QUANTITATIVE DETERMINATION OF AMINO ACIDS ON FILTER PAPER

STAINING IN TWO STAGES

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INTRODUCTION

With a view to many problems of clinical interest it was desirable to improve on the methods already known of the quantitative determination of amino acids on filter paper. Other methods for the determination of amino acid composition of protein hydrolyzates, which have been used with considerable success, were of no avail; the microbiological assay method being too time-consuming and needing constant control of the moulds, the different methods of column chromatography needing too much material. In the meantime, results with quantitative determination of amino acids on filter paper had much improved with the procedure as developed by MOORE AND STEIN[1]. With the introduction of a strongly reducing agent they arrived at equalizing the colour intensity given by minimal amounts of various amino acids with ninhydrin. In order to avoid uncertainty inherent to the ninhydrin reaction performed on paper, BOISSONAS[2] eluted the amino acids first and stained them with MOORE AND STEIN's reagent *in vitro*. Adapting this procedure for two-dimensional chromatography it could still be improved by avoiding any contamination by ammonium ions and by a careful outlining of the space allotted to each amino acid on the paper. So far the methods (NAFTALIN[3]) allowed only the location of the centre of each stained spot, but they left uncertain its exact contour. It was our aim to overcome such difficulties as mentioned by *doing the staining in two stages*.

EXPERIMENTAL

Materials

Human serum albumin was supplied by the central laboratory of the blood transfusion service of the Swiss Red Cross at Berne. It is gained there by CONH's ethanol fractionation. Electrophoresis showed that it consists of 99% albumin and 1% α-globulin.

Hydrolysis of protein

Protein samples are hydrolyzed by boiling them 24 hours under reflux in 5 ml of 5 N HCl per 20 mg of protein.

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Removal of hydrochloric acid

The above solution of protein hydrolysate is placed in a large watchglass and a gentle stream of warm air is passed over it. When evaporated to dryness a few ml of 10% propanol are added and the evaporation done again. The residue is dissolved in a proportional amount of 10% propanol to give a solution of a 0.4–0.8 mg/ml nitrogen content. The exact content is determined by micro-Kjeldahl.

Preparation of paper and starting point

On sheets of Whatman paper 1. 40 × 40 cm, the starting area is drawn as a circle of 1 cm ⊖. The centre of it is placed 10 cm from the right margin and 6 cm above the lower margin. A line parallel to the lower margin is drawn 33 cm above it (it will serve as a mark for the end of the propanol run). The solution to be analysed is placed from a micropipette with slow outflow in the centre of the starting circle. The outflow must be so regulated that the liquid does not overflow the circle. The total amount of amino acids placed there-upon must remain within 100 and 200 γ. Following this application the starting circle is covered with 5 μl of a freshly prepared 35% H_2O_2 solution containing 0.1% ammonium molybdate. Now the whole area surrounding the starting circle is exposed for about 30 sec to the vapours of a 25% solution of ammonia. As a result of the oxidation and following neutralisation the starting circle turns faintly pink. The oxidized sheet is used for the determination of S-containing amino acids, the others are determined on a second sheet where oxidation was omitted.

Development of the chromatogram

As first solvent a mixture of *n*-propanol and water, 7:3 by volume, is used. In order to perform the propanol run, which is ascending, the sheet of paper is rolled in a loose spiral. It is held and supported in this position by a rod frame of glass. Glass frame and paper are now placed in a glass cylinder, containing enough propanol to wet the paper immediately up to a height of about half a cm. The plexiglas cover of the glass cylinder contains a fixation for the upper end of the glass frame. After a run of about 28 hours at 15–18° C room temperature the propanol front reaches the pencil line, drawn 33 cm above the lower margin. The paper is withdrawn from cylinder and glass frame. A current of warm air dries it in a few minutes.

The second run, using the descending technique, is performed in a wooden chromatographic cabinet with paraffin-treated walls. On the bottom of it is placed a glass jar with phenol-saturated water. Prior to use, 4–6 drops of 25% ammonia are sprinkled on the bottom of the corners. Water saturated phenol is used as solvent and the run takes 16–17 hours. Now the (stiff) phenol-soaked paper is carefully lifted out and resuspended in an another wooden cabinet of similar dimensions. Here an electrically heated air current dries the paper throughly in 3 hours, whilst the phenol vapours are sucked out of the laboratory by an electrically driven fan.

For the ninhydrin staining the following solutions (modified MOORE AND STEIN reagent) are prepared:

1. Buffer solution:

Citric acid + H_2O	13.3 g
N NaOH	100 ml
$SnCl_2 + 2H_2O$	400 mg
Water	up to 250 ml

The buffer solution can be kept for two weeks when kept under paraffin oil.

2. Ninhydrin solution:

Ninhydrin (F. Hoffmann-La Roche, Bale)	4 g
methylcellosolve	100 ml

Highest grade purity of chemicals is needed to guarantee low blank values.

A mixture of equal volumes of solutions 1 and 2 is prepared and 1.5 ml of it are evenly sprayed on the chromatogram. The latter is placed for exactly 15 min in an oven at 60° C sharp.

Isolation of the spots

After development of the colour, the zone of each spot is carefully outlined on the paper with a pencil. An adequate margin is advantageous insofar as the mutual position of neighbouring amino acid spots allow it. As a rule there is no difficulty in singling out each spot, with only those of leucine and phenylalanine overlapping partly. However, the following spraying with KOH reveals phenylalanine, which takes on a somewhat yellowish colour, whereas leucine is purple. Lysine often leaves a trail behind and must be treated accordingly. On Fig. 1 the three circles B_1, B_2 and B_3 (area of each 10 cm^2) designate the areas used as blank values.

Removal of ammonia

Carboxyl groups of the filter paper have the tendancy to fix ammonia. Following the technique of BOISSONAS it is driven away by spraying the paper with KOH. To this end 12.5 ml of a 1% solution

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of KOH in absolute methanol are evenly sprayed on each side of the sheet of paper. The spray must be fine and preferably driven by compressed air under proper control from the operator. Holding the sheet against a window it should be uniformly transparent. Any flow of liquid must be avoided as it would distort the spots. After spraying, the sheet is placed for 15 min in an oven at 60° C under a steady current of dry air.

Second stage of staining

In order to keep a record of the chromatogram and to facilitate later calculation of the blanc value of each spot all the outlines of the spots are copied on a sheet of transparent paper. Thereupon each spot is carefully cut out and placed in a labelled test tube. 1 ml of the reagent mixture used before is added to each tube and a complete immersion of the papercuts ascertained. The test tubes are covered with aluminium caps and placed for 20 min in a boiling waterbath. Then they are rapidly cooled in running tap water.

Elution of the colour pigment

10 ml of 50% propanol are added to each test tube. They are well shaken at intervals and reading of the colour is performed after 90 min.

Reading of optical density in the Pulfrich step-photometer

After thorough mixing the content of the test tubes is transferred into the cell (2 cm depth and 8 ml total volume) of the step-photometer. As it was stated by MOORE AND STEIN[1] and again by MCFARREN AND MILLS[4] the filter S 57 gives the maximum absorption and the highest density readings. For proline the violet filter L 43 is best suited.

Calculation of result

Calculation is necessarily based on well reproducible standards. As a first step the purity of the amino acids was controlled by one-dimensional chromatography. On the basis of these results the intensity of colour given by known amounts of the different amino acids was determined. This had to be done because the leucine-factors as given by MOORE AND STEIN are apt to vary somewhat when different chemicals are used (BOISSONAS[1]). In order to know the exact position of every amino acid, a map of spots had been prepared previously.

Means of control

When the three blanks (Fig. 1b. B_1, B_2, B_3) are read for their optical density it is easy to judge whether secondary factors have interfered with the ninhydrin reaction. The maximal difference in optical density of the three blanks which is tolerated amounts to 0.02 units at the step-photometer. In this way any measurable interaction of ammonia ion is ruled out. The optical density of 1 ml of MOORE AND STEIN reagent is known, from the difference that is found by measuring the three blanks. The area of the spots can easily be measured on the copy of the chromatogram, which had been done on transparent paper. It is therefore possible to calculate for each spot the corresponding blank value. In this way the net optical density of each amino-acid spot is arrived at. Another means of control is given by the:

Determination of the total

After the first stage of staining, a circle of 10 cm^2 surface is drawn in a free zone of the chromatogram, as for a blank, and an amount of the *hydrolysate* equal to one-tenth of that which had been put on the starting-circle is carefully placed on it from a micropipette. This circle is then subjected to the rest of the treatment in the same way as an amino-acid spot. The resulting optical density will be a measure of the total α-amino N of the sample (Leucine factor to be used = 0.96). The total thus experimentally obtained must match with the one calculated from the sum of the different α-amino acids.

In order to prove that the two stage staining procedure is really additive, the following experiment was performed. On a sheet of Whatman No. 1, which had previously been dipped in phenol/water and dried, two rows of circles of 3 cm ⊖ were drawn some 8 cm apart. On each circle 0.08 ml *M* of an amino acid (see Table I) was placed by micro-pipette. Thereupon the sheet was treated like a chromatogram, but, before spraying with MOORE AND STEIN reagent, one row of the circles was covered with a thick sheet of paper. The amount of amino acid recovered from each circle was estimated in the usual way. The results are compiled in Table I.

This is proof that no amino acid is lost by staining first on paper and it further

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TABLE I

Amino acid	*Amount put on paper*	*Amount recovered from stained spots*	*Amount recovered from unstained spots*
	μg	*μg*	*μg*
	μg	μg	μg
GLY	6.0	6.0	5.8
ALA	7.1	7.6	7.6
VAL	9.4	9.7	9.5
LEU	10.5	10.5	10.5
SER	8.4	8.6	8.5
THR	9.5	9.5	9.2
ASP	10.7	10.8	10.5
GLU	11.8	11.4	11.8
HIST	12.4	12.4	12.1
LYS	11.8	11.4	11.4
ARG	14.0	13.6	13.6
TYR	14.5	13.6	13.6
PHE	13.2	14.0	13.7
PRO	9.2	8.9	9.0
CYS	9.5	9.0	9.5
MET	12.0	12.0	11.5

TABLE II

RECOVERY OF AMINO ACIDS FROM SYNTHETIC MIXTURE

Experiment I: 0.04 μM of different amino acids put on starting-circle, followed by chromatography.

Experiment II: 0.08 μM of proline and histidine treated as above.

Experiment III: 0.08 μM of cysteine and methionine on starting-cycle, oxidation by 0.1 % NH_4 molybdate in 35 % H_2O_2, followed by chromatography.

Exp. No.	*Amino acid*	*Amount put on the starting circle (μg)*	*Amount recovered from the corresponding spot (μg)*
1	LEU	6.0	5.5
	VAL	4.7	4.9
	ALA	3.6	3.8
	GLY	3.0	2.8
	TYR	7.3	7.0
	PHE	6.6	6.0
	GLU	5.9	6.3
	ASP	5.4	5.8
	LYS	5.9	5.0
	ARG	7.0	6.8
	SER	4.2	4.2
	THR	4.8	4.5
2	PRO	9.2	9.0
	HIST	12.4	4.0
3	CYS	9.5	10.0
	MET	12.0	11.5

shows that the elution is adequate. As we wished to know the percentage of amino-acid which is stained during the first step, 10.5 μg of leucine (0.08 μM) were put on paper and sprayed with MOORE AND STEIN reagent. After spraying with 1% KOH in methanol the resulting spot was cut out and eluted in a test tube by adding 0.5 ml of citrate buffer (pH 5.2), 0.05 ml methylcellosolve and 5 ml of 50% propanol. The optical density was read against a blank obtained from an equal surface of the same paper. It proved that 4 μg of leucine or 38% of the total amount had been stained in the first stage.

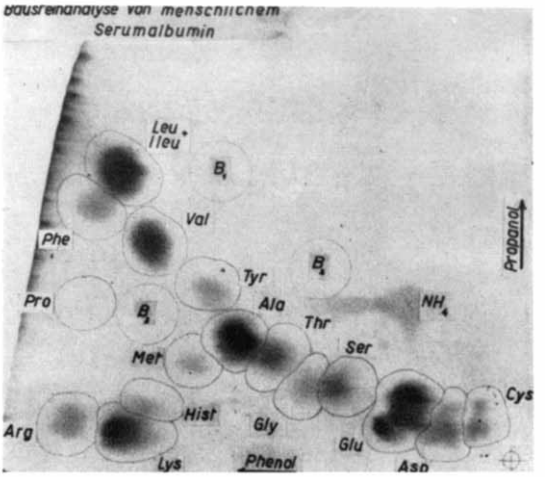

Fig. 1. Two-dimensional chromatogram of the hydrolysate of human serum albumin. Ascending run: propanol, descending run: phenol. B_1, B_2 and B_3 are areas of blanks. Quantitative assay.

It is useful to compare two-dimensional chromatograms of the same substance *i.e.* serum-albumin, but with different pairs of solvents. The spots in Fig. 1b, the method actually described, are coming near the ideal round spots and are therefore suitable for quantitative estimation of the amino acids.

TABLE III

AMINO ACID COMPOSITION OF NORMAL HUMAN ALBUMIN

	g per 100 g amino acids						*Mean deviation*			
						Mean	*Of each*		*Of mean*	
	1	*2*	*3*	*4*	*5*		*abs.* ±	% ±	*abs.* ±	% ±
GLY	1.0	1.05	1.0	0.95	0.95	0.99	0.04	4.0	0.017	1.7
ALA	8.0	8.4	8.4	7.6	8.4	8.14	0.35	4.4	0.15	1.8
VAL	7.0	6.4	6.3	6.0	6.0	6.34	0.42	6.6	0.18	2.9
LEU+ILEU	12.1	12.1	12.1	12.1	12.1	12.1	0.0	0.0	0.0	0.0
SER	2.9	2.8	2.6	2.4	2.4	2.62	0.23	8.8	0.10	3.9
THR	3.2	3.2	3.2	3.1	2.9	3.12	0.13	4.2	0.06	2.0
ASP	10.5	10.5	9.9	9.5	9.0	9.87	0.76	7.7	0.34	3.5
GLU	17.5	17.1	17.6	16.6	16.9	17.08	0.36	2.1	0.15	0.9
HIST	—	—	—	—	—	—	—	—	—	—
LYS	12.3	11.8	12.5	12.0	11.3	11.95	0.51	4.3	0.21	1.8
ARG	6.0	5.7	5.9	6.9	6.9	6.31	0.56	8.8	0.24	3.9
TYR	5.1	4.8	4.9	4.6	5.2	4.91	0.25	5.0	0.12	2.3
PHE	6.2	5.5	6.1	4.9	5.8	5.64	0.55	9.5	0.23	4.1
PRO	4.0	4.4	4.3	4.6	4.6	4.38	0.19	4.3	0.09	2.0
CYS	5.5	6.0	6.0	6.7	6.2	6.07	0.41	6.8	0.18	3.0
MET	—	—	—	—	—	—	—	—	—	—
Total						99.52				
Mean value								± 5.4		± 2.4

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Table II shows the results of three examples of numerous experiments on mixtures of pure amino acids. It is evident that, taking account of unavoidable errors of pipetting etc., the recovery of the amino acids normally occurring in a hydrolysate is quantitative for all of them except histidine.

The results obtained for human serum albumin, listed in Table III, are in fairly good accord with the values given by BRAND AND EDSALL[3]. Histidine is not listed; the difficulties encountered with this amino acid are being further investigated (compare TRISTRAM).

SUMMARY

A method is described for the quantitative determination of amino acids on filter paper. The staining with MOOR AND STEIN reagent is done in two stages, the first staining being done on the paper directly and the second in a glass tube with the eluted amino acid. As the method is a very sensitive one, different means of control are pointed out. As a result five quantitative determinations of the amino acid composition of normal human albumin are stated. The method should be useful for those already well acquainted with two-dimensional chromatography and where the available quantity of protein material is small.

RÉSUMÉ

Une méthode qui permet le dosage quantitatif des acides aminés sur papier filtre est décrite. La coloration par le réactif de MOORE ET STEIN se fait en deux étappes. La première coloration est exécutée directement sur le papier, la seconde dans une éprouvette après élution de chaque acide aminé. La méthode étant très sensible, plusieurs moyens de contrôle sont indiqués. Cinq déterminations quantitatives (exécutuées parallèlement) des acides aminés qui composent l'albumine humaine normale sont présentées comme résultat. La méthode devrait être utile aux chercheurs qui ont de l'expérience en chromatographie à deux dimensions et dans les cas où la quantité de matériel protéinique à disposition est limitée.

ZUSAMMENFASSUNG

Es wird eine Methode beschrieben welche die quantitative Bestimmung von Aminosäuren im Filterpapier gestattet. Die Farbreaktion mit MOORE UND STEIN Reagens wird in zwei Stufen vorgenommen. Zuerst direkt auf dem Papier und nochmals im Reagensglas mit dem Eluat jeder einzelnen Aminosäure. Da die Methode sehr empfindlich ist, werden mehrere Kontrollen angegeben. Als Resultat werden fünf parallel geführte quantitative Bestimmungen der Aminosäurebausteine von normalem menschlichen Serumalbumin aufgeführt. Die Methode dürfte sich als wertvoll erweisen wenn beim Ausführenden Erfahrung in zweidimensionaler Chromatographie vorausgesetzt werden darf und wenn für die Bestimmung nur wenig Protein erhältlich ist.

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