PAPER CHROMATOGRAPHIC SEPARATION OF AMINO ACIDS A SOLVENT TO REPLACE PHENOL*

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Good resolution of mixtures of *some* of the common amino acids can usually be obtained by a sensible choice of the various conventional solvent systems employed in two-dimensional paper chromatography. Yet, while most of the amino acids can be identified, these solvent systems fail to resolve complex mixtures of *all* of the common amino acids. Phenol has traditionally been employed as one of the solvents that enable separation of complex mixtures of the common amino acids by two-dimensional chromatography. However, the continued use of phenol is probably due to historical prominence rather than effectual attributes.

Some of the disadvantages of phenol are: (a) Phenol of sufficient purity is difficult to obtain, either in liquid or loose crystalline form. A chemical firm that supplied phenol in loose crystal form, and which we found to be of sufficient purity, has recently ceased to offer this product. (b) The purification of obtainable phenol is laborious. (c) Even with pure phenol, a solution of sodium cyanide, 8-hydroxyquinoline, or other preservatives must be placed in the chromatographic chamber to prevent phenolic decomposition during a run. (d) In our hands the amino acid spots have a tendency to "tail" and are less discrete in phenol than are the spots obtained with the reported solvent system. (e) Some of the common amino acids (*e.g.*, leucine and isoleucine) are not separated by phenol.

Because of these disadvantages we began a search for a solvent system which would replace phenol. After many trials, a solvent system consisting of *n*-butanol–methyl ethyl ketone–water (2:2:1 by vol.) proved to be the most promising. It did separate the leucines and most of the common amino acids. Nevertheless, with this system (in combination with *n*-butanol, acetic acid and water as the other dimension), glycine and serine ran together. WOLFE[1] used an *n*-butanol–methyl ethyl ketone–water (5:3:1 by vol.) solvent system containing 1 part of 17 *N* ammonia (in combination with *n*-butanol, acetic acid, water as the second dimension) to separate a mixture of all of the common amino acids. Although WOLFE's alkaline solvent system separates glycine and serine, he reported that asparagine partly overlapped lysine and arginine. We therefore endeavored to explore the possibilities of an *n*-butanol–methyl ethyl ketone–water system containing an alkaline component other than ammonia.

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HARDY *et al.*[2] reported good separation with such a system using the organic base, cyclohexylamine. Moreover, they reported that the introduction of cyclohexylamine had additional value in that it imparted variations in the usual color of several amino acids after treatment with ninhydrin. But, they also noted that solvents containing cyclohexylamine tended to turn the entire chromatographic sheet blue during the final drying unless the drying and development with ninhydrin were performed at a high temperature and for a short time (75° for 5 min). We found their findings to be essentially true; however, we experienced difficulty in preventing bluing of the entire paper even when the drying and development were performed in the suggested manner.

However, if the cyclohexylamine was placed in a separate vessel in the chamber, the vapors were sufficient to permit resolution and diverse coloration of the amino acids without the undesirable bluing of the paper. Even at lower temperatures of drying and development with ninhydrin (45° for 15 min) the blue discoloration did not occur. We noticed that if the completed chromatogram was exposed to strong light for a long period of time the paper would then develop a light blue cast. Since this would occur only after several hours of exposure to strong light, it posed no problem.

Our system consisted of *n*-butanol–methyl ethyl ketone–water (2:2:1 by vol.); and a beaker of cyclohexylamine (1 ml for every 25 ml of solvent mixture) was placed in the chamber to provide cyclohexylamine vapors. Further tests confirmed the value of this solvent system; it resolved most of the amino acids *and* also imparted distinctive colors to some of them. Because of the distinctive colors, this system or similar systems can also be used for single-dimensional chromatographic resolution of amino acids in mixtures that could not be resolved by phenol or other one phase systems[2]. Furthermore, when employed in quantitative two-dimensional chromatography, this type of system yields much better amino acid recoveries than phenolic systems[1].

The following procedure was employed for qualitative two-dimensional chromatography. A mixture of amino acids was spotted on Whatman No. 1 filter paper, 46 × 57 cm, and subjected to descending development in the first dimension (30 h) with *n*-butanol–acetic acid–water (25:6:25 by vol., WOIWOD[3]), and in the second dimension (20 h) with our solvent system*. The development took place at a temperature of 21° ± 1°. After final development the chromatograms were dried for 30 min at 45° and then dipped in a 0.25 % w/v solution of ninhydrin in acetone containing 7 % v/v glacial acetic acid[2], and finally heated for 15 min at 45°. The colors reported in Table I could be intensified, when necessary, if a hair drier was held directly over the spots soon after the final heating. R_F values (Table I) were determined in the usual manner. Since better resolution was obtained when the solvent front was allowed to run off the bottom of the paper, the distances traveled by the amino acids are also expressed as ratios of the distance between the origin and phenylalanine, *i.e.*, R_φ values (Table I).

* These times (30 and 20 h) permitted the solvent fronts to run off the bottom of the paper.

TABLE I

R_F AND R_{cp} VALUES OF AMINO ACIDS AND THE COLORS THEY DEVELOP IN *n*-BUTANOL–METHYL ETHYL KETONE–WATER PLUS CYCLOHEXYLAMINE VAPORS AFTER NINHYDRIN TREATMENT

(See text for details)

No.	*Amino acid*	R_F	R_{cp}	*Color*
1	Phenylalanine	0.63	1.00	Blue-grey
2	Tryptophan	0.60	0.96	Brown-grey
3	Leucine	0.57	0.91	Purple
4	Isoleucine	0.54	0.86	Purple
5	Threonine	0.52	0.82	Purple
6	Methionine	0.46	0.75	Purple
7	Tyrosine	0.43	0.69	Violet-grey
8	Valine	0.42	0.67	Purple
9	Serine	0.26	0.41	Purple
10	Histidine	0.25	0.40	Green-grey
11	Cysteic acid	0.24	0.39	Purple
12	Proline	0.24	0.38	Yellow
13	α-Alanine	0.22	0.35	Purple
14	Hydroxyproline	0.19	0.30	Carmine → beige†
15	Glycine	0.18	0.28	Purple → violet-grey†
16	Cystine	0.16	0.25	Brown-grey
17	β-Alanine	0.16	0.25	Royal blue
18	Asparagine	0.15	0.24	Beige
19	Glutamine	0.15	0.24	Purple
20	Cysteine	0.14	0.21	Violet-grey
21	Glutamic acid	0.12	0.19	Purple
22	Aspartic acid	0.11	0.18	Royal blue
23	Lysine	0.09	0.15	Purple
24	Arginine	0.07	0.11	Purple

† Original colors change to the indicated color after a few hours.

The photograph (Fig. 1) of a two-dimensional chromatogram illustrates the excellent resolution of a mixture of the common amino acids*. The only spots that lie somewhat close to one another are: 6 (methionine, purple) and 7 (tyrosine, violet-grey); and, 12 (proline, yellow) and 13 (alanine, purple). These spots are readily distinguished by their obvious color differences.

* Comparable chromatograms, using hydrolyzed and unhydrolyzed tissues, are currently being obtained in our studies of the amino acid composition of regenerating newt limbs.

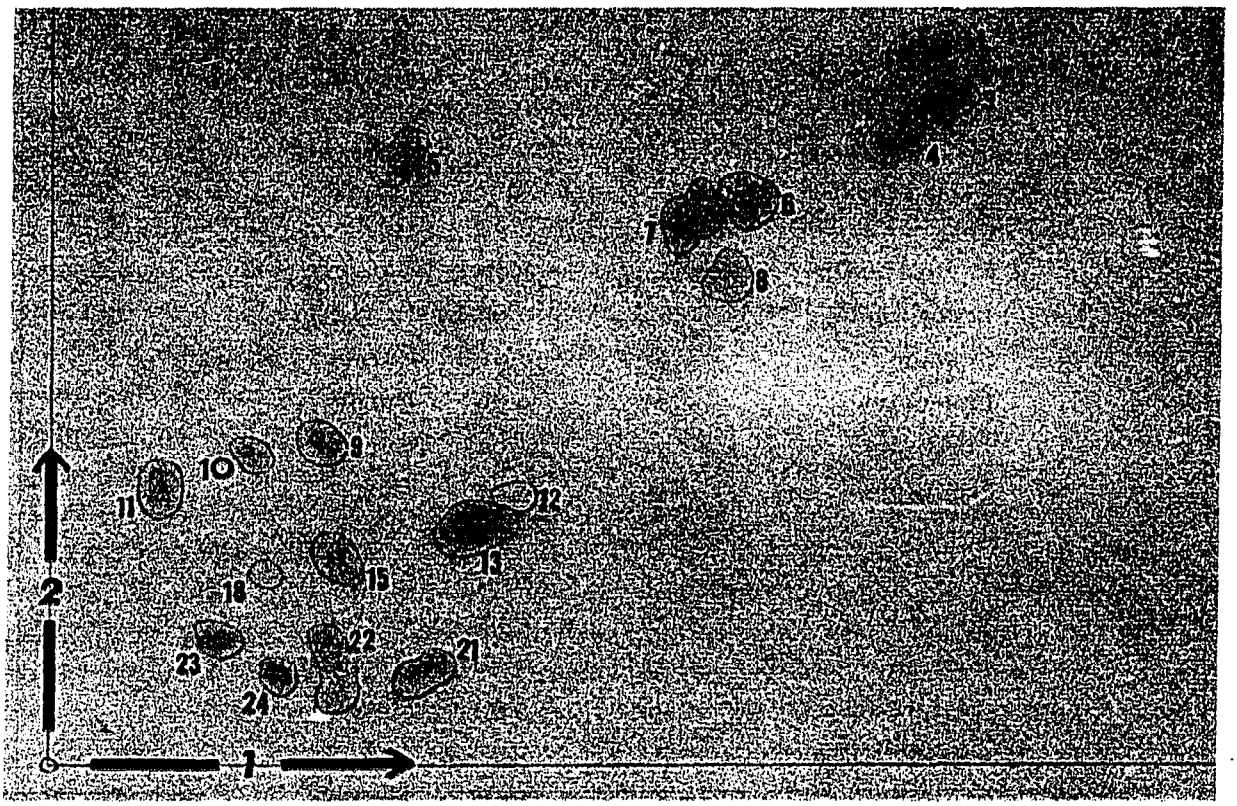

Fig. 1. Photograph of a typical two-dimensional chromatogram of a composite mixture of the common amino acids. Tryptophan and cystine were not spotted for this particular run; however they separate well and do not overlap any of the other amino acids (see R_F values in Table I). First dimension, *n*-butanol–acetic acid–water (25:6:25 by vol.), second dimension, *n*-butanol–methyl ethyl ketone–water (2:2:1 by vol.) plus cyclohexylamine vapors. See Table I for identity of spots.

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SUMMARY

The solvent *n*-butanol–methyl ethyl ketone–water (2:2:1) with an atmosphere saturated with cyclohexylamine yields excellent separation of mixtures of all the common acids, is readily reproducible, lacks the capriciousness of phenolic systems, and furthermore, imparts distinctive colors to many of the amino acids after ninhydrin treatment. These characteristic colors serve to confirm identification based on control runs for these amino acids and also serve as valuable "landmarks" facilitating the identification of adjacent amino acids that have the customary purple ninhydrin color.

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