# QUANTITATIVE ESTIMATION OF AMINO-ACIDS BY CIRCULAR PAPER CHROMATOGRAPHY

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Summary

A circular paper chromatographic method is described for the separation and estimation of amino-acids.

The accuracy of the method is illustrated by the analysis of edestin and bovine serum albumin, and its limitations and advantages are indicated.

Though the separation and estimation of amino-acids has been achieved, by using different solvent systems, in the two-dimensional[1-3] as well as the unidimensional chromatogram,[4-8] the methods employed are not without certain limitations. For example, in the two-dimensional technique, as only one sample can be mapped at a time, several maps, at different levels of concentration, have to be prepared to obtain satisfactory separation of all amino-acids, especially in the case of biological fluids of unknown amino-acid composition. Such a limitation renders the method time-consuming, and necessitates the use of large quantities of solvents. Some of the unidimensional techniques[4-5] require the use of large number of solvent systems, and consequently, of large number of chromatograms. On the other hand, it has been observed that the circular technique has the following distinct advantages.[9-11]

1. A number of samples of different concentrations can be analysed, under identical conditions, side by side with known standard materials.

2. Comparatively, the substances run quickly, and therefore, the time required for the development of the chromatogram is less. Further, the rate of flow of the developing solvent can be controlled, to some extent, by adjusting the size of the wick.

3. There is appreciable economy in the use of the solvents, and the necessary chromatographic assembly can easily be made.

4. The separation of the zones is sharp and well defined.

5. It is possible to work with relatively large quantities; as each substance runs into a large ring, and thus can be spread over a large area.

6. The interpretation of the results is considerably facilitated, as the known substances can be run in the same chromatogram.

In view of these advantages offered by the circular technique, it was considered desirable to develop the circular paper chromatographic method for the separation and estimation of amino-acids. In an earlier communication,[12] it has been shown as to how a mixture of amino-acids can be analysed by circular paper chromatography. Subsequently, it was realised that the method was fairly time consuming, and not quite easy of adoption for routine work. Attempts were therefore made to simplify the procedure, and to search for such solvents systems which, besides being the least in number, would give a good separation of the common amino-acids. For this purpose, as a first step, the Rf values of some common amino-acids were determined with a number of solvents, including the buffered solvents employed by Mc Farren,[5] various combinations of (i) pyridine and water, (ii) pyridine, water and acetic acid, (iii) propanol and water, (iv) propanol, water and acetic acid, (v) propanol buffered at different pH, (vi) ethanol and water, (vii) ethanol, water and acetic acid, (viii) ethanol, water and lutidine, (ix) ethanol, methanol and water, (x) ethanol and methanol buffered at different pH, (xi) methanol and water, (xii) methanol, isoamyl alcohol and water, (xiii) butanol and water, (xiv) butanol, water and acetic acid, (xv) acetone and water, and (xvi) phenol and water. It was found that, as judged by the difference in the Rf values of the amino-acids, among the solvents tested, those, consisting of butanol and water, and butanol, water and acetic acid in different proportions, showed a better promise for the separation of amino-acids. Accordingly, the resolving capacity of these solvents was further investigated, and the results obtained, briefly reported earlier,[13] are presented in this communication.

1. *Separation of the amino-acids with butanol saturated with water at room temperature* (68–77° *F.*)

From the Rf values of the amino-acids run at room temperature with this solvent (approximate composition, butanol 85%, water 15%), it was inferred that the use of this solvent could offer the means for effecting the separation of leucine, isoleucine, phenyl-alanine, tryptophane, tyrosine, proline, alanine, threonine, histidine and arginine; but, when the mixture of amino-acids was chromatographed, it was observed that the separation of the amino-acids was highly unsatisfactory, and in the case of some amino-acids, did not take place at all. To obviate this, the process of multiple running[14–16] was resorted to, and it was observed that the desired separation of the amino-acids was obtained, when the chromatogram was run thrice with this solvent. It was, however, felt that the movement of this solvent was rather slow, and that it might be hastened by the incorporation of more water into the solvent, which, in turn, might facilitate the separation of lysine and cystine also. Accordingly, the separation of amino-acids was investigated with solvents containing more water. The incorporation of more water into butanol was achieved by the addition of ethanol. It was observed that the introduction of more water into butanol resulted in comparatively faster flow of the solvent, and in the following order of separation of the amino-acids in the chromatogram run thrice with the solvent consisting of butanol, water and ethanol in the proportion of

20 : 5 : 1·3 : -leucine, isoleucine, phenyl-alanine, valine + methionine, tryptophane, tyrosine, proline, alanine, threonine, hydroxyproline, glycine + serine + glutamic acid, histidine, arginine, lysine and cystine + aspartic acid. Addition of further quantities of water, and an increase in the temperature cause an unsatisfactory separation of the amino-acids, and even an alteration in the order of separation, when phenyl-alanine does not separate well from iso-leucine, and aspartic acid takes the position of histidine, histidine and arginine come together, and lysine does not separate very distinctly from cystine. The best separation of the amino-acids is obtained when the chromatogram is run thrice at the temperature of 60–64° F. with the solvent consisting of butanol, water and ethanol in the proportion of 20 : 4·5 : 1.

2. *Separation of the amino-acids with the solvent consisting of butanol, water and acetic acid in the proportion of* 40 : 7 : 5

This solvent gives the separation of phenyl-alanine, valine, methionine, tryptophan, tyrosine, proline, alanine, hydroxy-proline, arginine and cystine, when the chromatogram is run twice with it at room temperature (68–77° F.). The separation of the amino-acids with this solvent is greatly influenced by the water content of the solvent, and the temperature at which the chromatogram is run. Similar order of separation is obtained in one run at 60–64° F. with the solvent containing butanol, water and acetic acid in the proportion of 40 : 7 : 5. Graded increase in the water content from 7 to 14 parts progressively decreases the sharpness of the separation of valine and methionine. Increase in temperature has a similar effect, and besides, causes an unsatisfactory separation of arginine from lysine and histidine. Such an effect of the increase in temperature on the separation of valine, methionine and arginine with this solvent can be considerably overcome by reducing the water content of the solvent or by running the chromatogram twice.

3. *Separation of the amino-acids with the solvent consisting of butanol, water and acetic acid in the proportion of* 40 : 4 : 15

This solvent, when run thrice at room temperature (68–77° F.), effects the separation of methionine, alanine, threonine, glutamic acid, hydroxyproline, glycine, serine, aspartic acid, arginine and cystine. Increase in the proportion of acetic acid upto 20 parts has no appreciable effect either on the order or the quality of separation, though a decrease in the concentration of acetic acid results in unsatisfactory separation of the amino-acids. Increase in water content from 5 to 9 parts gives better separation of cystine from lysine and histidine and a poor separation of arginine, aspartic acid, glycine, serine, glutamic acid and threonine. Rise in temperature brings down the sharpness of separation of all amino acids. The best separation of threonine, glutamic acid, glycine, serine and aspartic acid is obtained when the chromatogram is run thrice at 60–64° F. with the solvent consisting of butanol, water and acetic acid in the proportion of 40 : 4 : 15.

Thus, it can be seen that most of the common amino-acids can be separated with these three solvents in three different chromatograms. In Table I are summarised the data on the separation of amino-acids with these solvents.

TABLE I

*The separation of amino-acids with solvents, consisting of butanol, water and ethanol, and butanol, water and acetic acid in different proportions (v/v) at 60–64° F.*

| No. of solvent | Composition of the solvent (v/v) | No. of runs | Time of each run | Amino-acids that are separated from synthetic mixtures | Amino-acids that are estimated quantitatively |
|---|---|---|---|---|---|
| Solvent I | Butanol : Water : ethanol :: 20 : 4·5 : 1 | 3 | 18 hrs. | L, IL, Ph, Try, Ty, Pr, Al, Th, HPr, Hi, Ag, Ly (Fig. 1) | L, IL, Ph, Pr, Al, Th, Hi, Ag, Ly |
| Solvent II | Butanol : Water : acetic acid :: 40 : 7 : 5 | 1 | 18 hrs. | Ph, Va, Me, Try, Ty, Pr, Al. HPr, Ag, Cy (Fig. 2) | Ph, Va, Me, Try, Al, Ag, Cy |
| Solvent III | Butanol : water : acetic acid :: 40 : 4 : 15 | 3 | 16 hrs. | Me, Th, Glu, Gly, Se, AA, Ag, Cy (Fig. 3) | Th, Glu, Gly, Se, AA, Cy |

L = Leucine; IL = Isoleucine; Ph = Phenylalanine; Try = Tryptophane; Ty = Tyrosine; Pr = Proline; Al = Alanine; Th = Threonine; HPr = Hydroxyproline; Hi = Histidine; Ag = Arginine; Ly = Lysine; Va = Valine; Me = Methionine; Cy = Cystine; Glu = Glutamic acid; Gly = Glycine; Se = Serine; AA = Aspartic acid.

## MATERIALS REQUIRED

(*a*) *Chromatographic trays.*—These consist of wooden cabinets, 20″ × 20″ × 2″, which are covered with a glass shutter for watching the progress of the movement of the solvent. The cabinets, on the inside, are coated with paraffin, with a view to preventing the absorption of solvent vapours by the wood, and to facilitating the cleansing of the trays. Each tray is provided with paraffin-coated wooden support for keeping the chromatogram horizontally, and a petri-dish in the centre, which carries a small 20 c.c. beaker which holds the solvent.

(*b*) *Chromatographic paper.*—This is Whatman No. 1 filter-paper which, before using, is chromatographically washed first with N/100 HCl, air dried. and then washed twice with distilled water, and dried again.

*Details of the method.*—A sample of protein is hydrolysed with 6 N.HCl, employing. as suggested by Dustin *et al.*,[17] 20 c.c. of the acid for every 1 mgm. of the protein, with a view to reducing the decomposition of amino-acids in the presence of carbohydrates. The hydrolysate is then evaporated to dryness on a water-bath. The residue is extracted with 10% isopropanol, and its pH is adjusted to 6–7. The nitrogen content of the protein as well as its hydrolysate is determined. A suitable aliquot of this extract is then spotted, in three separate chromatographic papers, on the circumference of a circle of 1″ radius, drawn at the centre of a filter circle of 9″ radius. The volume of the aliquot is so adjusted that the concentration of any desired amino-acid does not exceed 7·5 $\gamma$. This may require the spotting of more than one aliquot of the same sample. After air drying the spot, each chromatogram is run at 60–64° F. with one of the three solvents, with the aid of a wick, made by rolling, along its length, a filter-paper, 2″ long and 1½″ broad, till the solvent boundary reaches the periphery of the paper. This takes about 16 to 18 hours. At the end of this period, the chromatogram is removed, and dried in the air. In the case of the chromatograms run with solvents I and III, the chromatogram is run twice more. After drying, each of the three chromatogram is sprayed with 0·4% solution of ninhydrin in 95% acetone. Acetone is allowed to evaporate at room temperature, and the ninhydrin colour is developed by heating the paper at 65° C. for 15 minutes. The coloured bands corresponding to each amino-acid, except those corresponding to proline and hydroxy-proline, are then cut out, and the colour of each band is extracted with 5 c.c. of 75% ethanol saturated with copper sulphate.[18] The addition of copper sulphate to the extracting alcohol makes the ninhydrin colour uniformly red. The colour intensity is then measured with spectrophotometer at 520 $m\mu$ or with Klett Summerson colorimeter, using the filter 54. The presence of hydroxyproline with glutamic acid does not interfere with the estimation of the latter. The yellowish colour of proline with ninhydrin, in the chromatogram developed with solvent I, is extracted with 50% *n*. propanol, and its concentration is determined spectrophotometrically at 350 $m\mu$. After the presence of hydroxyproline in the mixture is detected in the chromatogram run with solvents I and II, its concentration is determined by the method of Martin and Axelrod.[19] In Table II are given

TABLE II

*Readings (average of five) from the Klett colorimeter, with filter 54, for known quantities of amino-acids separated from a synthetic mixture*

| Name of the amino-acid | Colorimeter readings for | | | Colorimeter reading corresponding to 1 $\gamma$ of the amino-acid |
|---|---|---|---|---|
| | 2·5$\gamma$ | 5·0 $\gamma$ | 7·5 $\gamma$ | |
| *Amino-acids estimated in the chromatogram developed with solvent I* | | | | |
| Leucine .. .. | 43·23 | 88·80 | 133·4 | 17·69 |
| Isoleucine .. .. | 44·53 | 89·75 | 135·9 | 18·01 |
| Phenylalanine .. .. | 17·21 | 33·70 | 51·43 | 6·819 |
| Proline .. .. | .. | .. | .. | .. |
| Alanine .. .. | 66·19 | 132·0 | 195·6 | 26·25 |
| Threonine .. .. | 46·07 | 91·45 | 137·9 | 18·35 |
| Histidine .. .. | 20·44 | 41·09 | 61·53 | 8·2 |
| Arginine .. .. | 29·73 | 59·50 | 89·20 | 11·90 |
| Lysine .. .. | 30·07 | 60·14 | 90·21 | 12·03 |
| *Amino-acids estimated in the chromatogram developed with solvent II* | | | | |
| Phenylalanine .. .. | 16·04 | 32·8 | 49·13 | 6·531 |
| Valine .. .. | 46·34 | 92·3 | 135·4 | 18·27 |
| Methionine .. .. | 31·76 | 64·70 | 96·30 | 12·84 |
| Tryptophane .. .. | 13·00 | 26·00 | 38·06 | 5·137 |
| Tyrosine .. .. | 19·25 | 41·13 | 55·73 | 7·741 |
| Alanine .. .. | 65·19 | 129·4 | 193·6 | 25·87 |
| Arginine .. .. | 29·73 | 59·50 | 89·83 | 11·93 |
| Cystine .. .. | 12·99 | 26·23 | 38·00 | 5·147 |
| *Amino-acids estimated in the chromatogram developed with solvent III* | | | | |
| Threonine .. .. | 46·14 | 92·30 | 136·43 | 18·32 |
| Glutamic acid .. .. | 45·13 | 89·90 | 137·4 | 18·16 |
| Glycine .. .. | 49·15 | 98·3 | 147·0 | 19·63 |
| Serine .. .. .. | 50·46 | 99·90 | 151·9 | 20·15 |
| Aspartic acid .. .. | 25·37 | 50·45 | 74·93 | 10·05 |
| Cystine .. .. | 14·99 | 31·28 | 45·23 | 6·099 |

the readings from the Klett colorimeter for known quantities of amino-acids run under the condition described above.

*Accuracy of the method.*— To test the accuracy of the method, samples of edestin and bovine serum albumin have been analysed. The results obtained are

given in Tables III and IV, and are compared with some of the values published so far. It can be seen that the data compare well with the values reported by other workers.[20–24] The values of threonine and serine, as suggested by Rees,[25] have been divided respectively by 0·947 and 0·895. The values of glycine given in Tables III and IV have been corrected, as methionine, after three runs with solvent III, decomposes, and gives rise to four bands, the last of which falls with

TABLE III

*The amino-acid composition of edestin*

(Grams of amino-acid per 100 g. of protein)

| Amino-acid | Henderson & Snell (1948)[20] | Tristram (1949)[21] | Block & Bolling (1951)[22] (Recalculated to 18·6% N) | Thompson & Steward (1951)[23] | Authors |
|---|---|---|---|---|---|
| Cystine .. | .. | 1·4 | 1·3 | 0·80 | 2·87 |
| Lysine | 2·1 | 2·4 | 2·7 | 2·00 | 2·22 |
| Arginine .. | 17·4 | 16·7 | 16·6 | 14·80 | 14·01 |
| Histidine .. | 2·6 | 2·9 | 2·9 | .. | 2·74 |
| Aspartic acid | 13·4 | 12·0 | 12·0 | 13·20 | 12·85 |
| Serine .. | .. | 6·3 | 6·3 | 5·80 | 6·30 |
| Glycine .. | .. | .. | 5·1 | 5·10 | 5·55 |
| Glutamic acid | 19·4 | 20·7 | 20·7 | 23·2 | 19·32 |
| Threonine .. | 3·7 | 3·8 | 3·8 | 3·9 | 4·03 |
| Alanine .. | .. | 4·3 | 5·5 | 5·4 | 4·21 |
| Proline .. | 2·1 | 4·2 | 4·6 | 1·0 | .. |
| Tyrosine .. | 3·7 | 4·3 | 4·5 | 4·7 | 4·76 |
| Tryptophane .. | 0·9 | 1·5 | 1·5 | .. | 1·12 |
| Methionine .. | 4·6 | 2·4 | 2·3 | 2·90 | 2·90 |
| Valine .. | 6·6 | 5·7 | 6·3 | 5·5 | 5·61 |
| Phenylalanine | 5·2 | 5·4 | 5·8 | 4·7 | 5·10 |
| Isoleucine .. | 6·5 | 7·5 | 4·7 | 14·0 | 4·75 |
| Leucine .. | 7·5 | 4·7 | 7·7 | | 6·92 |

TABLE IV

*The amino-acid composition of bovine serum albumin*

(Grams of amino-acid per 100 g. of protein)

| Amino-acid | Redfield and Barron (1952)[4] | Stein and Moore (1949)[24] | Authors |
|---|---|---|---|
| Cystine .. .. | 5·5 | 5·54 | 6·11 |
| Lysine .. .. | 11·25 | 11·25 | 11·04 |
| Arginine .. .. | 5·38 | 5·29 | 5·28 |
| Histidine .. .. | 3·63 | 3·54 | 3·80 |
| Aspartic acid .. .. | 9·15 | 9·44 | 9·73 |
| Serine .. .. | 3·67 | 3·51 | 3·81 |
| Glycine .. .. | 1·42 | 1·38 | 1·40 |
| Glutamic acid .. .. | 15·25 | 14·49 | 14·54 |
| Threonine .. .. | 5·04 | 4·95 | 5·07 |
| Alanine .. .. | 5·41 | 4·99 | 5·17 |
| Proline .. .. | 3·68 | 4·00 | .. |
| Tryosine .. .. | 4·64 | 4·56 | 4·42 |
| Methionine .. .. | 0·74 | 0·71 | 0·76 |
| Valine .. .. | 4·68 | 5·01 | 4·95 |
| Phenylalanine .. .. | 5·69 | 5·87 | 5·83 |
| Isoleucine .. .. | 2·24 | 2·25 | 2·24 |
| Leucine .. .. | 10·25 | 10·58 | 10·23 |

that of glycine. In Table V are given the Klett colorimetric reading for all these four bands, when a known quantity of methionine is run with this solvent.

Thus, for determining the correct value of glycine, first the concentration of methionine in the sample is determined, and from the data given in Table V, the corresponding colorimetric reading for the 4th band, falling with that of glycine, is calculated, and then deducted from the colorimetric reading obtained for glycine from the chromatogram run with solvent III.

TABLE V

*The Klett colorimetric readings for the four bands arising as a result of the destruction of known quantities of methionine*

| No. of the band | Position of the band in the chromatogram run with solvent III | Colorimetric readings (average of 3) for the bands when the quantity of methionine run is | | | Colorimetric readings corresponding to 1 $\gamma$ of the amino-acid |
|---|---|---|---|---|---|
| | | 2·5 $\gamma$ | 5·0 $\gamma$ | 7·5 $\gamma$ | |
| 1 | Below the band of Ph + Va | 15·3 | 30·5 | 46 | 6·120 |
| 2 | Slightly above the band of Try + Ty + Al + Pr | 16·0 | 35·0 | 52 | 6·866 |
| 3 | Slightly below the band of Try + Ty + Al + Pr | 18·6 | 37·0 | 55 | 7·373 |
| 4 | With that of Gly | 5·0 | 10·0 | 15 | 2·000 |

*Limitations of the method.*—The method suffers from the following limitations:

(*a*) In quantities higher than 7·5 $\gamma$, the separation of leucine, isoleucine, valine, methionine, arginine, lysine, glutamic acid, glycine, serine and aspartic acid is not quite satisfactory for the quantitative estimation of these amino-acids.

(*b*) There is degradation of methionine, and the consequent development of additional bands in the chromatograms run with solvents I and III. In the chromatogram run with solvent I, the degradation products of methionine interfere with the estimation of tyrosine and tryptophane. For this reason, the estimation of tyrosine and tryptophane, in synthetic mixtures, is carried out in chromatogram run with solvent II. Similarly, in the chromatogram developed with solvent III, the degradation products of methionine interfere with the estimation of glycine, and this necessitates the use of a correction factor for the accurate determination of this amino-acid.

(*c*) The temperature has to be maintained rigidly between 60–64° F. for the satisfactory separation of the amino-acids.

(*d*) In the chromatogram developed with solvent III, sometimes, due to reasons not quite clear, the separation of threonine and glutamic acid is not very distinct. In such cases, threonine is estimated in the chromatogram developed with solvent I, and its colorimetric reading is deducted from the colorimetric reading for the bands of threonine and glutamic acid in the chromatogram developed with

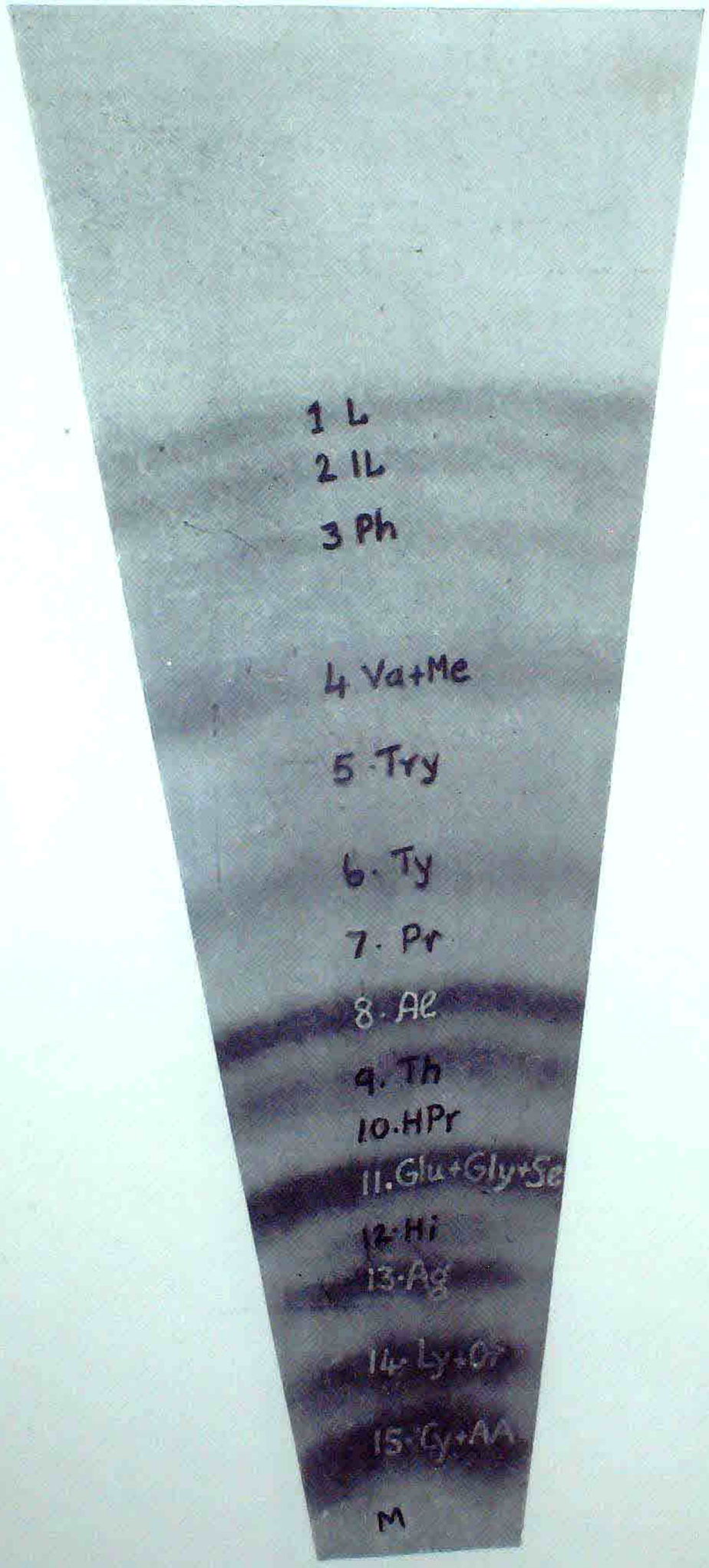


FIG. 1. Solvent used: Butanol: Water: Ethanol:: 20 : 4·5 : 1·0. Number of runs=3. Time of each run=18 hours. Order of separation: L-IL Ph-(Va+Me)-Try-Ty-Pr-Al Th-HPr-(Glu+Gly+Se)-Hi-Ag-Ly (in the absence of Or)-(Cy+AA).

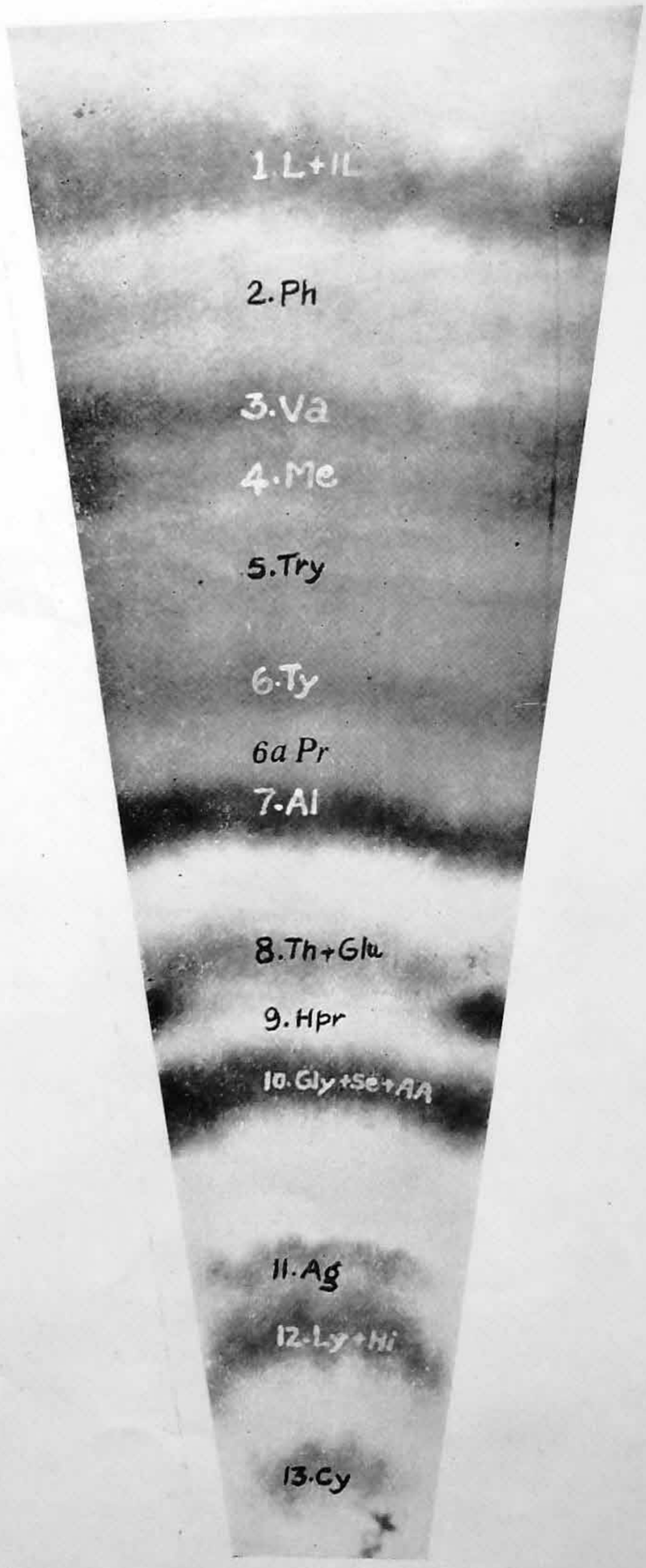


FIG. 2. Solvent used: Butanol : Water : Acetic acid : : 40 : 7 : 5. Number of runs=1. Time of run = 18 hours. Order of separation : (L + IL)-Ph-Va-Me-Try-Ty-Pr-Al-(Th + Glu)-Hpr-(Gly + Se + AA)-Ag-(Ly + Hi)-Cy.

solvent III, to give the colorimetric reading for glutamic acid. The colorimetric reading, for a given quantity of threonine, is the same in the chromatogram developed with solvents I and III, and the colorimetric reading for the combined bands of threonine and glutamic acid in the chromatogram run with solvent III is the sum of the colorimetric readings for threonine and glutamic acid. Similarly, sometimes, lysine does not separate distinctly from cystine and aspartic acid in the chromatogram run with solvent I. In such cases, histidine is estimated in the chromatogram developed with solvent I, and its colorimetric reading is deducted from the colorimetric reading for the combined bands of lysine and histidine in the chromatogram run with solvent III to give the colorimetric reading for lysine.

(*e*) The estimation of some of the amino-acids is vitiated by the presence of other amino-acids. For example, in the chromatogram developed with solvent I, $\alpha$-amino-butyric acid comes with tyrosine, $\gamma$-amino-butyric acid with threonine, and ornithine with lysine, and in the chromatogram developed with solvent II, $\alpha$-amino-butyric acid goes with tryptophane, $\gamma$-amino-butyric acid with tyrosine, $\beta$-alanine with alanine, ornithine with lysine and histidine, and $\alpha$-*e*-diamino-pimelic acid with cystine, and in the chromatogram run with solvent III, $\alpha$-amino-butyric-acid comes with methionine, citrulin with serine, and $\alpha$-*e*-diamino-pimelic acid with cystine.

*Advantages of the method.*—1. As judged by the data given in Tables III and IV, the method is accurate, and renders it possible to carry out the amino-acid analysis of protein hydrolysates in only 3 separate chromatograms.

2. Comparatively, the method takes less time. Considering that, on an average, 9 aliquots can be separately spotted on a circle of 1″ radius in a filter circle of 9″ radius, 3 protein hydrolysates can be analysed for 18 amino-acids in triplicates in a period of 4 working days.

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