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AN IMPROVED TECHNIQUE FOR THE ANALYSIS OF AMINO ACIDS AND RELATED COMPOUNDS ON THIN LAYERS OF CELLULOSE

PART I. QUALITATIVE SEPARATION

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SUMMARY

Using thin layers of cellulose powder and a new pair of solvent systems, forty amino acids and related compounds have been separated unambiguously on a single glass plate by two-dimensional chromatography. The positions of a further twenty-three compounds are also recorded.

INTRODUCTION

In 1966 JONES AND HEATHCOTE[1] described a simple and rapid technique which enabled twenty-four common amino acids to be completely resolved on a two-dimensional thin-layer chromatogram in about 6 h. Even pairs of amino acids, which had previously been troublesome to resolve when present at the same time, such as leucine and isoleucine, valine and methionine, and glycine and serine were among the amino acids separated by this technique. Although this represented an improvement over the multi-dimensional technique of VON ARX AND NEHER[2], it had nevertheless some minor disadvantages. One was that the resolution of arginine and lysine was not entirely satisfactory and another was that glutamic acid and aspartic acid, when present as hydrochlorides, sometimes appeared as double spots of different ionic species. A third disadvantage was that the system required a partially saturated atmosphere for successful development and was therefore subject to variations in temperature.

The present paper describes the separation achieved by an improved solvent system which is based essentially on that of JONES AND HEATHCOTE, but which eliminates these defects. The separations are carried out in a saturated development tank using sufficient of the volatile polar component, methanol, to counteract demixing. The new solvent system gives a slightly better spread of the amino acids over the plate and, as well as improving the resolution of the basic amino acids, produces a better separation of alanine and tyrosine. The unambiguous resolution of a mixture of forty of the more common amino acids and related compounds is described and the R_F values of a total of sixty-three compounds are listed.

MATERIALS AND METHODS

Equipment

The equipment used was obtained from Shandon*. Thin-layer plates were spread using the Shandon Unoplan apparatus, in which the plates are pressed against two guide rails to give a level surface for spreading.

Cellulose layer

The cellulose powder used in this investigation was MN-300 (without binding agent)**. The cellulose as bought contains coloured impurities which, if not removed, interfere with the separation of the amino acids. It is therefore essential to wash the cellulose prior to preparation of the layers. The method employed in the present paper is a modification of that used by REDGWELL AND BIELESKI[3]. The cellulose (50 g) was slurried with 200 ml of 80% methanol. The slurry was poured into a Buchner funnel and then washed with the following series of solvents in order:

2-propanol–acetic acid–water (60:20:20, v/v) (300 ml);
methanol–water (25:75, v/v) (200 ml);
methanol–1 *N* hydrochloric acid (60:40, v/v) (200 ml);
water (200 ml);
methanol (200 ml).
The cellulose was dried overnight *in vacuo*.

Amino acid standard solutions

Stock solutions of amino acids (0.025 *M*) were made in aqueous 2-propanol (10%, v/v) with the addition of the minimum quantity of dilute hydrochloric acid to effect solution in the case of tyrosine, etc. These solutions were kept in the refrigerator when not in use.

Detection reagents

Ninhydrin–cadmium acetate chromogenic reagent. This reagent was the one devised by HEATHCOTE AND WASHINGTON[4], and was made by dissolving 0.5 g of cadmium acetate in 50 ml of water to which 10 ml of glacial acetic acid had been added. Propanone was then added until the total volume was 500 ml. Portions of this solution were taken before use and sufficient solid ninhydrin was added until the final concentration was 0.2% (w/v).

Ehrlich's reagent. This reagent was prepared as described by SMITH[5]. It consists of *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid 10% (w/v). This was added to four times its volume of propanone.

*Solvents for chromatographic development****

The 2-methylpropanol-2 was of GPR grade, and the butanone and propanone of MFC grade. All other solvents were of Analar grade.

Preparation of thin layers

The plates were first cleaned entirely free from grease by immersing them

* Shandon Scientific Co., 65 Pound Lane, London NW10, Great Britain.
** Macherey Nagel and Co., agents Camlab (Glass) Ltd., Cambridge, Great Britain.
*** Hopkin and Williams Ltd., Chadwell Heath, Essex, Great Britain.

overnight in Haemosol solution and rinsing well with distilled water afterwards. This prevents fissure of the layers, which may occur during their preparation.

A mixture containing 70 ml of water and 10 ml of ethanol was added slowly, with stirring, to 15 g of washed cellulose powder. The crude paste was then homogenized with a high-speed electric propeller stirrer for 60 sec to form the slurry. With the plates in position and the bag inflated, the spreader (slit width 400 μ) containing the slurry of adsorbent was drawn carefully with uninterrupted movement over the plates. When the surfaces of the spread layers became matt the plates were placed in a chromorack and stored horizontally overnight to dry in the laboratory cupboard.

The dry cellulose layers were found to have a thickness of 150 μ. According to WOLLENWEBER[6] this appears to be the optimal thickness for most separations.

Application of amino acids

Spots were placed at a position 1.5 cm up from the lower edge of the layer and 1.5 cm inside from the left hand edge of the layer. The solutions were applied to the layer using 1 μl capillary pipettes (Microcaps) and the spots were dried in a stream of warm air. The solvent front in each dimension (13 cm from the origin) was also marked. When thin layers are spread using the Shandon equipment, a margin of unspread plate is produced along two opposite sides of the layer. When running two-dimensional chromatograms the first dimension was developed with the margin, so that the development was "along the grain" of the layer.

Development in the first dimension

The double spots corresponding to different ionic species of glutamic and aspartic acids which can appear on chromatograms of the hydrochlorides developed with weakly acid systems were removed by replacing the formic acid used in the earlier system[1] with hydrochloric acid. Furthermore the addition of butanone to this solvent system produces a better separation of tyrosine and alanine. The composition of the solvent system finally chosen for development in the first dimension (solvent No. 1) was 2-propanol–butanone–1 N hydrochloric acid (60:15:25, v/v).

Tank saturation was achieved by employing the method of SANKOFF AND SOURKES[7]. In this procedure, solvent from the previous day's run was allowed to remain in the tank. Prior to development, the old solvent was discarded and replaced by 100 ml of fresh solvent.

Two plates were positioned in the tank with their lower edges dipping into the above solvent. The time taken for the solvent front to ascend to the finishing line was about 2.5 h at ambient temperature (22°). The plates were then removed from the tank, dried in a stream of cold air for 15 min and heated in a convection oven at 60° for a further 15 min to remove the final traces of hydrogen chloride. It is essential to remove most of the hydrogen chloride from the plate before heating since, if it is placed directly in the oven, charring of the cellulose occurs. Before development in the second dimension the plates were allowed to cool and the slight yellow band at the solvent front was isolated by breaking the layer with the sharp end of a spatula.

Development in the second dimension

The solvent designed for development in the second dimension (solvent No. 2)

was a modification of the one used by JONES AND HEATHCOTE[1]. It consisted of 2-methylpropanol-2-butanone–propanone–methanol–water–(0.88)ammonia (40:20:20:1:14:5, v/v). The addition of propanone improved the separation of the basic amino acids and the methanol was added to counteract the effects of demixing. Tank saturation was again achieved by the method of SANKOFF AND SOURKES[7] using 170 ml of the solvent. The plate was developed at right angles to the first dimension by the ascending technique until the solvent front was 13 cm above the origin. At ambient temperature this occurred over a period of about 2.5 h. The solvent was removed by heating the plate in a convection oven for 15 min at 60°.

Detection of amino acids and related compounds

In order that this separation technique might be applied to biological fluids and protein hydrolysates, the positions of some relevant compounds which might be associated with amino acids were also determined.

The amino acids and amines were localized using the ninhydrin–cadmium acetate reagent. After spraying with the reagent, the plates were heated at 60° for 15 min in a convection oven. The positions were then noted and the R_F values determined. For the detection of imidazoles the plates were sprayed with Ehrlich's reagent. The majority of colours developed within 30 min at room temperature. After allowing the plate to stand at room temperature for 24 h the positions of the spots were noted.

RESULTS

Table I gives the R_F values of the amino acids and related compounds after chromatography using the above two-dimensional solvent system. Fig. 1 is a map

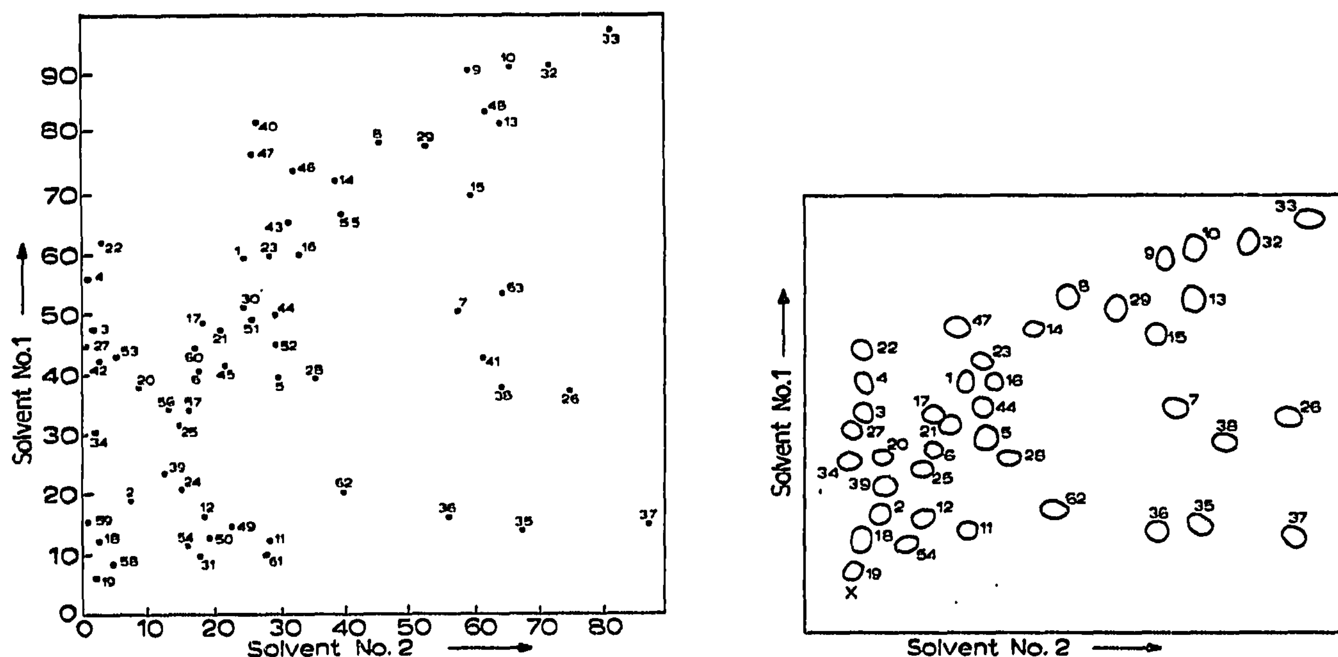

Fig. 1. Map of 63 amino acids and related compounds separated by TLC. For key, see Table I.

Fig. 2. Separation of ninhydrin-positive compounds by TLC. For key, see Table I.

TABLE I

R_F × 100 VALUES OF AMINO ACIDS AND RELATED COMPOUNDS ON THIN LAYERS OF CELLULOSE

No.	*Amino acid*	*R_F × 100 value*	
		Solvent No. 1	*Solvent No. 2*
1	Alanine	57	23
2	Arginine	19	6
3	Aspartic acid	48	1
4	Glutamic acid	56	1
5	Serine	39	27
6	Glycine	37	16
7	Threonine	51	61
8	Valine	79	44
9	Isoleucine	90	63
10	Leucine	90	69
11	Histidine	11	26
12	Lysine	16	17
13	Phenylalanine	82	67
14	Tyrosine	72	35
15	Tryptophan	70	54
16	Proline	58	30
17	Hydroxyproline	48	17
18	Cysteine	12	5
19	Cystine	6	3
20	Cysteic acid	53	8
21	β-Alanine	46	19
22	α-Aminoadipic acid	62	3
23	β-Aminoisobutyric acid	60	26
24	Asparagine	21	14
25	Glutamine	31	13
26	Ethanolamine	39	81
27	Phosphoethanolamine	43	0
28	Taurine	41	33
29	Methionine	78	51
30	γ-Amino-*n*-butyric acid	51	23
31	Hydroxylysine	10	17
32	Norleucine	92	73
33	2-Amino-octanoic acid	93	81
34	Argininosuccinic acid	31	0
35	Cadaverine	14	70
36	Putrescine	16	60
37	Histamine	15	85
38	Kynurenine	38	67
39	Homoarginine	23	12
40	*p*-Aminohippuric acid	82	25
41	4-Amino-5-imidazole carboxylic acid	43	63
42	δ-Aminolaevulinic acid	41	2
43	α-Amino-*n*-butyric acid	65	29
44	Methionine sulphone	50	27
45	Methionine sulphoxide	39	20
46	α-Aminoisobutyric acid	74	30
47	ε-Aminocaproic acid	76	23
48	Ethionine	83	66
49	1-Methylhistidine	14	18
50	3-Methylhistidine	12	14
51	Sarcosine	48	24
52	Homoserine	45	27
53	3,4-DOPA	43	6

TABLE I (*continued*)

No.	Amino acid	$R_F \times 100$ value	
		Solvent No. 1	Solvent No. 2
54	Ornithine	11	15
55	Pipecolic acid	66	36
56	Citrulline	34	12
57	Penicillamine	34	15
58	Djenkolic acid	9	5
59	2,6-Diaminopimelic acid	16	1
60	Formiminoglycine	45	16
61	2,4-Diaminobutyric acid	10	25
62	Glucosamine	20	38
63	Epinephrine	51	52

showing the position of sixty-three amino acids and related compounds. Fig. 2 shows the actual sizes and shapes of spots of a mixture of amino acids and related compounds separated by means of the above solvent system.

DISCUSSION

It will be seen that the new solvent systems reported in the present paper give a large spread of amino acids and related compounds over the plate. This is extremely useful for qualitative screening examinations. The use of uniphasic solvent systems and also of a completely saturated system both contribute to the highly reproducible R_F values which are obtained. Thus, with mixtures in which the amino acid composition is known to be within defined limits, *e.g.* protein hydrolysates, it is possible to identify the amino acids from the R_F value of the spots. Ninhydrin–cadmium acetate is a particularly sensitive reagent, the detection limits for most amino acids, after two-dimensional chromatography, being about 5×10^{-4} μmole. Some compounds, such as taurine, threonine and cadaverine, are slow to develop and the plates may have to be left overnight for the spots to appear if the amount present approaches the lower limit of detection.

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