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AN IMPROVED TECHNIQUE FOR THE ANALYSIS OF AMINO ACIDS AND RELATED COMPOUNDS ON THIN LAYERS OF CELLULOSE

II. THE QUANTITATIVE DETERMINATION OF AMINO ACIDS IN PROTEIN HYDROLYSATES

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SUMMARY

A simple and accurate method has been devised for the quantitative analysis of protein hydrolysates using thin-layer chromatography. Ninhydrin–cadmium acetate is the staining reagent and, using reflectance densitometry, calibration graphs have been drawn for twenty-two naturally occurring amino acids. The equation of the line for each amino acid has been calculated and used for the determination of the amount of amino acid present in protein hydrolysates. The results obtained agree well with those obtained by the 'Technicon' Automatic Amino Acid Analyzer and with those given in the literature.

INTRODUCTION

Several methods are available for the quantitative evaluation of thin-layer chromatograms. Methods involving direct, *in situ*, determination of the compounds on the TLC plate are simpler and less tedious to carry out than determination after elution from the adsorbent. The most simple direct methods are those relying on minimum spot visibility[1] and measurement of spot area[2–4], which do not require an expensive instrument in order to obtain favourable results. However, a method which depends on both the area and on the intensity of colour is preferable to a method depending on only one of these parameters, as is the use of an instrumental method of determination which does not depend on subjective measurement.

Because of its simplicity, densitometry has recently been employed by several authors[5–7] for the quantitative determination of substances resolved by TLC. The results obtained by the above authors have shown the method to be both efficacious and reproducible.

In Part I (ref. 8) of this series of papers we described an improved method for the separation, on thin layers of cellulose, of forty amino acids and related compounds.

The present paper describes a method for the quantitative determination of amino acids in protein hydrolysates by direct reflectance densitometry, after their separation using a modification of the solvent system used in Part I. In order to give stable and reproducible colours the detection reagents are supplemented with a salt of the complex-forming cation, Cd^{2+}. A total of twenty-two compounds have been examined and standard calibration curves have been prepared. The standards have been used to determine the amount of each amino acid present in samples of hydrolysed protein. The results obtained compare favourably with those obtained from ion-exchange analysis and also with the values given in the literature.

EXPERIMENTAL

Apparatus

The thin-layer equipment used throughout this work was supplied by Shandon*. The solutions were applied to the thin layers by means of capillary pipettes (Microcaps)** of 1 μl capacity. The automatic recording and integrating double-beam reflectance densitometer, "Chromoscan", with thin-layer attachment was used for the quantitative evaluation of thin-layer chromatograms***. The results obtained by densitometry following chromatographic separation were compared with those obtained by ion-exchange chromatography using the Technicon§ automatic amino acid analyser.

Materials and methods

Adsorbent. Cellulose powder MN300 (without binder)§§ washed by the procedure given in Part I (ref. 8) was used.

Solvents. The solvents§§§ used throughout this work were of Analar grade with the exception of 2-methyl-2-butanol (G.P.R.) and butanone and propanone (M.F.C.).

Amino acids. The amino acids§§§ used were the natural L-forms with the exception of DL-threonine and DL-serine.

Proteins. The casein was supplied by British Drug Houses, Laboratory Chemicals Division, Poole, England (Package No. 0197143). The ash and moisture contents were found to be 1.9% and 4.8%, respectively. The gelatin was a good grade, acid-processed gelatin of high viscosity kindly supplied by Messrs. B. Young and Co. Ltd., Bermondsey, London. The ash and moisture contents were determined by analysis to be 1.2% and 15.0%, respectively.

Preparation of cellulose thin layers. Plates (20 × 20 cm) were spread with washed cellulose using the equipment supplied by Shandon and the procedure described in Part I (ref. 8) of this series of papers.

Standard solutions. Stock solutions of amino acids (0.025 *M*) were made up in aqueous 2-propanol (10%) as directed by SMITH[9]. These stock solutions were diluted stepwise to 0.0005 *M* as required; when not in use, they were kept in the refrigerator.

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** Drummond Scientific Co., Philadelphia, Pa., U.S.A.
*** Joyce Loebl and Co. Ltd., Gateshead-on-Tyne, Great Britain.
§ Technicon Instruments Co. Ltd., Hanworth Lane, Chertsey, Surrey, Great Britain.
§§ Macherey Nagel and Co. Ltd., Agents Camlab (Glass) Ltd., Cambridge.
§§§ Hopkin and Williams Ltd., Freshwater Road, Chadwell Heath, Essex.

Application of solutions to the thin layers. The starting point, 1.5 cm from the edges of the layer at the bottom left-hand corner, was positioned by marking the edges with a soft pencil. Care was taken not to disturb the surface of the layer in order to prevent distortion of the spots during chromatography. The limit of the solvent front (13 cm from the origin) in each dimension was marked in the same manner. To keep the area of the initial spot constant, the whole of the volume (1 μl) of solution was applied at once. This was accomplished by lightly touching the layer with the tip of the capillary tube. When it was necessary to apply volumes greater than 1 μl, the solution was applied successively in 1 μl portions. Between each application, the spot was dried in a stream of warm air.

Development. In Part I of this series of papers, after much experimentation, a solvent system was chosen which was capable of resolving large numbers of amino acids. However, in quantitative work it is not always necessary to make this factor the most important one since, in any particular protein hydrolysate for example, one can expect there to be fewer than twenty of the common amino acids present.

For the quantitative work, which will now be described, it was necessary to aim at producing the most concise spots as well as achieving adequate separation. The solvent system finally chosen in Part I was used as a basis for this work and indeed the solvent for development in the first dimension could not be improved upon.

The solvent systems used were: 2-propanol–butanone–1 *N* hydrochloric acid

TABLE I

$R_F \times 100$ VALUES OF PROTEIN AMINO ACIDS ON THIN LAYERS OF CELLULOSE

Solvent systems: 1st dimension: 2-propanol–butanone–1 *N* hydrochloric acid (60:15:25). 2nd dimension: 2-methyl-2-butanol–butanone–propanone–methanol–water–ammonia (0.88) (50:20:10:5:15:5).

No.	*Amino acid*	$R_F \times 100$	
		First dimension	*Second dimension*
1	Alanine	57	18
2	Arginine	19	8
3	Aspartic acid	48	2
4	Glutamic acid	56	2
5	Serine	39	23
6	Glycine	37	14
7	Threonine	51	48
8	Valine	79	35
9	Isoleucine	90	51
10	Leucine	90	55
11	Histidine	11	21
12	Lysine	16	13
13	Phenylalanine	82	55
14	Tyrosine	72	27
15	Tryptophan	70	49
16	Proline	58	24
17	Hydroxyproline	48	12
18	Cysteine	12	5
19	Cystine	6	2
20	Cysteic acid	53	6
21	Methionine	78	41
22	Hydroxylysine	10	14

(60:15:25) for development in the first dimension, and 2-methyl-2-butanol–butanone–propanone–methanol–water–ammonia (0.88) (50:20:10:5:15:5) for development in the second dimension.

Development in both dimensions was carried out in a saturated atmosphere exactly as described in Part I. The R_F values for the protein amino acids are given in Table I.

Chromogenic reagents. The ninhydrin–cadmium acetate reagent is similar to the one used by HEATHCOTE AND WASHINGTON[10], for the determination of amino acids on paper chromatograms. For consistent results it was desirable to raise the concentration of acetic acid in the reagent above that employed by the above authors as this gave the complex greater stability. KRAUSS[11] employed 5% acetic acid in the reagent but we found that 4% acetic acid was sufficient to give reproducible results.

The reagent was prepared by dissolving 0.5 g of cadmium acetate in 50 ml of water to which 20 ml of glacial acetic acid had been added. Propanone was then added to bring the total volume to 500 ml. Portions of this solution were taken before use and sufficient solid ninhydrin was added until the final concentration was 0.2% (w/v). The colour was developed by heating the plates, after spraying with reagent, at 60° for 15 min. Consistent results were obtained by allowing the heated plates to stand in the dark for 4 h prior to scanning.

Isatin–cadmium acetate was used for the detection of proline and hydroxyproline since these amino acids give yellow colours with the above reagent. It was prepared by adding 0.2 g of isatin to 100 ml of the above stock cadmium acetate solution. The colours were developed by heating the sprayed plates at 90° for 10 min. The plates were then stored in the dark at room temperature for 1 h before being scanned.

The above spray reagents were applied to the thin-layer plate using a Shandon atomiser at a distance of 30 cm until the plates just appeared translucent, excess of reagent being avoided to preclude any diffusion of the amino acid spots.

A separate plate was used for the determination of proline and hydroxyproline as this led to more consistent results than did overspraying the ninhydrin reagent with the isatin reagent.

Hydrolysis of proteins. The protein (10 mg) was hydrolysed at 110° with 6 *N* hydrochloric acid (2 ml) for 24 h in a sealed tube under an atmosphere of nitrogen. After this time the hydrolysate was evaporated *in vacuo* to dryness. A few milliliters of water were added and the solution was again taken to dryness. The process was repeated twice in order to remove most of the hydrochloric acid.

Densitometry. The characteristics of the Joyce Loebl "Chromoscan" densitometer have been discussed by DALLAS[7] and by SHELLARD AND ALLAM[6].

Throughout this work the conditions of operation of the instrument were standardised in order to make the procedure as simple as possible. Thus, since the ninhydrin–cadmium acetate reagent gave a red colour with most amino acids, a light filter of 490 nm was used to give maximum absorbance. Likewise for the blue complex produced by proline and hydroxyproline with the isatin–cadmium acetate reagent, a filter of wavelength 620 nm was used. The aperture used throughout was 1005 (10 mm × 0.5 mm) slit, and the gear ratio was 1:2. In all cases the direction of scan was opposite to the direction of development in the second dimension. The recommendation by DALLAS[7] to place two thicknesses of Whatman No. 3MM filter

paper immediately behind the plates was adopted throughout the work. The integrator on the instrument was not used because we could not obtain consistent results even when scanning the same spot. Instead, the area under the densitometric curve was obtained from the product of the altitude and the width of the curve at half the maximum height.

RESULTS AND DISCUSSION

Preparation of standard curves

After separating mixtures of amino acids by the above procedure, each plate was stained with reagent and the developed spots were scanned with the densitometer. The area under the response curve was measured for each amino acid at twelve different concentrations over the range of 5×10^{-4} μmole to 5×10^{-2} μmole; five separate replicas on five separate plates were made. When the mean response area was plotted against the concentration of amino acid, a curve was obtained in each case. The most useful form of the data, however, was obtained by plotting response area against the square root of the concentration of amino acid, as described by SHELLARD AND ALLAM[6] for alkaloids. Fig. 1 shows the results obtained for a typical selection of the amino acids. The graphs were found to be linear over the concentration range of analytical interest (0.05–5 μg) but the gradient was found to be different for each amino acid. This was largely to be expected in view of the results of HEILMANN *et al.*[12], who showed that the colour yields of the ninhydrin–cadmium acetate reagent for each amino acid were different.

From the standard graph of each amino acid, the equation of the line was determined (see Table II for data).

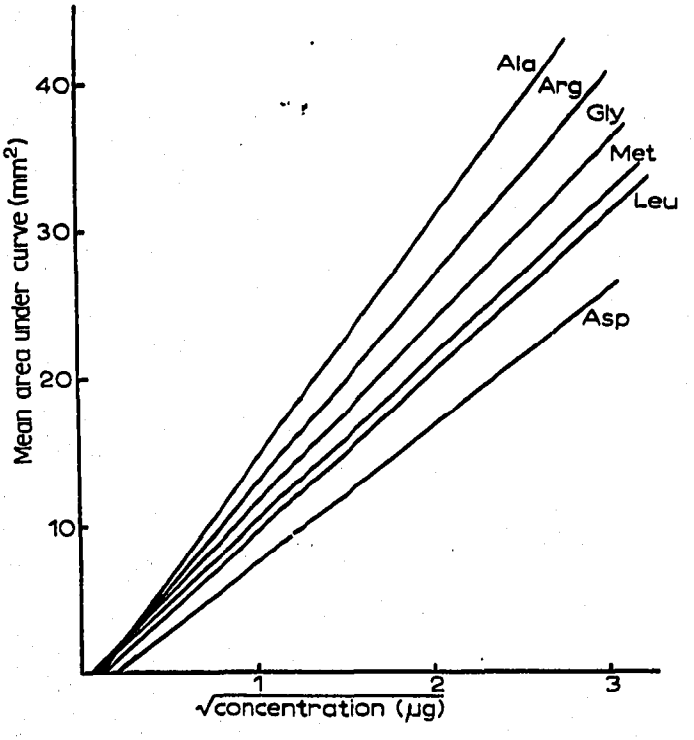


Fig. 1. Typical colour yields of amino acids with ninhydrin–cadmium acetate.

TABLE II

LINE CONSTANTS FOR AMINO ACIDS DETECTED WITH NINHYDRIN–CADMIUM ACETATE REAGENT.
Adsorbent: Cellulose powder. Solvent systems as in Table I.

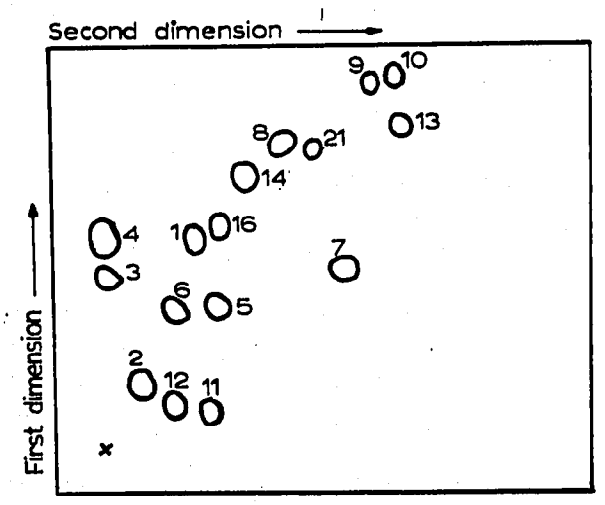
Amino acid	*Slope*	*Intercept*	*Amino acid*	*Slope*	*Intercept*
Alanine	1600	−240	Tyrosine	1000	−200
Arginine	1333	− 67	Tryptophan	542	−142
Aspartic acid	875	−200	Proline[a]	1890	−334
Glutamic acid	1375	−175	Hydroxyproline[a]	273	− 50
Serine	1083	−150	Cysteine	875	−175
Glycine	1125	− 75	Cystine	786	−128
Threonine	1600	−480	Cysteic acid	1125	−125
Valine	1430	−240	Methionine	1000	− 75
Isoleucine	1457	−307	Hydroxylysine	818	−172
Leucine	1000	−200			
Histidine	833	−100			
Lysine	1428	−171			
Phenylalanine	875	−200			

[a] Detected with isatin–cadmium acetate reagent. Method of scanning: reflectance; aperture (slit): 1005; Gear: 1:2.

If $A = m\sqrt{c} + b$ is the equation of a straight line then $(A - b)/m = \sqrt{c}$, where A is the area under the densitometric curve in mm^2, b is the intercept of the line on the ordinate in mm^2, m is the gradient of the line in $mm^2/\sqrt{\mu g}$, and c is the amount of amino acid in μg.

Determination of the amino acid

The equation of the line was used to determine the amount of a particular amino acid present in acid hydrolysates of gelatin and casein which had been chromatographed using the above solvent systems. Figs. 2 and 3 show the resolution of amino acids obtained in 15 μg samples of gelatin and casein hydrolysates, respectively.



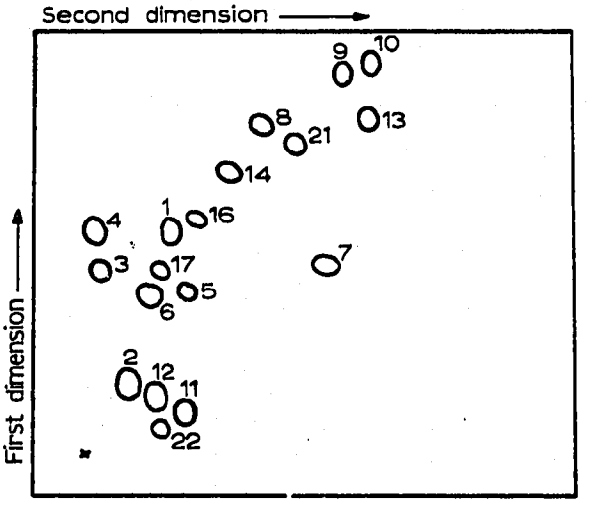


Fig. 2. Separation of amino acids present in a casein hydrolysate. For key see Table I.
Fig. 3. Separation of amino acids present in a gelatin hydrolysate. For key see Table I.

TABLE III

THE AMINO ACID COMPOSITION OF A GELATIN HYDROLYSATE BY THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

Amino acid	*Grams of amino acid per 100 g of gelatin on a moisture- and ash-free basis*		
	TLC	*Ion-exchange (Technicon)*	*Literature (Ref. 14)*
Alanine	10.6	10.3	9.3
Arginine	8.0	7.6	8.55
Aspartic acid	6.1	6.4	6.7
Glutamic acid	13.0	12.6	11.2
Glycine	28.6	31.1	26.9
Histidine	0.3	0.3	0.73
Hydroxylysine	1.5	1.4	1.2
Hydroxyproline	14.5	14.0	14.5
Isoleucine	1.0	0.8	1.8
Leucine	3.2	3.9	3.4
Lysine	4.4	4.5	4.6
Methionine	0.7	0.8	0.9
Proline	15.3	15.0	14.8
Phenylalanine	2.3	2.0	2.5
Serine	3.2	3.6	3.18
Threonine	1.8	2.2	2.2
Tyrosine	0.2	0.4	1.0
Valine	3.8	3.5	3.3

TABLE IV

THE AMINO ACID COMPOSITION OF A CASEIN HYDROLYSATE BY THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

Amino acid	*Grams of amino acid per 100 g of casein on a moisture and ash-free basis*		
	TLC	*Ion-exchange (Technicon)*	*Literature (Ref. 15)*
Alanine	3.0	3.3	3.2
Arginine	3.8	3.9	4.1
Aspartic acid	7.6	7.3	7.1
Glutamic acid	22.0	20.8	22.4
Glycine	2.4	2.2	2.0
Histidine	3.1	3.2	3.1
Isoleucine	5.6	5.0	6.1
Leucine	8.8	8.2	9.2
Lysine	7.8	8.1	8.2
Methionine	2.9	3.1	2.8
Phenylalanine	4.8	5.0	5.0
Proline	10.1	10.5	10.6
Serine	6.4	6.3	6.3
Threonine	5.5	6.9	4.9
Tyrosine	5.8	4.9	6.3
Valine	6.5	6.3	7.2
Cystine	0.4	1.4	0.4

TABLE V

RECOVERY OF THE INDIVIDUAL AMINO ACIDS FROM GELATIN AFTER 24-HOUR HYDROLYSIS

Amino acid	*% recovery*	
	Ion-exchange (Technicon)	*TLC*
Alanine	98.5	99.1
Arginine	99.2	95.4
Aspartic acid	101.4	97.6
Glutamic acid	97.0	101.4
Glycine	97.0	98.2
Histidine	100.7	94.2
Hydroxylysine	97.6	95.1
Hydroxyproline	98.1	93.7
Isoleucine	96.3	98.1
Leucine	95.0	99.4
Lysine	99.2	96.3
Methionine	98.5	101.4
Proline	96.9	100.3
Phenylalanine	102.1	95.8
Serine	98.1	96.4
Threonine	101.1	92.8
Tyrosine	96.9	95.8
Valine	98.7	99.9

The results obtained by the densitometric procedure were compared with those obtained by the ion-exchange technique of PIEZ AND MORRIS[13] using the Technicon automatic amino acid analyser and also with the values quoted in the literature. Table III shows the results obtained for a 24-h acid-hydrolysed sample of gelatin and Table IV shows the corresponding results for casein. The results of experiments on the recovery of added known amounts of amino acids from the gelatin hydrolysate are given for both TLC and ion-exchange chromatography (Technicon) in Table V.

It can be seen from Tables III and IV that the figures for both methods of analysis agree, in the main, to within ± 10%, a variation similar to that obtained by HEATHCOTE AND WASHINGTON[10] and by ATFIELD AND MORRIS[16] using paper chromatography and electrophoresis, respectively. The largest percentage differences occurred with those amino acids which were present only in small amounts. This may be due to the difference in sample size, 15 μg for densitometry and 500 μg for the auto analyser. However, the overall agreement between the values obtained by densitometry and ion-exchange is remarkably good. Considering that the proteins analysed by other workers[14,15] were not the same preparations as those analysed by ourselves, the agreement with literature values is also good.

It is perhaps worthwhile to compare the relative attributes of the two methods. The main disadvantage of TLC is that the operation is essentially manual and certain skills must be acquired by the operator. For the occasional lone sample the automatic ion-exchange method is the quicker (24 h) compared with TLC, which requires 2 days for one sample. However, during this time an operator can analyse at least thirty samples on plates. Perhaps the main advantage of TLC with regard to biological peptides and proteins is that much smaller amounts are required, TLC being at least ten times, and often one hundred times, more sensitive. The usual precautions to be

observed for TLC are concerned chiefly with achieving a constant thickness of the layer and constant initial spot size. However, the conditions for the generation of the colour produced by the staining reagent are of even greater importance in quantitative work and the suitability of the ninhydrin–cadmium acetate reagent in this connection has been demonstrated.

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