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Separation of amino acids by thin-layer chromatography

Many procedures have been described[1–4,*] for the separation of amino acids by thin-layer chromatography (TLC) on chromatoplates coated with either silica gel or cellulose or a mixture of both. However, none of these procedures are so satisfactory as to be of use in different kinds of investigations. Regardless of the medium used, success in separation greatly depends on the choice of solvent system.

The work reported here is an attempt to find a suitable medium and a solvent system for the separation of not only standard amino acids but also free amino acids present in extracts of various plant materials. Standard amino acids and purified plant extracts were separated on chromatoplates coated with cellulose and on Eastman Kodak Chromagram sheets.

Materials

Glass plates of 20 × 20 × 0.4 cm were used. The adsorbents applied were: Silica Gel G (Camag) and Cellulose MN 300 (Macherey, Nagel and Co.). Chromagram sheets were supplied by Eastman Kodak Co., Rochester, N.Y., U.S.A. The spreader was of the standard Desaga type.

Preparation of chromatoplates. (1) 25 g of silica gel were vigorously shaken with 80 ml of distilled water and blended in a Waring blender for 1 min. (2) A mixture of 10 g of Cellulose MN 300 and 4 g of silica gel was blended with 80 ml of distilled water for 40 sec in a Waring blender. (3) 15 g of cellulose and 90 ml of distilled water were efficiently blended for 2 min in a Waring blender. The slurry in each case was poured into a Desaga spreader and applied to the plates to a thickness of 250 μ. The plates were air dried for 10–15 min at room temperature to set and then arranged on a rack and dried in an oven at 90–110° for about 30 min. They were cooled and used immediately or stored for some time. No precautions are needed when preparing cellulose-coated plates since the cellulose adheres very firmly to the surface of the glass plates. (4) Chromagram sheets coated with a silica gel layer of 100 mμ thickness were dried at 110° for 30 min.

Solvents. The solvent systems used are listed in Table I. With the exception of solvent system D, developed in our laboratory, the solvent systems have been previously reported in the literature.

Preliminary work indicated that solvent system D gave the best separation of amino acids. The solvents were used in quantities of 100–120 ml. Usually fresh solvent systems were used for every four to six plates or with each new run.

Procedure

Aliquots equivalent to 2–10 μg of amino acids, singly or in groups of four, were applied to the chromatoplates and Chromagram sheets with a Hamilton syringe and developed by the ascending technique. In the first dimension the solvent was allowed to ascend up to a height of 15 cm in the direction in which the adsorbent was spread on the plates. The chromatoplates or the Chromagram sheets were then

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* *Editor's note.* There are many more of course.

TABLE I

SOLVENT SYSTEMS

No.	Solvent system		Reference
	1st dimension	*2nd dimension*	
A	Chloroform–methanol–ammonium hydroxide (2:2:1)	Phenol–water (3:1)	5
B	*n*-Butanol–methyl ethyl ketone–acetic acid (2:3:1) and 4 ml of cyclohexylamine	*n*-Butanol–acetic acid–water (12:3:5)	6 (modified)
C	Butanol–acetic acid–ammonium hydroxide–water (10:10:5:2)	Isopropanol–formic acid–water (20:1:5)	6 (modified)
D	Butanol–acetone–ammonium hydroxide–water (10:10:5:2)	Isopropanol–formic acid–water (20:1:5)	

removed from the tank and completely dried overnight at room temperature. The next day they were developed in the second dimension and the solvent was again allowed to ascend to a distance of 15 cm. Developing the plates twice in each solvent system has sometimes given a much better separation. Care was taken to see that the plates and the sheets were completely dry between the runs. The developing chambers were saturated before each run.

Detection. Fresh solutions of 0.2 % ninhydrin in absolute ethanol, 10 ml of acetic acid and 2 ml of 2,4,6-collidine were used. The compact amino acid spots with specific colors appeared after heating the plates or the Chromagram sheets at 40–60° for 10–15 min. Freshly sprayed plates showed different colors, which facilitated the identification. However, the spots turned to a bluish-violet color when left overnight at room temperature.

Results and discussion

The color reactions and the mean R_F values for 25 standard amino acids on cellulose plates are presented in Table II. Neither the separation of standard amino acids nor that of purified plant extracts on silica gel plates was satisfactory. Their R_F values were low, with a great tendency to cluster close to the original spot. Similarly mixed layers of both silica gel and cellulose did not yield satisfactory results under our conditions, though claimed otherwise by some workers[3] who reported that the use of either silica gel or cellulose-coated plates was unsatisfactory for the separation of amino acids. This, of course, depends on the choice of a proper solvent system. The work reported here clearly emphasizes this aspect.

The distribution of standard amino acids on cellulose plates after two-dimensional TLC, using solvent system D and double development, is shown in Fig. 1. Prior saturation of the chromatographic tanks by lining the walls with 10-cm-high Whatman No. 1 filter paper on all four sides promoted separation and helped reduce boundary or border effects. The variability in R_F values experienced when using

TABLE II

R_F VALUES[a] AND COLORS OF AMINO ACIDS

The amino acids are run individually in single dimension and two-dimensionally. Solvent systems: 1st dimension, butanol–acetone–ammonia–water (10:10:5:2); 2nd dimension, isopropanol–formic acid–water (20:1:5).

No.	*Amino acid*	*R_F value*		*Color with ninhydrin*
		1st dimension	*2nd dimension*	
1	Phenylalanine	0.90–0.96	0.88–0.96	Dull brown
2	Tryptophan	0.85–0.95	0.83–0.93	Dull brown
3	L-Leucine	0.83–0.93	0.81–0.90	Bright purple
4	Isoleucine	0.81–0.91	0.80–0.90	Bright purple
5	DL-Threonine	0.78–0.88	0.80–0.90	Bright purple
6	Methionine	0.73–0.83	0.80–0.90	Bright purple
7	Tyrosine	0.64–0.74	0.63–0.74	Dull brownish purple
8	L-Valine	0.64–0.73	0.60–0.70	Bright purple
9	L-Serine	0.60–0.70	0.60–0.70	Bright purple
10	Histidine	0.57–0.67	0.55–0.66	Dull brown
11	DL-Serine	0.50–0.60	0.50–0.60	Bright purple
12	L-Proline	0.46–0.56	0.46–0.56	Yellow with purple margin
13	L-Lysine	0.45–0.55	0.45–0.55	Dull purple
14	DL-Alanine	0.43–0.53	0.42–0.52	Bright purple
15	Glycine	0.40–0.50	0.40–0.50	Dull purple
16	Hydroxyproline	0.39–0.47	0.40–0.50	Mustard yellow
17	γ-Aminobutyric acid	0.36–0.46	0.40–0.50	Bright purple
18	Asparagine	0.35–0.45	0.38–0.48	Bluish
19	β-Alanine	0.35–0.41	0.37–0.48	Brownish purple
20	Cystine	0.33–0.43	0.32–0.35	Brownish purple
21	L-Cystine	0.34–0.44	0.31–0.34	Brownish purple
22	Cysteic acid	0.20–0.50	0.23–0.52	Very dark purple
23	Arginine	0.21–0.30	0.21–0.31	Bright purple
24	Glutamic acid	0.17–0.27	0.17–0.27	Dark purple
25	Aspartic acid	0.13–0.23	0.13–0.23	Peacock blue

[a] Mean of 15–20 determinations.

cellulose plates is very insignificant. The sensitivity of the ninhydrin reaction is also very good on cellulose plates especially, the color darkening the next day.

In most solvent systems described for TLC or PC, several fast-moving amino acids like leucine, isoleucine and methionine and valine have a tendency to overlap. One of the objectives of this study was to resolve these spots. It is clear from Figs. 1 and 2 that with a proper solvent system it was possible to separate these amino acids. Thus with just one plate and one solvent system, without the combination of TLC and electrophoresis as recommended in ref. 4, it was possible to separate all the amino acids.

When studying the chromatographic behavior of different amino acids, it is important to take into consideration the chemical constitution of the amino acids. Since this aspect has been elegantly described in ref. 2, no attempt was made to repeat that type of work here. Some compounds like cysteic acid, alanine and γ-aminobutyric acid, though separating well on cellulose, show a distinct tailing in the first solvent system. However, when developed in the second solvent system, the spots are more compact.

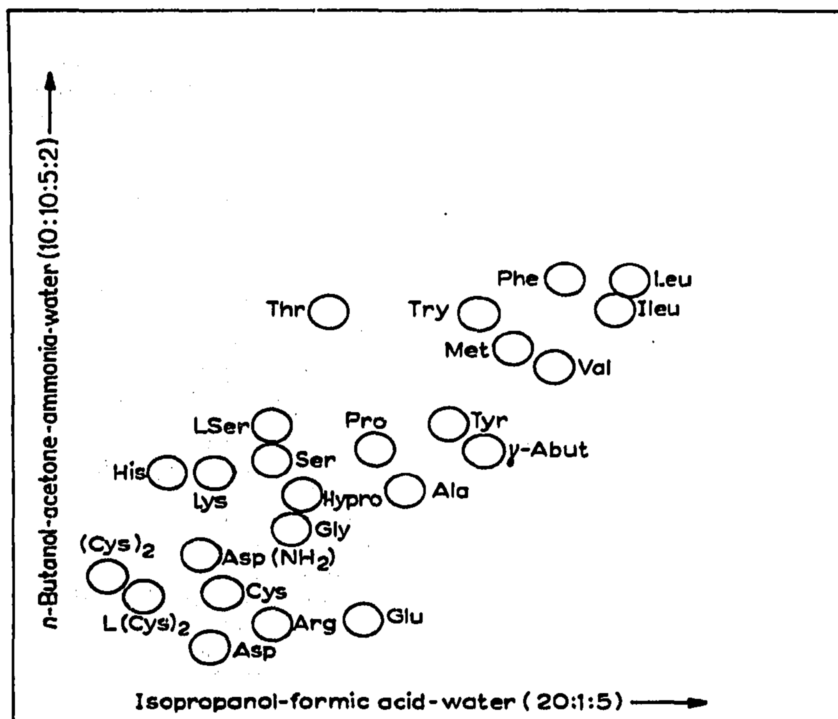

Fig. 1. Diagram of the separation of 24 standard amino acids on cellulose-coated 20 × 20 cm thin-layer chromatoplates. 10 μl of the mixture was applied per spot.

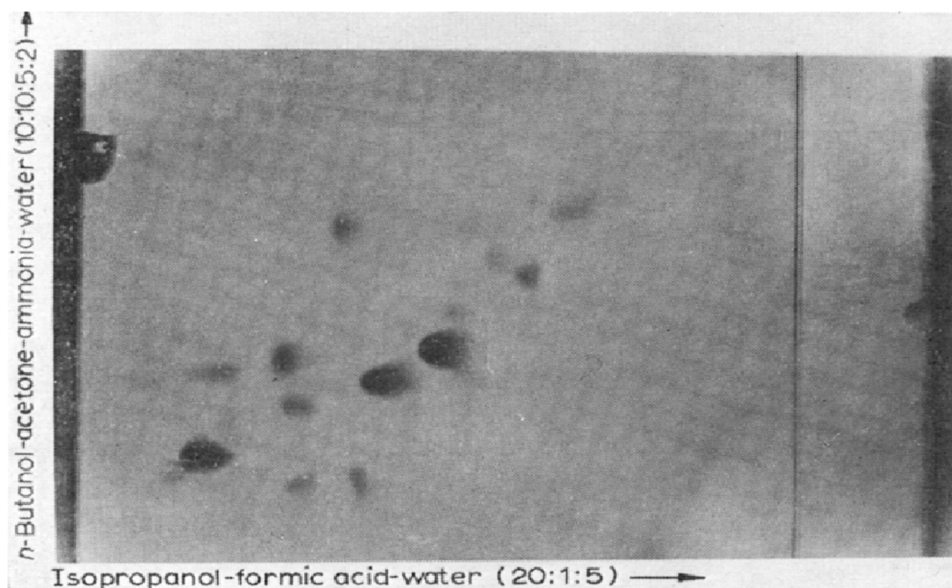

Fig. 2. Photograph of the separation of purified plant extracts on cellulose-coated 20 × 20 cm thin-layer chromatoplates. 10 μl are equivalent to 10 mg of soybean cotyledons.

Separation of plant extracts

The procedure described here was applied very successfully to the separation of free amino acids from different plant extracts. While separation of a mixture of synthetic amino acids by TLC may give good results, the separation of crude plant

extracts is usually inadequate due to many interfering substances. Desalting and purification of plant extracts from germinating soybean cotyledons, hypocotyl and roots were achieved on ion-exchange columns[7] before application of TLC. This gave excellent results (Fig. 2).

Eastman Chromagram sheets, 100-μ thick, have given a fairly good separation of standard amino acids spotted in quantities of up to 5–10 μg. However, this layer is less satisfactory for the separation of amino acids in plant extracts since only a very robust layer can handle aliquots of solutions up to 50 μl to be applied quantitatively in one spot.

Conclusion

The procedure described above is simple and especially very good for the separation of free amino acids in purified plant extracts, by two-dimensional TLC on cellulose-coated plates using a single solvent system.

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