AN IMPROVED METHOD FOR THE DETERMINATION OF AMINO ACIDS BY SPECTRAL REFLECTANCE

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INTRODUCTION

The promise of such advantages as reduced tailing, increased sensitivity, and greater speed and resolution[1] has aroused considerable interest in the application of thin-layer chromatography to the analysis of amino acids. In an effort to enhance the utility of the technique for this purpose, the authors have devised a procedure for the *in situ* determination of amino acids resolved on chromatoplates by means of spectral reflectance[2]. The precision attained with this procedure was found to be limited by elements associated with the color development process, such as the incomplete reaction of the acids with the ninhydrin reagent and the leaching out of the acids during the spraying operation.

Since the degree of precision thus achieved was less than that provided when the same procedure without the chromogenic step was applied to a stable system[3], it was felt that substantial improvement in the method would result if the color were developed without sprays. Accordingly it was decided to investigate the possibility of adapting a non spray method, suggested by EL KHADEM *et al.*[4] for the identification of amino acids and sugars separated on paper chromatograms, to the problem at hand. By adding the detecting reagents to the solvent mixtures, these investigators succeeded in eliminating not only the spraying operation but also the drying step preceding it.

EXPERIMENTAL

The amino acids used for this study (DL-alanine, L-arginine, L-glutamic acid, glycine, L-leucine, L-lysine, DL-methionine, DL-phenylalanine, DL-serine and DL-valine) were of Calbiochem A Grade purity. Stock solutions of the acids containing 500 mg in enough distilled water to make 50 ml of solution were applied as spots by means of a Hamilton microsyringe in 5 μl increments. The 20 × 5 × 0.35 cm plates used for one-dimensional analysis as well as the 20 × 20 × 0.35 cm plates employed for the two-dimensional resolutions were coated with Merck silica gel G according to the procedure given in an earlier paper[2]. After resolutions had been achieved, the plates were heated in a mechanical convection oven at 60° for 30 min to dry them and to develop the colors.

Both one- and two-dimensional chromatograms were used in investigating the applicability of four solvent mixtures: (1) *n*-propyl alcohol–water–acetic acid (64:36:20); (2) *n*-butyl alcohol–water–acetic acid (60:20:20); (3) phenol–water (75:25); and (4) *n*-propyl alcohol–34% ammonia (67:33). The first three solvent systems were

employed in conjunction with one-dimensional analyses carried out by the ascending technique. Systems three and two, and four and one were paired off during the two-dimensional analyses with the first of each pair being used for the initial development. Chromatograms were dried at 60° for 30 min prior to development in the second dimension. Successful resolutions of mixtures of the ten amino acids were achieved in 10 h or less during which the solvent fronts were permitted to move 18 cm in each dimension by the ascending technique.

The acids were identified by using R_F values[1] or, in ambiguous situations, by simultaneously running standards for comparison purposes. A Beckman Model DU Spectrophotometer fitted with a standard attachment for the measurement of diffuse reflectance was employed for the quantitative evaluation of the spots, which were scraped off the chromatographic plates and worked up into 40 mg analytical samples. The cells used to hold the samples and reference material as well as the procedure followed in preparing material for examination have been described elsewhere by the authors[2,3].

RESULTS AND DISCUSSION

Identification of amino acids

When solvent mixtures containing 0.2 % ninhydrin were used to develop one-dimensional chromatograms, it was not possible to observe the coloration noted by EL KHADEM *et al.*[4] with paper chromatograms when the solvent front reached the amino acids. The spots that appeared after drying, however, were sharply defined and exhibited no tailing. An increase in the ninhydrin concentration to 0.4 % did succeed in producing a faint pink color during the development of the plates, which made it

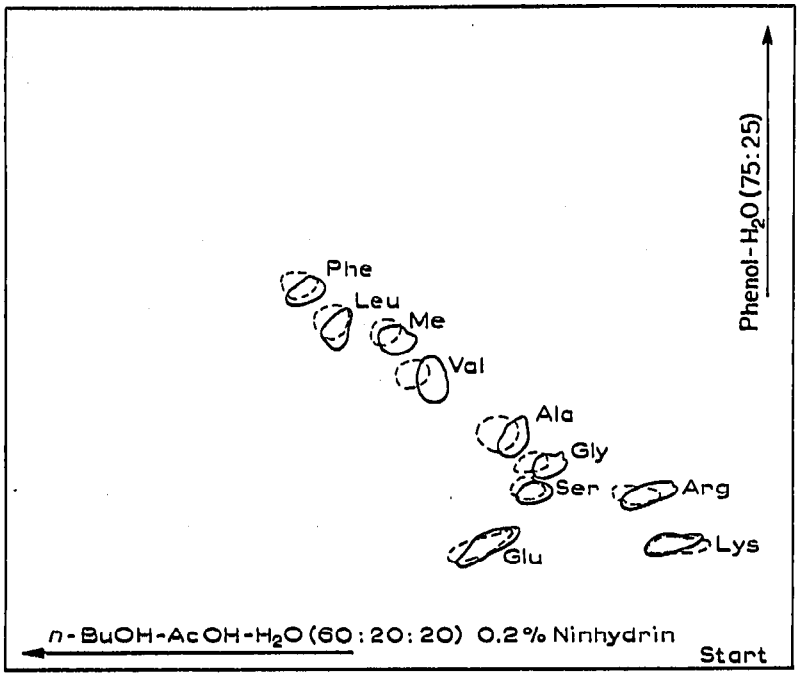

Fig. 1. Two typical chromatograms obtained by using solvent mixture three for the initial development and 0.2 % ninhydrin in solvent mixture two for the second development. Trial one, dotted line; trial two, solid line.

possible to follow the movement of the spots, as well as a greater sensitivity. This last was offset somewhat by the appearance of some tailing.

A clean separation of a mixture consisting of 3 μg of each of the ten amino acids was achieved in 8 to 10 h when solvent three without ninhydrin was used for the initial development and solvent two which was 0.2 % with respect to ninhydrin was employed in the second dimension. Some idea of the degree of resolution attained as well as the feasibility of using R_F values for the identification of the acids can be gained from a consideration of Fig. 1, which shows one chromatogram obtained by means of this procedure superimposed on a second. It should be remembered that the thickness of the adsorbent layer was somewhat greater than usual so as to provide enough material for the determination of the acids, and that the adsorbent was applied not with a precision applicator but manually with masking tape and a glass rod.

Similar success, insofar as resolution is concerned, was realized when solvent mixture four was employed for development in the first dimension and 0.2 % ninhydrin in solvent mixture one for development in the second. As may be seen in Fig. 2,

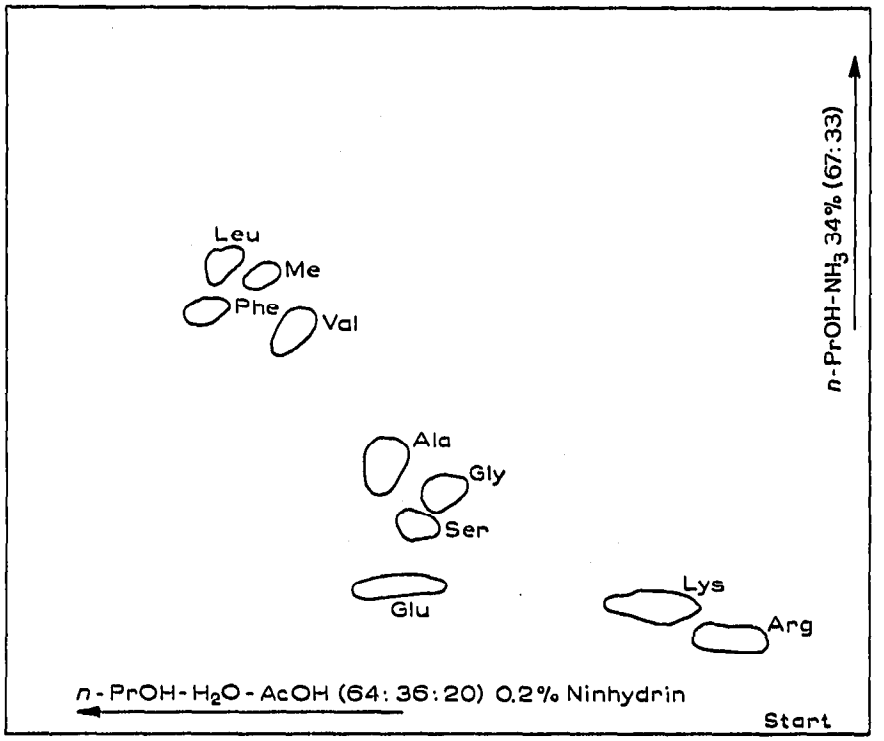

Fig. 2. Typical chromatogram obtained by using solvent mixture four for the initial development and 0.2 % ninhydrin in solvent mixture one for the second development.

which depicts a typical chromatogram obtained with these solvent systems, it is possible to make use of ammoniacal solvents in these separations provided that they are employed for the first development only and provided that the ammonia is removed by a drying operation preceding the second development. Enough adsorbed ammonia remains, however, to impart a brownish or purplish tinge to the entire plate. This results in a lack of contrast between the color of the adsorbent and that of the ninhydrin complexes of the amino acids which renders ammoniacal solvents unsuitable for use in conjunction with the method described herein.

Determination of amino acids

The reproducibility that can be expected of the method was determined by chromatographing four 5 μg replicates of each acid over a distance of 15 cm in one dimension by the ascending technique and preparing them for analysis according to the procedure outlined in the experimental section. When solvent mixture one to which 0.2 % ninhydrin had been added was employed to develop the plates, an average standard deviation of 0.49 % R was obtained for the ten sets. As may be seen in Table I, which

TABLE I

REPRODUCIBILITY OF REFLECTANCE READINGS OBTAINED AT 515 mμ FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF AMINO ACIDS CHROMATOGRAPHED IN ONE DIMENSION USING SOLVENT MIXTURE NO. 1 (0.2 % NINHYDRIN)

Amino acid	*Range* (% R)	*Mean* (% R)	*Std. dev.* (% R)
Alanine	72.8–73.6	73.0	0.39
Arginine·HCl	80.3–81.6	80.8	0.59
Glutamic acid	80.5–81.7	81.2	0.51
Glycine	77.3–77.9	77.5	0.27
Leucine	73.8–74.5	74.2	0.30
Lysine·HCl	79.0–80.9	79.8	0.84
Methionine	78.3–79.4	78.8	0.58
Phenylalanine	81.3–82.6	82.3	0.71
Serine	75.5–76.4	76.1	0.41
Valine	74.2–74.7	74.4	0.27
		Av. std. dev.	0.49

summarizes the results of this experiment, the largest standard deviation found for any one set was the 0.84 % R value observed with lysine. Similar data were obtained using solvent mixture two which was 0.2 % with respect to ninhydrin.

These results represent a considerable increase in precision over that provided by spray methods. An average standard deviation of 1.45 % R and a maximum standard deviation for a single set of 2.32 % R were found for a previous study[2] conducted with three 30 μg replicates of the same ten acids. The two studies differed principally in the solvent systems used and in that the ninhydrin was applied as a spray. Since the results of this research indicate that precision changes of the magnitude being discussed were not observed when the solvent systems were varied, one must ascribe the increase in reproducibility to the elimination of the spraying operation. This conclusion is in accord with the findings reported by JELLINEK AND FRIDMAN[5] who carried out a critical evaluation of the errors in a densitometric analysis of glycine on paper chromatograms.

The effect produced by increasing the ninhydrin concentration was ascertained by repeating the preceding experiment with solvent three to which 0.4 % ninhydrin had been added. As indicated in Table II, the reproducibility attained was only slightly less than that observed with 0.2 % ninhydrin solutions. An average standard deviation of 0.53 % R was obtained for the ten sets; the largest standard deviation found for any one set was 0.87 % R. There was, however, an increase in sensitivity from the 1.0 μg value found for most of the acids with solvents one and two that had

TABLE II

REPRODUCIBILITY OF REFLECTANCE READINGS OBTAINED AT 515 mμ FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF AMINO ACIDS CHROMATOGRAPHED IN ONE DIMENSION USING SOLVENT MIXTURE NO. 3 (0.4 % NINHYDRIN)

Amino acid	*Range* (% *R*)	*Mean* (% *R*)	*Std. dev.* (% *R*)
Alanine	50.2–51.1	50.8	0.60
Arginine·HCl	58.3–59.8	59.0	0.66
Glutamic acid	57.7–58.9	58.3	0.57
Glycine	55.8–56.9	56.3	0.56
Leucine	51.0–51.6	51.2	0.30
Lysine·HCl	56.3–57.6	56.9	0.61
Methionine	55.9–57.0	56.7	0.57
Phenylalanine	59.0–60.9	60.1	0.87
Serine	54.2–54.8	54.4	0.30
Valine	52.7–53.5	53.2	0.34
		Av. std. dev.	0.53

been made 0.2 % with respect to ninhydrin to the 0.5 μg value observed with the 0.4 % ninhydrin solution of solvent three. These last results are essentially the same as those obtained when the ninhydrin was applied as a spray[2].

As expected there was some decrease in reproducibility where the ten acids were chromatographed in two dimensions, though the precision was still considerably better than that achieved in one dimension with the use of sprays. The results obtained when four 5 μg replicates were chromatographed in the first dimension with solvent mixture three and in the second dimension with solvent mixture two to which 0.2 % ninhydrin had been added are presented in Table III. The average standard deviation for these data was 0.77 % *R*, with no standard deviation in excess of 1.18 % *R* being found for any of the acids.

TABLE III

REPRODUCIBILITY OF REFLECTANCE READINGS OBTAINED AT 515 mμ FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF AMINO ACIDS CHROMATOGRAPHED IN THE FIRST DIMENSION WITH SOLVENT MIXTURE NO. 3 AND IN THE SECOND DIMENSION WITH SOLVENT MIXTURE NO. 2 (0.2 % NINHYDRIN)

Amino acid	*Range* (% *R*)	*Mean* (% *R*)	*Std. dev.* (% *R*)
Alanine	67.2–68.1	67.8	0.41
Arginine·HCl	78.3–80.4	79.3	0.90
Glutamic acid	78.7–80.3	79.5	0.78
Glycine	74.3–75.3	74.8	0.41
Leucine	68.8–70.8	69.8	0.74
Lysine·HCl	77.9–80.4	79.3	1.15
Methionine	75.8–77.2	76.6	0.65
Phenylalanine	80.3–82.4	81.1	0.90
Serine	70.6–73.0	72.3	1.18
Valine	70.2–71.5	70.9	0.60
		Av. std. dev.	0.77

Despite the increased reproducibility resulting from the elimination of the spraying operation, elements associated with the ninhydrin reaction continued to be the chief factors limiting the precision of the method. Among the most important of these was the close dependence of the color stability of the ninhydrin complexes of the adsorbed amino acids upon the nature of the solvent system used to develop the chromatoplates and on the temperature at which the developed plates were kept[6]. The effect of temperature was such that developed plates which were stored any length of time had to be maintained below 10° to reduce color density changes to a level consistent with precision requirements of the order discussed above. Under these temperature conditions the solvent mixture containing phenol was found to be preferable to those containing acetic acid from the standpoint of color stability. Over a 24 h period no variation in excess of 1 % R was observed in the case of plates developed with solvent mixture three while plates developed with mixtures one and two exhibited variations as large as 3 % R. This increased stability associated with the phenolic solvent was apparent even when it was employed as the first solvent in the development of a two-dimensional chromatogram. To insure maximum precision, therefore, it is essential that plates on which unknowns and reference standards are being resolved be processed at the same time and under identical conditions. By proceeding in this manner, it is possible to cancel out not only variations related to the ninhydrin reaction but also those which occur during the drying of the chromatograms because of oxidation and volatilization of the amino acids.

Errors associated with the other operations constituting the procedure were of secondary importance. Variations attributable to the packing of the sample cell were found to amount to an average standard deviation of 0.2 % R for a stable system whose analysis involved no chromogenesis[3]. Although excessive tailing and poor resolutions can curtail the accuracy considerably, especially if large concentrations of acids are involved, such errors can be avoided to a large degree by choosing suitable separation procedures; by increasing the thickness of the adsorbent layer; and by extending the development time. When working with more than 20 to 30 μg of acid, it was necessary to make the analytical sample larger than 40 mg to accommodate the increased amount of test material as the areas of the spots were approximately proportional to the concentration. To determine the loss of precision that might be attributed to tailing, plates on which were paired developed and undeveloped spots consisting of 30 μg of the same acid were sprayed, dried at approximately 55°, and subjected to analysis by spectral reflectance. In the case of no acid were differences greater than those ascribable to the ninhydrin reaction found between paired spots. This observation is in keeping with results reported for paper chromatography[7,8].

The effect upon reproducibility of varying the slit width of the spectrophoto-

TABLE IV

REPRODUCIBILITY OF REFLECTANCE MEASUREMENTS AS A FUNCTION OF SLIT WIDTH. READINGS OBTAINED AT 515 mμ WITH A SAMPLE OF GLYCINE COMPLEXED WITH NINHYDRIN

Slit width (mm)	0.25	0.3	0.4	0.5	0.6	0.7
Band width (mμ)	5	6	8	10	12	14
Mean of four readings (% R)	67.5	67.5	67.6	67.7	67.5	69.1
Standard deviation (% R)	0.05	0.00	0.05	0.08	0.14	0.22

meter was ascertained by measuring the reflectance at 515 mμ of a 10 μg sample of glycine complexed with ninhydrin relative to a standard consisting of adsorbent removed from the same plate. Four consecutive measurements were made at each of several slit openings using the blue-sensitive phototube at load resistor setting 2 and at sensitivity setting 3. A consideration of the results obtained, which are set forth in Table IV, revealed that standard deviations of 0.05 % R or less can be expected for slit widths in the range 0.25–0.4 mm. There is a decrease in precision with widths greater than this while smaller widths are not suited for the measurement of diffuse reflectance. The band widths isolated at the various slit settings were obtained from dispersion data provided for the Beckman DU spectrophotometer.

Finally the probable relative error in the measurement of the concentrations of alanine, leucine, serine and valine was determined by making use of the precision data obtained with four 5 μg replicates of the acids and listed in Table I, and of the calibration curves for these same acids which were presented by the authors in an earlier paper[2]. A similar investigation was carried out to ascertain the relationship

TABLE V

PROBABLE RELATIVE ERROR IN THE MEASUREMENT OF THE CONCENTRATIONS OF SOME AMINO ACIDS

	Alanine	*Leucine*	*Serine*	*Valine*
Range (% R)	72.8–73.6	73.8–74.5	75.5–76.4	74.2–74.7
Mean (% R)	73.0	74.2	76.1	74.4
Standard deviation (% R)	0.39	0.30	0.41	0.27
Equivalent change in measured concentration of acid (μg)	0.18	0.15	0.25	0.14
Probable % relative error	3.6	3.8	5.0	2.8

between the probable relative error and the concentration of glycine. In this instance solvent one which was 0.2 % with respect to ninhydrin was employed in conjunction with four replicates of acid at each concentration investigated. Data relative to these two studies are presented in Tables V and VI, respectively. The change in measured concentration equivalent to the deviations observed for the various acids was obtained from the appropriate calibration curves and expressed as a probable % relative error in concentration. For the five acids at 5 μg concentration this figure ranged from

TABLE VI

PROBABLE RELATIVE ERROR IN THE MEASUREMENT OF THE CONCENTRATION OF GLYCINE AS A FUNCTION OF CONCENTRATION

	Concentration of glycine (μg per spot)			
	2	5	10	20
Range (% R)	84.3–86.0	77.3–77.9	63.6–65.1	55.3–57.1
Mean (% R)	85.1	77.5	64.4	56.3
Standard deviation (% R)	0.92	0.27	0.66	0.76
Equivalent change in measured concentration of glycine (μg)	0.18	0.20	0.50	1.40
Probable % relative error	9.0	4.0	5.0	7.0

a low value of 2.8 % for valine to a high of 5.0 % for serine. In the case of glycine minimal values were obtained in the intermediate concentration range. The relatively large 9.0 % value observed at the 2 μg concentration may be attributed to the fact that this concentration approached the 1 μg sensitivity limit for glycine as well as to the increased contribution of volumetric and gravimetric errors associated with such operations as the preparation of the standard solutions. At the opposite end of the scale, the 7.0 % figure found for 20 μg concentrations can be ascribed to the flattening of the calibration curve that occurs at high concentrations. Of the acids investigated, this effect is particularly noticeable in the case of glycine[2].

CONCLUSIONS

The application of a nonspray method devised by EL KHADEM *et al.*[4] for use in paper chromatography to the determination by reflectance spectrophotometry of amino acids resolved on thin-layer plates has enhanced the utility of the latter technique considerably. By dissolving ninhydrin in the solvent mixtures employed to develop the chromatograms, one drying and the subsequent spraying of the plates are eliminated. Despite this modification, the R_F values of the amino acids are not altered with the result that values obtained by means of the conventional spray method can still be utilized for purposes of identification. The nonspray procedure can be applied successfully to two-dimensional chromatography by adding the ninhydrin to the second solvent system. When no quantitative work is contemplated, ammoniacal solvents can be employed for the first development provided that the ammonia is removed by drying prior to the development in the second direction.

Depending on the concentration of ninhydrin used, the sensitivity of the method is between $5 \cdot 10^{-9}$ and $1 \cdot 10^{-8}$ moles for most amino acids[2]. Although this is somewhat less than that afforded by the *in situ* estimation of amino acids separated on paper, this slight decrease in sensitivity is more than compensated for by an increase in precision and accuracy. The elimination of the spraying operation with its attendant irregularities, such as leaching out of the amino acids, has resulted in a precision which is comparable to that reported for reflectance measurements made of spots on paper, and which is better than that reported for transmission measurements made of these same spots through the paper[9]. The comparison becomes all the more favorable when one considers that the latter data were obtained with a stable system —copper rubeanate—and with undeveloped spots. The overall accuracy attained in the measurement of the concentrations of amino acids equals or exceeds that achieved when such techniques as total scanning and the electronic integration of curves are employed in conjunction with paper chromatograms[5,10,11].

Although the present study was restricted to amino acids, there appears to be no reason why an appropriate modification of the procedure described herein cannot be applied to other systems. For example, EL KHADEM *et al.*[4] have also employed the nonspray technique for the separation of sugars on paper while BEVENUE AND WILLIAMS[12] have devised a method for the direct estimation by reflectance spectrometry of sugars separated by paper chromatography. It should be possible to make use of these results in conjunction with such procedures for the resolution of sugars on thin-layer plates as those proposed by STAHL AND KALTENBACH[13] and by PASTUSKA[14].

SUMMARY

A nonspray method is described whereby amino acids resolved on thin-layer plates can be estimated by spectral reflectance. In addition to facilitating the determination the elimination of the spraying operation with its attendant irregularities has resulted in a substantial increase in precision and accuracy. In this respect the method is competitive with paper chromatography, used in conjunction with such techniques as total scanning and the electronic integration of curves.

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