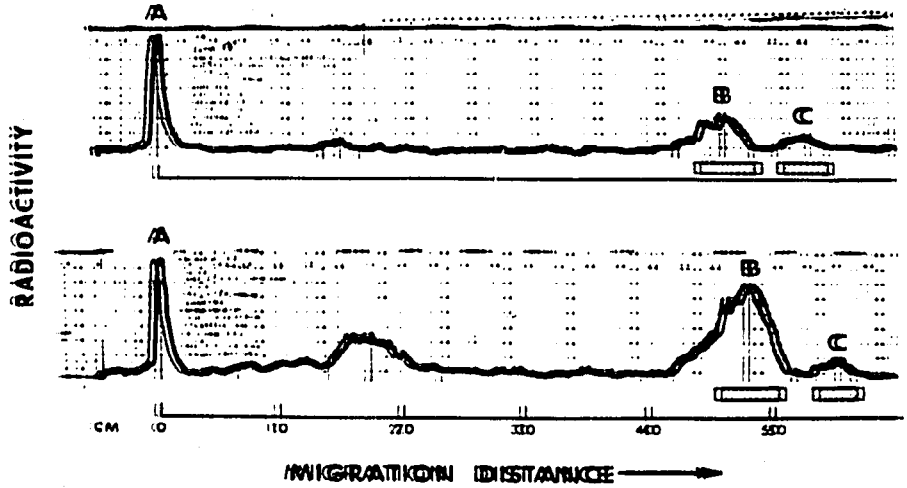

Fig. 2. Radioactive scans of chromatograms showing separation of iV-^{14}C (B) and V-^{14}C (C) from enzymatically produced methylation mixtures. V and iV, as markers (17 μg of each), were added before chromatography. The origin is designated by a radioactive marker (A). Positions of the separated compounds viewed under UV light, are indicated below each scan. 40 h migration time.

distance of isomer separation was ascertained from the UV fluorescence of the chromatographed spots or from a radioactive scan. Two such scans are seen in Fig. 2 and indicate the degree of separation of V-^{14}C and iV-^{14}C in experimental mixtures containing other radioactive products as well.

With the O-methylgallic acids, of low R_F value, development for 72 h was used (with extension wicks B and D in Fig. 1). The results are shown in Table I. Separation of these close-R_F compounds with a clear zone distance of 1.0 cm between them was achieved.

Discussion

By use of the described simple technique, separation of compounds as close in properties as even the monomethylated gallic acids was achieved. The distance of separation of the spots was sufficient so that isolation without cross-contamination and even recovery and quantitative determination of the separated compounds could be accomplished, such as by scraping off the TLC adsorbent layer for scintillation counting.

The length of TLC strip employed and distance of compound separation is limited only by feasibility, depending on R_F ranges and diffusion rates of the compounds, volatility of the solvent mixture, etc. With the present examples, the distance of travel of compounds was as much as 55 cm and the time of travel as much as 72 h to achieve the desired separation of isomers in a convenient manner.

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