THE DETERMINATION BY REFLECTANCE SPECTROPHOTOMETRY OF AMINO ACIDS RESOLVED ON THIN-LAYER PLATES

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INTRODUCTION

The obvious advantages afforded by paper chromatography in the analysis of amino acids has resulted in an extensive literature in the field[1]. A considerable number of these publications deal with problems involving the quantitative estimation of the acids[2] following their resolution. In this connection, much interest has been shown in the *in situ* determination of the acids as a means of eliminating tedious elution procedures and thus expediting the analyses. POLSON *et al.*[3] made measurements by visually comparing the colors resulting from the addition of ninhydrin to standard and unknown amino acid concentrations. BULL *et al.*[4] devised a procedure for the estimation of amino acids separated by paper chromatography which involved direct photometry of the spots. The accuracy attained by this and some of the other direct photometric methods[5–7] developed subsequently was of the order of ± 5 % to ± 12 %. LUGG AND MCEVOY-BOWE[8,9] achieved increased precision by employing a specially designed densitometer having a small aperture and by taking account of texture irregularities in the chromatographic paper.

The promise of reduced tailing, increased sensitivity, and greater speed and resolution[10] has induced many investigators to resort to the thin-layer technique in the analysis of amino acids. Unfortunately, since methods developed for the *in situ* estimation of acids separated on paper are not applicable to chromatoplates, there is need for a procedure whereby this can be accomplished in separations involving thin plates. The possibility of using reflectance measurements for this purpose was suggested by a study of the application of spectral reflectance to thin-layer chromatography[11]. Results of this research demonstrated that the components of dye mixtures resolved on thin-layer plates can be determined with a precision of approximately ± 5 % by direct examination of the plates. If the reflectance measurements are carried out on spots removed from the plates and packed in an appropriate cell, the degree of precision attained is identical to that afforded by transmittance.

EXPERIMENTAL

The amino acids studied (DL-alanine, L-arginine, L-glutamic acid, glycine, L-isoleucine, L-leucine, L-lysine, DL-methionine, DL-norleucine, DL-norvaline, DL-phenylalanine, DL-serine, DL-threonine and DL-valine) were of Calbiochem A Grade purity. Stock solutions containing 500 mg of the acids per 50 ml of solution were used in making up

the dilution series employed in this research. Distilled water served as the solvent throughout and the solutions were applied as spots by means of a Hamilton microsyringe in 5 μl increments. The 20 × 5 × 0.35 cm plates were coated with adsorbent by distributing a Merck silica gel G–water (4:10) mixture with a glass rod which rested on one thickness of masking tape affixed to the ends of the plates. This technique gave a uniform coating 0.2–0.3 mm thick. The plates were dried at 180° for 2 h and stored in a desiccator. The amino acids were chromatographed in *n*-propanol–water (64:30) by the one-dimensional ascending technique described by BRENNER AND NIEDERWIESER[10], and the plates were then dried at 60° for 30 min in a mechanical convection oven.

The spray reagent, consisting of 90 g of *n*-butanol, 10 g of phenol and 0.4 g of ninhydrin, was one described by BULL *et al.*[4]. An even dispersion of the spray was achieved by using an atomizer in conjunction with compressed air at a distance of 30 to 40 cm from the plate. After the gas pressure was adjusted to forestall impairment of the adsorbent surface, the plates were sprayed until they first appeared translucent. An excess of spray was avoided to preclude leaching out of the amino acids. The plates were next exposed to a stream of cold air for 5 min, dried in a mechanical convection oven at 60° for 15 min, and then stored in the dark at about 10° in a refrigerator until required for the determination. These drying conditions were selected after a consideration of the results of various investigations[6,12,13] which indicated that drying temperatures exceeding 60° substantially reduced recoveries of amino acids from paper chromatograms.

Direct spectral examination of these plates was accomplished with a Beckman Model DK-2 Spectrophotometer fitted with a standard reflectance attachment. A Beckman Model DU Spectrophotometer, likewise equipped for the measurement of diffuse reflectance, was employed to examine spots scraped off the chromatoplates. The cells used to hold the sample and reference material as well as the technique utilized in preparing material for examination have been described elsewhere by the authors[11]. With these cells 40 mg of the material being studied sufficed to give a thin layer, of an approximate thickness of 0.3 mm and an approximate diameter of 1.8 cm, dimensions which have been found to be optimum for the analysis. The reference standard in all cases consisted of adsorbent from the plate under investigation.

For the analysis of spots removed from chromatoplates, the 40 mg comprising the sample were weighed to ± 0.3 mg and then ground in a small agate mortar for two periods of 1 min each to insure homogeneity and uniform particle size. Samples were weighed and worked up in a low humidity, air-conditioned room to avoid the condensation of moisture. With the exception of this period, the samples were stored in a large desiccator from the time they were removed from the refrigerator until they were introduced into the reflectance attachments of the spectrophotometers. In addition to the calcium chloride desiccant, the desiccator contained dry ice as a coolant to ensure color stability.

RESULTS AND DISCUSSION

Color development

The necessity for storing the samples at reduced temperatures was pointed up by the results of a study of the variation of the color developed using the ninhydrin spray described above with time and temperature. A dilution series of leucine ranging from 5.0 to 50 μg was employed for this study with readings being taken at 520 mμ. Spots

were removed from the chromatoplates and prepared for analysis by the procedure outlined above. As may be seen in Fig. 1, the color intensity of the ninhydrin complex of leucine decreased by as much as five reflectance units within 24 h when the samples were kept at room temperature (28°). To attain the degree of precision inherent in the reflectance technique[11], the storage temperature had to be maintained below 10°. When

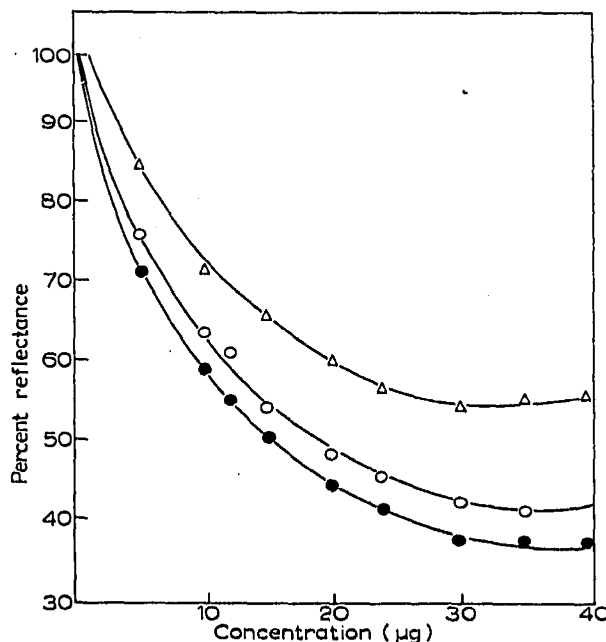

Fig. 1. % reflectance at 520 mμ of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration and time. ●–● after 2 h; ○–○ after 24 h; △–△ after 3 days at room temperature (28°).

this precaution was observed with a similar dilution series, the average change over a 24 h period was found to be 1.0 reflectance unit and the greatest decrease noted was of the order of 2.0 reflectance units. The data for this study are presented in Table I. Similar results were obtained with all of the amino acids listed above except phenylalanine and glutamic acid. In these two instances changes ranging up to three and five reflectance units, respectively, were noted.

The ninhydrin spray reagent used initially was a modification of one described by BRENNER AND NIEDERWIESER[10]. It was prepared by mixing 50 parts by volume of a 0.2 % solution of ninhydrin in absolute alcohol with 3 parts by volume of a 1 % solution of $Cu(NO_3)_2 \cdot 3H_2O$ in absolute alcohol and then adding 20 ml of glacial acetic acid to 100 ml of the resulting mixture. The spray described by BULL *et al.*[4] was found to provide greater sensitivity, however, and was employed in the acquisition of the data presented in this report. Fig. 2, which depicts % reflectance and absorbance at 520 mμ of the ninhydrin complex of leucine adsorbed on silica gel as a function of concentration, shows clearly the increased color intensity resulting from the use of the latter spray. Both sprays yield analytically useful data which take the form of a straight line up to concentrations of 30 μg when absorbance is plotted *versus* the square root of the concentration. Sensitivities found for leucine—0.5 μg for the BULL spray and 2 μg for the modified BRENNER-NIEDERWIESER spray — were consistent with the 0.5 μg value reported by PRATT AND AUCLAIR[14] for a 0.1 % solution of ninhydrin in

TABLE I

% REFLECTANCE AT 520 $m\mu$ OF NINHYDRIN COMPLEX OF LEUCINE ADSORBED ON SILICA GEL AS A FUNCTION OF CONCENTRATION AND TIME FOR SAMPLES STORED BELOW 10°

Concentration of dilution series (µg leucine)	*Time*			*Range in reflectance units*
	2 h (% R)	*6 h (% R)*	*24 h (% R)*	
0.5 × 10	71.7	71.9	70.9	1.0
1.0	59.3	59.3	58.7	0.6
1.2	56.0	55.9	55.2	0.8
1.4	52.4	51.4	51.2	1.2
1.6	50.0	49.2	48.5	1.5
2.0	46.4	45.8	44.4	2.0
2.2	45.0	44.6	44.0	1.0
2.4	43.1	42.2	41.7	1.4
2.6	41.8	41.8	42.2	0.4
2.8	40.1	39.5	38.9	1.2
3.0	39.2	38.9	37.8	1.4
3.2	38.5	38.5	38.1	0.4
3.4	38.0	37.8	37.4	0.6
3.6	37.8	37.2	37.2	0.6
4.0	37.2	36.3	36.0	1.2
5.0	36.8	36.3	36.3	0.5
Average range in reflectance units:				1.0

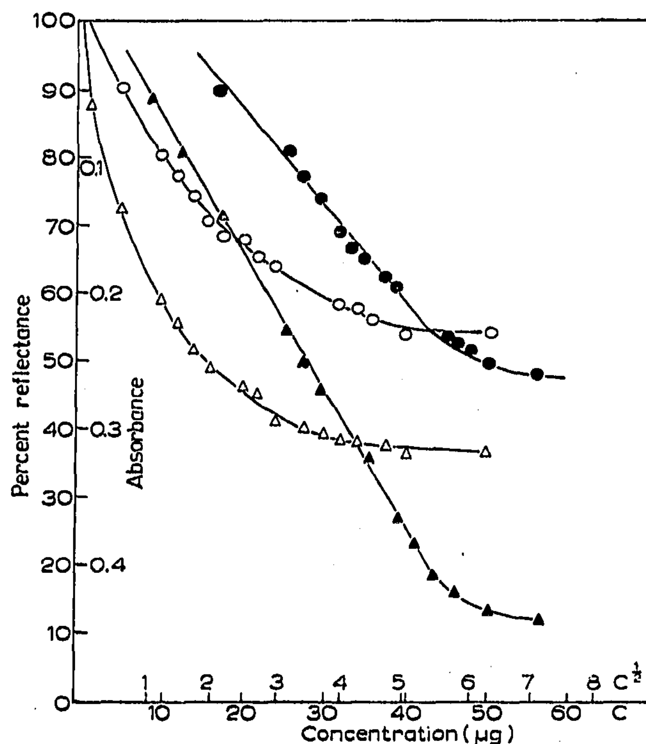

Fig. 2. % reflectance and absorbance at 520 $m\mu$ of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration. Modified BRENNER–NIEDERWIESER spray: ○–○ % reflectance *vs.* C; ●–● absorbance *vs.* $C^{1/2}$, BULL *et al.* spray, △–△ % reflectance *vs.* C; ▲–▲ absorbance *vs.* $C^{1/2}$.

n-butanol used in conjunction with paper chromatograms. The difference can be ascribed to the three- or four-fold dilution of the ninhydrin complex with silica gel adsorbent that occurs during the preparation of the sample for analysis.

The procedure outlined above for the development and measurement of the color due to the ninhydrin complex was suggested by the results of a study of the effects of temperature and time of development, and of post-development storage time, upon color intensity. Chromatoplates, spotted with identical amounts of leucine (30 μg) and sprayed with the BULL reagent, were developed at different temperatures (27°, 60° and 90°) for varying periods of time (10, 15 and 20 min) and then read at 1, 4 and 24 h intervals following their storage in the dark at room temperature. The results of the study are summarized in Table II as mean values of % reflectance readings

TABLE II

% REFLECTANCE AT 530 mμ OF NINHYDRIN COMPLEX OF LEUCINE ADSORBED ON SILICA GEL AS A FUNCTION OF TEMPERATURE AND TIME OF COLOR DEVELOPMENT, AND OF POST-DEVELOPMENT STORAGE TIME (AT 27°)

	Development temperature						
	90° ± 2°			*60° ± 1°*			*27° (room temperature)*
Development time (min)	*10*	*15*	*20*	*10*	*15*	*20*	
Storage time (h)	*(% R)*	*(% R)*	*(% R)*	*(% R)*	*(% R)*	*(% R)*	*(% R)*
1	47.6	46.6	46.9	44.9	43.9	45.7	
4	54.2	52.7	53.2	49.4	48.2	51.3	41.3
24	81.6	79.0	80.2	72.5	69.1	72.3	53.8

obtained from three replicates for each set of experimental conditions. Although plates read an hour after the ninhydrin color had been developed yielded the lowest reflectance readings regardless of the other variables, a longer interval was utilized since it lends itself more to serial analyses. Because it afforded greater precision, development at 60° for 15 min was preferred to development at room temperature despite the fact that the latter method produced a more intense color. Indeed, of the readings taken at the 4-h interval, only those obtained at room temperature exhibited a range in excess of 1.5 reflectance units. There the ranges found corresponded to 3.0 reflectance units. These results agree substantially with those obtained by MCFARREN *et al.*[6] with paper chromatograms. Such was not the case with an attempt to enhance color intensity by treating the chromatoplates with steam for 5 min following development at 60° for 10 min. Whereas BULL *et al.*[4] employed this technique successfully with paper chromatograms, similar treatment of thin-layer plates had the reverse effect. In one case the % reflectance was found to increase from 43.9 to 55.2.

Direct examination of chromatoplates

The quantitative potential of the direct examination technique is indicated by Fig. 3, which shows the reflectance spectra of various concentrations of leucine adsorbed on silica gel and sprayed with ninhydrin reagent. Since it has already been shown[11] that the precision provided by this procedure is of the same order as that afforded by direct

transmission methods applied to paper chromatography[4–7], no further work was done on this aspect of the research.

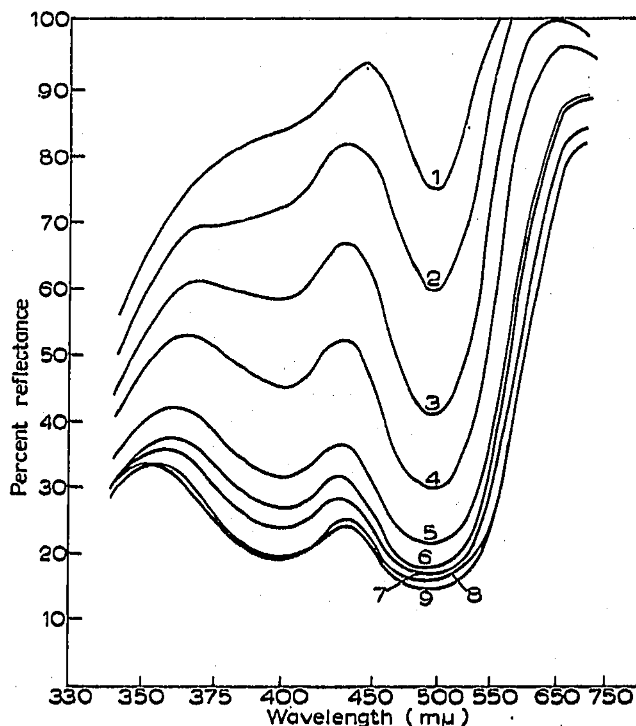

Fig. 3. Reflectance spectra of various concentrations of leucine adsorbed on silica gel and sprayed with ninhydrin reagent. Concentrations in mg: (1) 2.00; (2) 5.00; (3) 10.0; (4) 15.0; (5) 20.0; (6) 25.0; (7) 30.0; (8) 35.0; (9) 40.0.

Examination of spots removed from chromatoplates

A considerable increase in precision was achieved by analyzing spots removed from chromatoplates. The reproducibility one can anticipate for readings obtained for different spots of the same concentration of various amino acids adsorbed on silica gel and sprayed with ninhydrin reagent is indicated in Table III. In the case of each acid three 30 μg replicates were chromatographed over a distance of 15 cm in one dimension and prepared for analysis according to the procedure outlined in the experimental section. For this study the size of the analytical samples was increased to 60 mg and the reflectance at 515 mμ was determined 12 h after the ninhydrin color had been developed. An average standard deviation of 1.42 % was obtained for the fourteen sets; the largest standard deviation found for any one set was the 2.32 % value observed with alanine. This degree of precision was attainable, however, only with sets subjected to the procedure at one and the same time. Particularly large deviations resulted when plates were sprayed at times differing by an hour or more. In fact the precision of the determinations was limited by elements associated with the generation of the ninhydrin color and not by deviations arising from the packing of the sample cell, which were of secondary importance. This was in contrast to results obtained for a stable system whose analyses involved no chromogenesis[11]. On the other hand, when standards were processed along with samples under investigation, it was possible to achieve this order of precision with spots containing as little as 2 μg

of an amino acid. The best possible results were obtained when sets containing 3 to 4 replicates were chromatographed in the same chamber.

The possibility of using a single standard curve in the determination of several amino acids was suggested by a consideration of the color densities and standard deviations recorded in Table III. Accordingly the relationship between reflectance and

TABLE III

REPRODUCIBILITY OF REFLECTANCE READINGS OBTAINED AT 515 mμ FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF AMINO ACIDS ADSORBED ON SILICA GEL AND SPRAYED WITH NINHYDRIN REAGENT

Compound	*Range (% R)*	*Mean (% R)*	*Std. dev. (% R)*
Alanine	45.4–47.0	45.8	2.32
Arginine·HCl	60.4–62.2	61.2	1.37
Glutamic acid	60.9–63.0	62.3	1.94
Glycine	68.5–71.0	69.6	1.83
Isoleucine	47.4–49.0	48.0	1.80
Leucine	48.8–49.6	49.3	0.88
Lysine·HCl	59.8–60.2	60.0	0.33
Methionine	54.2–55.7	55.0	1.38
Norleucine	48.4–49.2	49.0	0.34
Norvaline	46.8–47.9	47.6	1.47
Phenylalanine	69.5–71.2	70.5	1.26
Serine	53.2–54.9	54.3	1.76
Threonine	60.0–61.8	61.3	1.85
Valine	48.4–49.8	49.2	1.47
		Av. std. dev.:	1.42

the concentration of the ninhydrin complexes of various amino acids adsorbed on silica gel was determined by means of the procedure employed for the precision study. Reflectance readings were taken 6 h after the ninhydrin color had been developed for dilution series covering the 0.5 to 50 μg range. Some of the standard curves obtained during the course of this study are shown in Fig. 4. It was found that one standard curve could indeed be used for the determination of norvaline, valine, norleucine, isoleucine and leucine without affecting the precision significantly. As may be seen in Fig. 4, the largest spread found between the curves for norvaline and leucine, which bracketed those for the other group members, only amounted to two reflectance units. Although standard curves for the other nine acids in general resembled those shown in Fig. 4, no other grouping analogous to the norvaline–leucine set was found. This was not surprising since the acids differed not only in their color development characteristics but also in their sensitivities. Sensitivities (in μg) found for the acids were as follows: alanine, glutamic acid, isoleucine, leucine, norleucine, norvaline, serine and valine —0.5; glycine —0.8; methionine —2.5; threonine —5; lysine —8; arginine —10; and phenylalanine —12. The interchange that occurred in the relative positions of the curves for glycine and threonine, which are included in Fig. 4, with increased concentration is indicative of some of the complexities encountered. A more useful form of these data is obtained when absorbance is plotted *versus* the square root of the concentration, as is done in Fig. 5, since this results in a linear relationship in the concentration range of analytical interest.

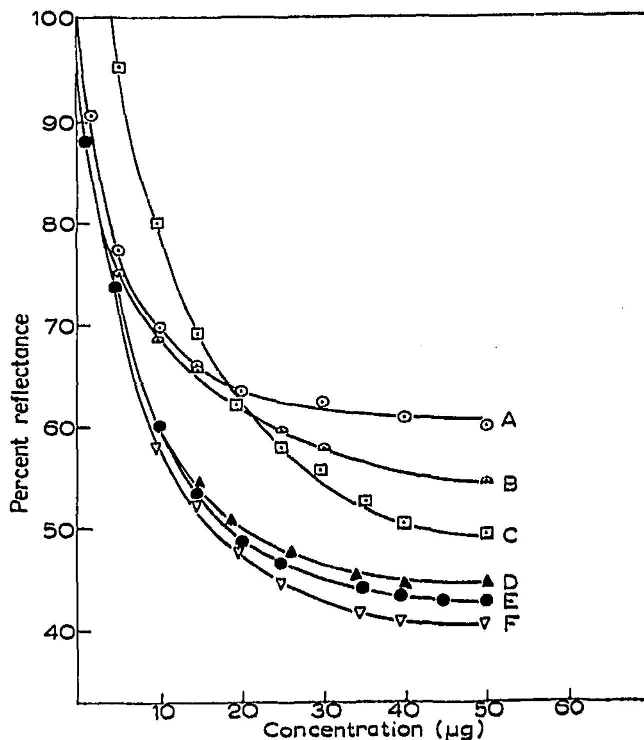

Fig. 4. % reflectance at 515 mμ of ninhydrin complexes of various amino acids adsorbed on silica gel as a function of concentration. (A) Glycine. (B) serine. (C) Threonine. (D) Leucine. (E) Norvaline. (F) Alanine.

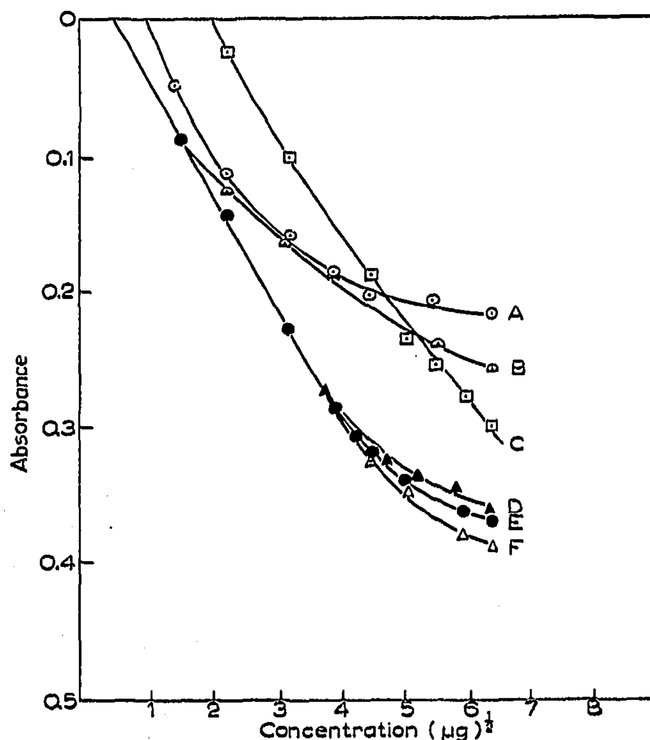

Fig. 5. Absorbance at 515 mμ of ninhydrin complexes of various amino acids adsorbed on silica gel as a function of the square root of concentration. (A) Glycine. (B) Serine. (C) Threonine. (D) Leucine. (E) Norvaline. (F) Alanine.

CONCLUSIONS

When specified precautions are observed in the generation of the ninhydrin color, amino acids resolved on chromatoplates can be determined by reflectance spectrophotometry. Direct examination of the plates yields results having the same range of precision, 5 to 12 %, as is reported for direct photometric methods developed for paper chromatograms. A degree of precision approaching that afforded by transmittance is achieved if the reflectance measurements are carried out on spots removed from the chromatoplates. Unlike analyses dealing with stable systems and involving no chromogenesis, the precision attained is limited by elements associated with the color development process and not by deviations arising from the preparation of the analytical sample. The dilution of the ninhydrin complex with silica gel adsorbent that occurs during the sample preparation reduces the sensitivity of the reflectance method to approximately half that reported for transmission measurements carried out with paper chromatograms, but the advantages afforded by the thin-plate technique more than compensate for this reduced sensitivity. These include more rapid resolution, increased precision and no requirement that the substrate be treated to compensate for textural irregularities. Over the concentration range best suited for analysis, 2 to 30 μg, a linear relationship is observed with most of the acids studied when absorbance is plotted against the square root of the concentration. Although the present study was restricted to one-dimensional thin-film chromatography, there appears to be no reason why spectral reflectance cannot be applied to the two-dimensional process with equal success.

SUMMARY

A procedure was devised whereby amino acids resolved on chromatoplates can be determined by spectral reflectance. Direct examination of plates yielded a degree of precision comparable to that afforded by direct transmission methods applied to paper chromatography. Precision approaching that afforded by transmittance is attained if the reflectance measurements are carried out on spots removed from the plates and specified precautions are observed in the generation of the ninhydrin color.

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