A mixed layer for separation of amino acids by thin-layer chromatography

Both cellulose and silica gel layers have been used in the separation of amino acids by thin-layer chromatography (TLC). Neither layer is completely satisfactory for separating amino acids present in crude plant extracts. In attempting to improve separation techniques for such extracts we have found that a mixture of cellulose and silica gel forms layers superior to those prepared from either component.

Method

A mixture of 10 g MN 300 cellulose (Macherey Nagel), 4 g silica gel H or G (Merck's Kieselgel H or G, Stahl) and 80 ml of distilled water is blended for 30 sec at approximately 20,000 r.p.m. in a Virtis homogenizer. Efficient blending is of major importance. The uniform suspension is spread over thin-layer plates in the usual way; in this study 250 μ thick layers on 20 × 20 cm glass plates were prepared

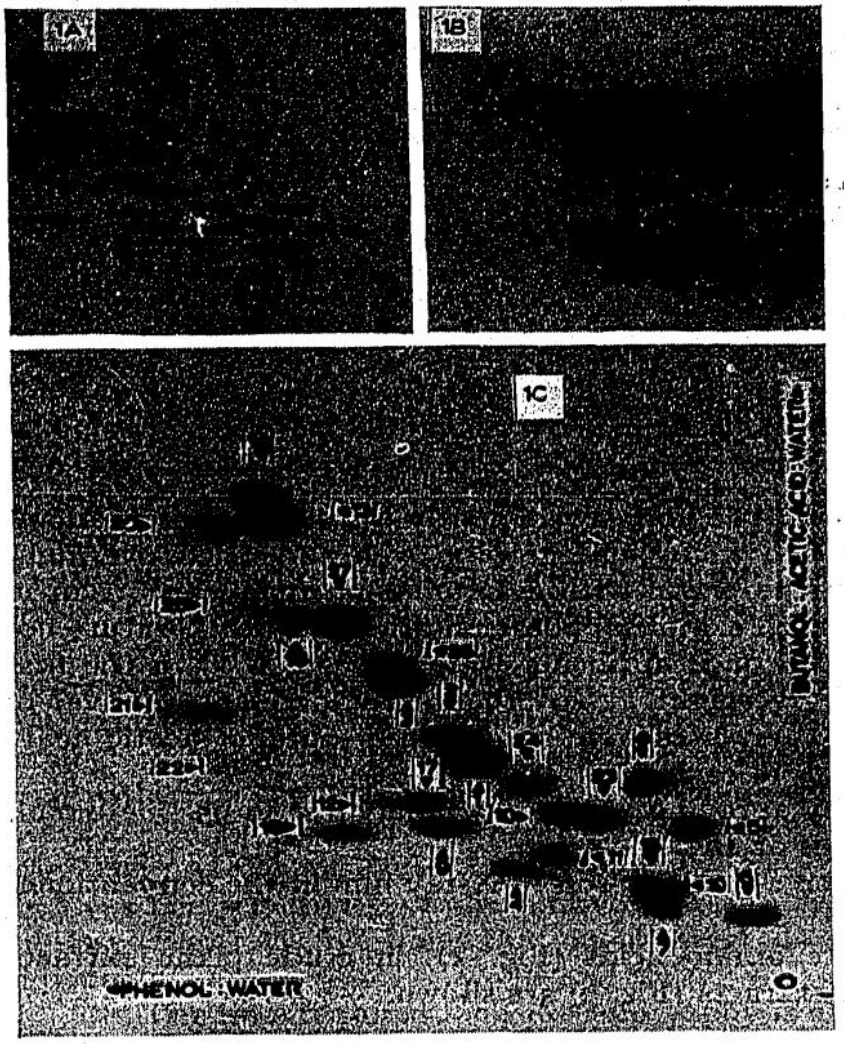

Fig. 1. Thin-layer chromatograms of a standard mixture of twenty-seven amino acids (as listed in Table I). The amount of each amino acid on each chromatogram is 1 μg. All chromatograms made on 20 × 20 cm plates. The key and R_{alanine} value for each amino acid is listed in Table I. 1A = cellulose layer (× 0.4); 1B = silica gel H layer; 1C = cellulose/silica gel H layer.

TABLE I

$R_{alanine}$ VALUES IN PHENOL–WATER (A) AND BUTANOL–ACETIC ACID–WATER (B)

The standard mixture of amino acids listed in Table I was prepared by dissolving each in 30% ethanol, with the exception of cystine and tyrosine, which were made up in 0.1 *N* HCl. Each amino acid was present at a final concentration of 1 $\mu g/\mu l$.

Key	*Amino acid*	$R_{alanine}$	
		A	*B*
1	Alanine	1.00	1.00
2	β-Alanine	1.07	1.09
3	γ-Amino-*n*-butyric acid	1.24	1.34
4	Arginine	0.87	0.51
5	Aspartic acid	0.29	0.68
6	Cysteic acid	0.09	0.29
7	Cystine	0.38	0.31
8	Glutamic acid	0.43	0.89
9	Glutamine	1.11	0.70
10	Glycine	0.72	0.76
11	Histidine	0.74	0.57
12	Hydroxyproline	1.30	0.80
13	Isoleucine	1.65	2.07
14	Leucine	1.69	2.18
15	Lysine	0.45	0.42
16	Methionine	1.60	1.35
17	Methionine sulphone	1.17	0.81
18	Methionine sulphoxide	1.43	0.68
19	Ornithine HCl	0.35	0.41
20	Phenylalanine	1.86	2.06
21	Pipecolic acid	1.89	1.23
22	Proline	1.79	0.98
23	Serine	0.69	0.74
24	Threonine	0.82	0.90
25	Tryptophan	1.72	1.79
26	Tyrosine	1.27	1.44
27	Valine	1.42	1.60

with Shandon thin-layer equipment. The plates were gently warmed under a radiant heater until set, then transferred to a chromatography oven and dried overnight at 40°. The sample is applied to the origin with a Drummond microcap, 3 cm in from the corner of the plate, and each sample application dried at a temperature not exceeding 40°. Plates are developed once in phenol–water (80:20, w/v) in the first dimension (6 h), and then dried overnight at 40° to ensure adequate removal of phenol. In the second dimension the plates were run twice in butanol–acetic acid–water (5:1:4, v/v/v top phase) (4 h in each run).

Amino acids are detected by spraying with 0.5 % ninhydrin in 95 % ethanol, drying the plate and then heating it at 105° for 5 min.

Autoradiographs are made on Kodak no-screen X-ray film in the conventional manner.

Typical separations of pure amino acids (Fig. 1), in crude plant extracts (Fig. 2), and in radioactive plant extracts (Fig. 3) are shown.

Discussion

Amino acids in standard solutions separate well on silica gel, though they have relatively low R_F values and so cluster near the origin (Fig. 1B). Similarly, amino

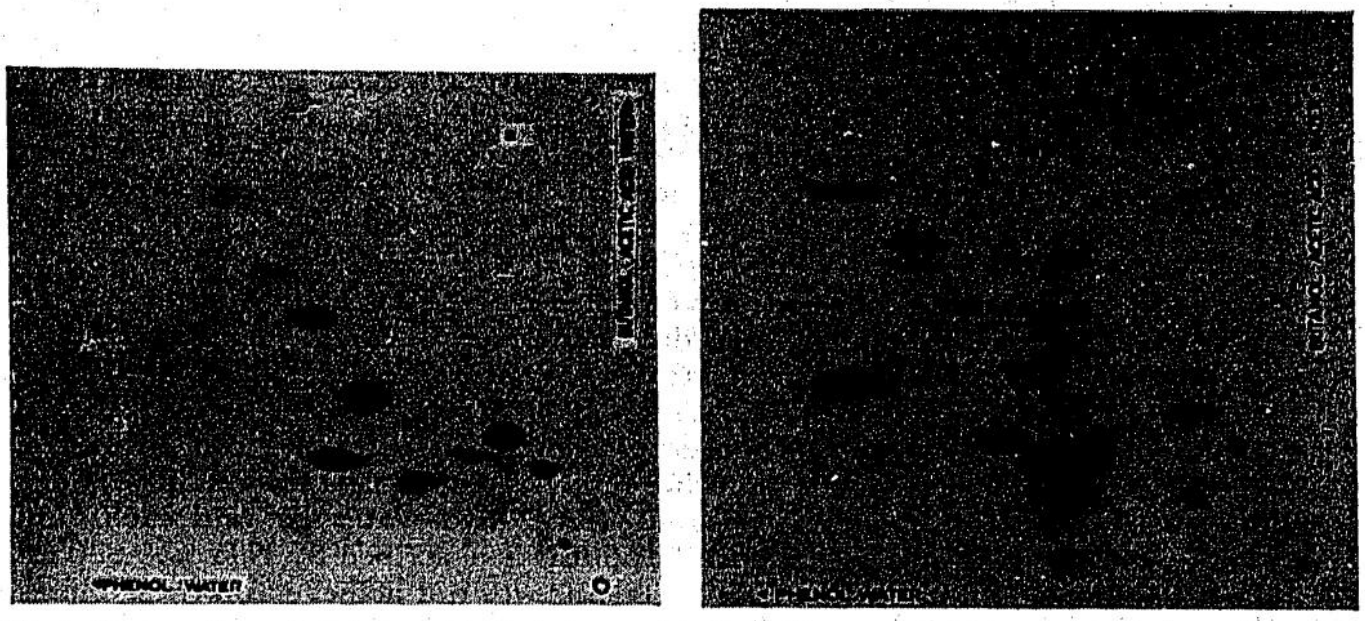

Fig. 2. Separation of amino acids from a crude plant extract on a cellulose–silica gel H layer. The volume of the extract applied is 5 μl (equivalent to 5 mg fresh weight of *Spirodela* plants extracted with 87 % ethanol).

Fig. 3. Autoradiograph of a thin-layer chromatogram of ^{14}C-labelled compounds from a crude ethanolic extract of *Spirodela* plants. The plants were exposed to a mixture of ^{14}C-labelled amino acids (5 μC in 0.25 ml) for 21 h. They were then washed, extracted and chromatographed as in Fig. 2. The plate was autoradiographed for 18 h.

acids separate reasonably well on cellulose though they show marked tailing in the first solvent (Fig. 1A). However, in each case the presence of a relatively low level of other compounds in an extract causes gross interference through distortion of the chromatographic pattern and extensive trailing of the compounds.

In contrast, the mixed layer has, with pure compounds, the virtues of both adsorbents and the faults of neither. Spots are compact, well separated and of relatively high R_F value (Fig. 1C). Other compounds present in crude extracts do not interfere with the separation at even quite high loadings (Figs. 2 and 3). For example, crude extract equivalent to 20 mg *Spirodela* tissue was successfully chromatographed on a standard plate. Untreated urine samples (10 μl) were also successfully chromatographed on the mixed layer. The presence of urea and other salts drastically interfered with such separations attempted on cellulose layers, silica gel layers, or paper sheets.

The mixed layer had other advantages:

(a) The sensitivity of the ninhydrin reaction is approximately twice that on cellulose (Fig. 1A), five times that on silica gel alone (Fig. 1B), and fifty times that on paper.

(b) The time required for autoradiography is about one-twentieth that required for the same amount of extract separated by paper chromatography (this appears to be true of most TLC methods).

(c) The layer is robust, and can be handled easily, autoradiographed readily, and even written on with a soft pencil. This robustness allows aliquots of solution up

to 50 μl to be applied quantitatively to a single spot. Radioactive spots can be cut out, removed and mounted by a simple stripping technique[1].

(d) Pairs of compounds which are not usually resolved but which can be separated on this layer, include methionine–valine and leucine–isoleucine. Separation of asparagine from arginine was incomplete on the mixed layer. These compounds can be separated in both standard amino acid mixtures and crude extracts by using phenol saturated with a solution of 6.3 % sodium citrate and 3.7 % Na H_2PO_4[2] in the first dimension.

Although the method described above was the most satisfactory, other variants were tested. The use of Whatman CC41 cellulose in place of MN 300 cellulose resulted in more diffuse spots and less sharp separation. Whatman SG41 silica gel could be substituted for silica gel H or G.

Small plates, 10 × 7.5 cm (photographic quarter plates) have been used in place of the 20 × 20 cm plates for routine analysis and for rapid test procedures (phenol development time 90 min, butanol–acetic acid–water 40 min each run). These plates were coated with a "Perspex" applicator described by NYBOM[3]. Separation of methionine–valine and of leucine–isoleucine was not achieved on these plates. Nevertheless, the separation was still superior to that obtained on large plates of silica gel or of cellulose.

It has been claimed that phenol brings about the breakdown of some amino acids[4] and several mixtures have been suggested as possible substitutes for this solvent[5–8]. Some of these were not satisfactory[7,8] when tested on thin-layer plates while others[5,6] gave reasonable results. However, none gave the resolution obtained with phenol. With TLC there is good reason to believe that decomposition of amino acids will be less than with paper chromatography[1].

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