THE RAPID RESOLUTION OF NATURALLY OCCURRING AMINO ACIDS BY THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

The use of unidimensional chromatography is seldom sufficient to resolve complex mixtures of naturally occurring amino acids. Even with two-dimensional chromatography it has not hitherto been possible to separate all the common amino acids satisfactorily on one chromatogram.

Over the past few years much work has been carried out on the application of thin-layer chromatography to the separation of amino acids[1–7]. However, despite the superiority of this technique over paper chromatography, great difficulty has been encountered in separating adequately all the natural amino acids on a single chromatogram. In particular, the pair of amino acids leucine/isoleucine have rarely been resolved under such conditions. Nevertheless, by the use of a series of different solvent pairs and chromatograms, it is possible, by successive partial separations, to analyse a complex mixture. This technique was employed by FAHMY, NIEDERWIESER, PATAKI AND BRENNER[4], and also by VON ARX AND NEHER[7]. The properties of filter paper and cellulose powder resemble each other in that chromatograms run on these two solid phases may be developed by partition chromatography. Solvent systems which are capable of separating amino acids on paper are also effective for their separation on thin layers of cellulose, the latter separation being usually superior because of the inherent advantages of thin-layer chromatography.

Some success has previously been achieved in separating the amino acids leucine and isoleucine by paper chromatography[8]. Because of this, and the fact that TEICHERT *et al.*[5], WOLLENWEBER[6], and VON ARX AND NEHER[7] have shown that thin layers of cellulose may be applied to the separation of amino acids, cellulose powder was chosen as the adsorbent in the present work. This paper describes the unambiguous separation of 23 amino acids which are commonly present in proteins. A brief preliminary report of the work has already appeared[9].

EXPERIMENTAL

Materials and equipment

Equipment. Throughout this work Shandon** equipment was used, the layer-spreading equipment being particularly useful because it enables layers of adsorbent slurry to be spread on to glass plates of different thickness at the same time.

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Adsorbent powder. The thin layers used in the present investigation were prepared from Cellulose Powder MN 300 manufactured by Macherey, Nagel & Co.*

Standard solutions. Standard amino acid solutions were prepared as suggested by SMITH[10]. Sufficient of each amino acid was accurately weighed out to produce a 0.05 *M* solution in 25 ml of aqueous propan-2-ol (10 %, v/v). The quantities of amino acids were dissolved in 10–15 ml of the propan-2-ol solution with the addition, if necessary, of the minimum quantity of hydrochloric acid to dissolve those amino acids insoluble in water. The final volumes were then made up to 25 ml.

Ninhydrin–collidine chromogenic reagent. This reagent was similar to that used by BRENNER AND NIEDERWIESER[3]. It consisted of ninhydrin (0.3 g), glacial acetic acid (20 ml), and collidine (2,4,6-trimethylpyridine) (5 ml) in sufficient ethanol to produce a final volume of 100 ml.

Solvents for chromatographic development. All solvents used in this work were of Analar grade**, with the exception of *tert.*-butanol, which was GPR grade**.

PREPARATION OF CELLULOSE THIN LAYERS

TRUTER[11] states that slurries of cellulose may be obtained by dispersing cellulose powder (1 g) in acetone (6 ml). However, thin layers produced in this way were found to be thicker than those produced from water slurries of cellulose powder, and they also adhered to glass less firmly. They had the further serious disadvantage that when samples, in aqueous solution, were applied to the layer, fissure occurred around the circumference of the sample spot during drying thus isolating the spot from the rest of the layer.

The most satisfactory thin layers of cellulose powder were produced in a manner similar to that employed by VON ARX AND NEHER[7]. A quantity of cellulose powder (15 g) of MN 300 was slurried with a mixture containing 70 ml of water and 10 ml of ethanol. From this quantity of slurry five 20 × 20 cm glass plates were coated with layers of slurry each 300 μ in thickness. When the surfaces of the thin layers became matt (this occurred after about 20 min), the plates were transferred to a chromorack and stored overnight at room temperature.

During this time the cellulose thin layers came to equilibrium with atmospheric moisture at room temperature. This is important, since cellulose layers as used, constituted partition chromatographic systems, the water adsorbed on the layers acting as the liquid phase on a solid cellulose support. RANDERATH[12] correlates paper chromatography and cellulose thin-layer chromatography with partition systems.

The final thickness of a cellulose thin layer (when dry) is much less than the initial thickness of the layer, when water is used to form the slurry. Layers initially spread to a depth of 300 μ produce cellulose layers only 100 μ thick when dry.

DEVELOPMENT OF TWO-DIMENSIONAL CHROMATOGRAMS

Sample spots were so positioned on thin layers that development of the first dimension was always "along the grain" of the layer, thus maintaining a standard procedure (see Fig. 1). Amino acid solutions, 1 μl or less, were placed on the thin

* Macherey, Nagel & Co., agents Camlab (Glass) Ltd., Cambridge.
** Hopkin & Williams Ltd., Freshwater Road, Chadwell Heath, Essex.

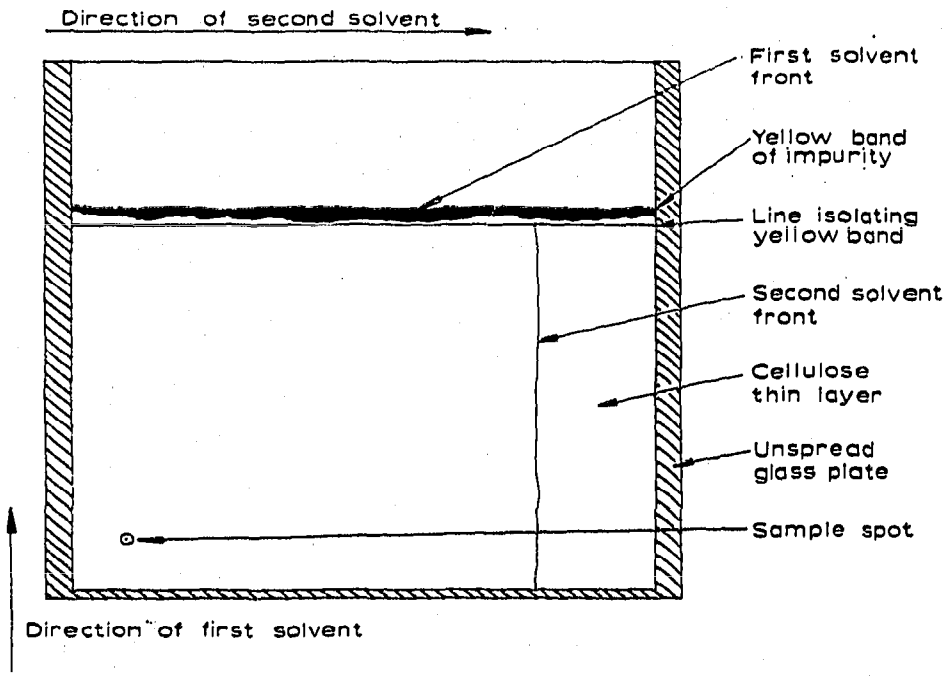

Fig. 1. Two-dimensional thin-layer chromatography on cellulose thin layers.

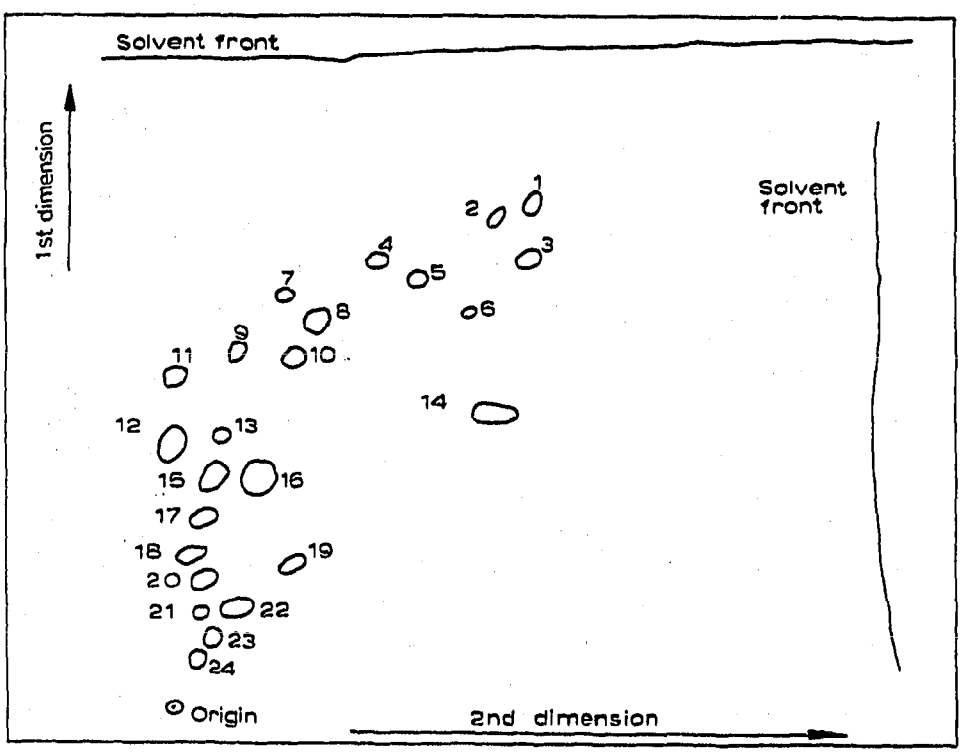

Fig. 2. Amino acid separations on thin layers of cellulose powder. 1 = leucine; 2 = isoleucine; 3 = phenylalanine; 4 = valine; 5 = methionine; 6 = tryptophan; 7 = 2-amino-*n*-butyric acid; 8 = tyrosine; 9 = α-alanine; 10 = proline; 11 = glutamic acid; 12 = aspartic acid; 13 = hydroxyproline; 14 = threonine; 15 = glycine; 16 = serine; 17 = glutamine; 18 = arginine; 19 = taurine; 20 = lysine; 21 = ornithine; 22 = histidine; 23 = cysteic acid; 24 = cysteine. Solvents: 1st dimension: propan-2-ol–formic acid–water (40:2:10, v/v); 2nd dimension: *tert.*-butanol–methyl ethyl ketone–0.88 NH_3–water (50:30:10:10, v/v).

layer, using 1-μl capillary tubes, at a point 1.5 cm from the two edges of the layer in the bottom left-hand corner of the plate. Each spot was dried in a stream of warm air after application to the layer. The solvent for development in the first dimension consisted of propan-2-ol–formic acid–water (40:2:10, v/v)[7]. The thin layer was placed into a tank immediately after the solvent had been added, so that the tank atmosphere was not saturated with solvent vapour prior to development. This was found to be important with both solvent systems if good separation of the amino acids was to be obtained. Ascending development of the chromatogram was allowed to continue at room temperature until the solvent front had travelled a distance of 12–13 cm above the origin (initial sample spot position). This took place over a period of approx. 3 h. The solvent was blown off the layer with warm air until no odour of formic acid could be detected. The latter process required 3–4 min.

It was found that an undulating yellow-coloured band, caused by impurities, was formed along the solvent front. This yellow band held back the solvent front, during development in the second dimension, along the portion of the thin layer which was covered by the band. However, it was possible to isolate the yellow band from the portion of the layer carrying amino acids by breaking the layer, with a scribe, along a line parallel to the edge of the glass plate, just below the yellow band, as illustrated in Fig. 1.

The second solvent, a modification of one of the solvents devised by BOISSONNAS[8], consisted of *tert.*-butanol–methyl ethyl ketone–ammonia (0.88)–water (50:30:10:10, v/v). Once again the plate was developed by the ascending technique until the solvent front was 12–13 cm above the origin, which occurred in this case over a period of about 2.5 h. Excess solvent was removed from the layer by warm air until no odour of solvent could be detected.

DETECTION OF AMINO ACIDS ON THIN-LAYER CHROMATOGRAMS

After two-dimensional development, the amino acids on the chromatograms were detected by means of the ninhydrin–collidine chromogenic reagent. The reagent was sprayed on to the thin layers, which were then held in a stream of warm air. Heating was continued until coloured spots, denoting the positions of amino acids, appeared. As each coloured spot appeared its centre was marked to facilitate calculation of the R_F values.

PAPER CHROMATOGRAPHY

Although this paper is mainly devoted to work carried out by thin-layer chromatography, it is of interest to mention that the solvent systems used for the development of two-dimensional thin-layer chromatograms were also successfully applied to amino acid separations by paper chromatography. Using 10-in. sq. of Whatman No. 1 paper, conventional two-dimensional ascending chromatography was carried out with the same solvent systems as used for thin-layer chromatography. The times required were 8 h for the development of the first dimension and 5 h for the second dimension. Good separations of the amino acid pairs leucine/isoleucine, valine/methionine, and glycine/serine were produced, but the overall separation was inferior to that resulting from thin-layer chromatography.

TABLE I

R_F AND $R_{Leucine}$ VALUES OF AMINO ACIDS SEPARATED BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY

Amino acid		*Colour with ninhydrin–collidine chromogenic reagent*	*First dimension IFW*[a]		*Second dimension TMAW*[b]	
			$R_F \times 100$	$R_{Leucine} \times 100$	$R_F \times 100$	$R_{Leucine} \times 100$
α-Alanine	(Ala)	Violet	55	71	10	19
2-Amino-*n*-butyric acid	(Abut)	Violet	64	82	16	32
Aspartic acid	(Asp)	Green	41	61	0	0
Arginine	(Arg)	Violet	24	31	3	6
Cystine group						
Cystine	$(CyS)_2$	Grey	0	0	0	0
Cysteine	(CySH)	Violet	8	10	3	6
Cysteic acid	$(CySO_3H)$	Blue	11	14	5	11
Glutamic acid	(Glu)	Violet	52	67	1	2
Glutamine	$(Glu(NH_2))$	Violet	29	38	4	9
Glycine	(Gly)	Brown	36	46	7	13
Histidine	(His)	Grey/Brown	15	20	9	18
Hydroxyproline	(Hyp)	Yellow	42	54	7	15
Isoleucine	(Ile)	Violet	76	97	45	90
Leucine	(Leu)	Violet	78	100	52	100
Lysine	(Lys)	Violet	20	25	4	9
Methionine	(Met)	Violet	66	85	36	68
Ornithine	(Orn)	Violet	15	20	4	9
Phenylalanine	(Phe)	Brown	69	89	51	99
Proline	(Pro)	Yellow	54	70	17	33
Serine	(Ser)	Violet	36	46	13	25
Taurine	(Taur)	Violet	22	28	17	33
Threonine	(Thr)	Violet	45	59	45	88
Tryptophan	(Trp)	Violet	60	78	42	81
Tyrosine	(Tyr)	Brown	60	78	21	41
Valine	(Val)	Violet	69	89	30	58

[a] IFW = propan-2-ol–formic acid–water (40:2:10, v/v).
[b] TMAW = *tert.*-butanol–methyl ethyl ketone–ammonia (0.88)–water (50:30:10:10, v/v).

RESULTS

The colours of the amino acids produced by the ninhydrin–collidine chromogenic reagent and their R_F and $R_{Leucine}$ values in both dimensions are given in Table I. The coloured spot arising from the presence of threonine appeared only slowly, as did those for other amino acids when present in small amounts (less than 1 μg). The identification of several amino acids, *e.g.* proline, histidine, was made easier by the fact that they produced characteristically coloured spots.

The quality of any separation cannot be judged purely from a knowledge of the R_F values of the spots, because it is the actual size of the spots which determines the true effective separation of neighbouring spots. Fig. 2 shows the actual sizes of the spots and the effective separations produced by thin-layer chromatography.

DISCUSSION

The results of the presents work show that twenty-three naturally occurring amino acids have been unambiguously resolved on a single thin-layer chromatogram. It has not been possible in the past to achieve this. Even pairs of amino acids, which have previously been troublesome to resolve when present at the same time, such as leucine/isoleucine, valine/methionine, and glycine/serine are among the twenty-three amino acids separated.

The necessity for the tank atmosphere to be unsaturated with respect to solvent vapour before development of cellulose thin-layer chromatograms was stated by VON ARX AND NEHER[7] and has been verified by the present work. Indeed prior saturation of the tank atmosphere resulted in inferior separations of the amino acids being produced.

The thin-layer technique which has been described has been used satisfactorily for the analysis of acid-hydrolysed peptides and desalted urine samples.

SUMMARY

Using a two-dimensional thin-layer chromatographic technique, with thin layers of cellulose powder, twenty-three naturally occurring amino acids have been separated unambiguously on a single chromatogram. The separation takes only six hours and includes a resolution of the amino acids leucine and isoleucine.

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