*Onderstepoort Journal of Veterinary Research,*
*Volume* 25, *Number* 1, *January,* 1951.
The Government Printer, Pretoria.

# A Photometric Method for Quantitative Paper Partition Chromatography of Amino Acids.

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DETERMINATION of the concentration of amino acids in solutions by filter paper partition chromatography (Polson, Mosley and Wyckoff 1947; Polson 1948) is based upon the visual estimation of the intensity of the spots resulting from serial dilutions of the mixture under examination compared with that of spots from similar dilutions of mixtures of known concentration. This method gives results comparable in accuracy with those obtained from other methods of amino acid estimation. Very often, however, the intensity of a spot on the chromatogram of the unknown solution falls between that of two spots on the standard chromatogram so that the exact position becomes difficult to determine. The need thus arose for a method of greater accuracy. Colorimetric examinations of acetone extracts of the spots prepared under standard conditions is one such method, but this necessitates tedious extraction in a soxhlet apparatus. (Polson *et al.* 1947). In this paper a photometric method is described whereby the intensity of spots may be measured directly and the amino acid concentration determined accurately.

*Apparatus.*

The apparatus used is shown in Fig. 1. Essentially it is designed to project and focus a spot of light of a desired shape on to a photocell. The 1 cm. straight filament of a lamp S fed by a stable voltage supply is imaged by the lens $L_1$ on to the lens $L_2$ at approximately unit magnification. A mask M corresponding in shape to that of the spot to be measured is interposed behind $L_1$. The photo-electric cell P is placed at such distance from the lens $L_2$ that the image of the mask M is focussed on to its surface. The image of M must be slightly bigger than the chromatographic spot to be examined. Unit C consists of two thin glass plates between which the filter paper is flattened and held in a plane parallel to the photocell P. An iris diaphragm I is inserted into the system as a means of varying the intensity of light that falls on the spot without changing the size or shape of the light spot.

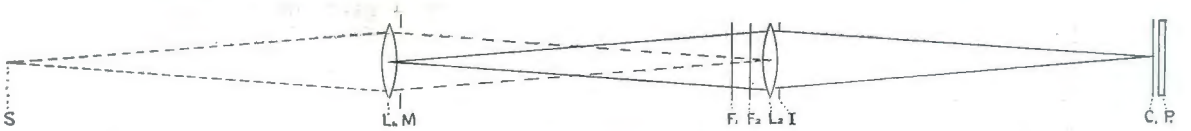


FIG. 1.—Photo-electric colorimeter for measuring intensities of chromatographic spots.

Received for publication on 22nd March, 1950.—Editor.

Inclusion of a Neutral Wratten filter $F_1$ in the system suggested itself as an additional means of controlling the intensity of the light. To obtain maximum sensitivity spectral transmission curves of a number of chromatographic spots were made on a recording spectrophotometer. Curves obtained, of which that for alanine is shown in Fig. 2, indicated the suitability of Wratten filter No. 58 to limit the wave length to the region of 560 millimicrons. Fortunately the barrier layer type photocell used has its maximum sensitivity in this region. The filter is placed at $F_2$ in Fig. 1.

*Materials.*

$L_1$and $L_2$ are ordinary spectacle lenses of focal length 15 cm. and diameters equal to that of the photocell used. S is a 12 volt 48 watt motorcar headlamp with a filament length of 1 cm. The photocell used is a barrier layer type, the output of which can be measured directly by a sensitive galvanometer suitably damped.

*Operation.*

Strips of the chromatogram are pulled between the glass plates across the photocell. Galvanometer deflections of the spots and of the clear filter paper on either side of each spot are read. The galvanometer deflection for each spot is divided by the average of the two galvanometer deflections of the clear filter paper on either side of that spot. The values so obtained are plotted as ordinates against the dilution factor as abscissae for the standard and for the unknown chromatogram. This rather cumbersome method was adopted in an attempt to minimise the effects of variations in the light transmission properties of the filter paper. If homogeneous filter paper is used the galvanometer readings of the spots may be plotted directly against the dilution factor using the average reading of the filter paper as the O concentration. To obtain a conveniently large reading on the galvanometer for the clear filter paper the intensity of the light spot on the photocell is adjusted with the iris diaphragm.

*Results.*

Three representative diagrams from which amino acid concentrations were determined are given in Figs. 3 to 5 in which curves drawn through the circles depict data from standard chromatograms, those through the dots from solutions under investigations. The procedure for determining the concentration of the particular amino acid is to pin-point the position of equal galvanometer deflection on the abscissae of each pair of curves. Knowing the concentration in the standard solution at that dilution the unknown can be calculated.

*Accuracy of the Method.*

Consideration of the location of the points through which the curves are drawn in the figures is an index of the accuracy of the method. This is supported by quoting two examples selected at random from a number of determinations. A solution of aspartic acid undiluted and diluted 1 to 4 gave concentrations of 2·96 and 0·78 per cent. respectively; a solution of serine diluted 1 to 4 and 1 to 20 gave concentrations of 2·97 and 0·60 per cent. respectively.

*Sources of Error.*

If the concentration of an amino acid in the highest dilution in the hydrolysate is greater than that in the lowest dilution of the standard its concentration cannot be determined. It thus becomes necessary to dilute the hydrolysate to a point

where the concentration of the unknown amino acid falls within the range of dilutions of the standard. Satisfactory results were obtained when the undiluted standard solution contained 1·25 mg./c.c. of each amino acid to be determined.

For accurate estimations careful attention must be paid to several important points.

(1) *Constant Temperature of the Chromatographic Tank.*—The extreme importance of this factor cannot be over-emphasised if well defined circular spots are to be produced. When phenol is used saturation of the solvent with water is maintained at a given temperature. If the temperature falls even slightly a watery phase separates. The reverse is the case with collidine in that a watery phase separates with a rise in temperature. The effect of this watery phase is to produce a waterlogged condition of the filter paper as a result of which ill-defined spots of a streaky appearance are produced.

(2) *Uniform Application of Ninhydrin.*—The conventional method of spraying the paper with a solution of ninhydrin in butanol is unsatisfactory. A convenient alternative is to pull the paper through the reagent in a trough under a glass tube sealed off at the ends after being filled with mercury. By this means a uniform distribution of ninhydrin over each horizontal section of the paper is assured.

(3) *Stains on the Filter Paper Caused by Solvents.*—Some solvents, notably the cresols, phenol and collidine, produce patchy brown discoloration of the filter paper. This makes the paper quite unsuitable for accurate photo-electric measurements. The difficulty is overcome by washing the paper overnight in the chromatographic tank in pure butanol prior to development with ninhydrin.

(4) *Amino Acid Concentration.*—Dilutions of the solutions under investigation must be adjusted to obtain gradients in colour intensity of the spots. The intensity of the spots on the chromatogram of the unknown hydrolysate should differ as little as possible from that of the standard used for comparison.

*Experimental Results.*

In Table 1 the results obtained from an analysis of silk fibroin are given. The amino acids aspartic and glutamic acid, serine, glycine, threonine and alanine were determined from a chromatogram run in phenol saturated with water, the mixture containing 0·1 per cent. $NH_3$; tyrosine, leucines and valine from a chromatogram developed in benzyl alcohol saturated with water; arginine from a chromatogram that was first developed in p. cresol-water to which was added 3 per cent. $NH_3$ and then in the opposite direction in benzyl alcohol (Polson l.c.).

TABLE 1.

*Amino acid composition of HCl-hydrolysate of silk fibroin.*

| *Amino acid.* | *G. of amino acid from 100 g. fibroin.* |
|---|---|
| Aspartic Acid | 3·81 |
| Glutamic acid | 2·65 |
| Serine | 11·0 |
| Glycine | 40·9 |
| Threonine | 3·50 |
| Alanine | 34·5 |
| Tyrosine | 10·7 |
| Valine | 1·36 |
| Leucines | 1·88 |
| Argenine | ·876 |



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Estimations of the two main amino acid components in hydrolysates of silk fibroin have been performed by several workers. In the following table some values thus determined are given.

TABLE 2.

*Concentrations of Glycine and Alanine in Hydrolysates of Silk Fibroin as Determined by Different Workers.*

| *Authors.* | *Glycine.* | *Alanine.* | *Method.* |
|---|---|---|---|
| Bergmann and Niemann (1937–38)..... | 43·8 | 26·4 | Selective precipitation. |
| Polson, Mosley and Wyckoff (1947)..... | 42·4 | 34·0 | Visual chromatography. |
| Shankman, Camien and Dunn (1947)..... | 43·6 | — | Microbiological. |
| Rockland and Dunn (1949)..... | 43·4 | 34·9 | Photoelectric chromatography. |

The values of glycine and alanine in Table 1 compare very well with those in Table 2.

SUMMARY.

Due to the difficulties involved in the visual estimation of amino acids in protein hydrolysates from serial dilutions on chromatograms a very simple photoelectric method was devised to facilitate the accurate estimation of amino acid concentration. Sources of errors which must be avoided are temperature fluctuations of the chromatographic tank, non uniform application of ninhydrin to the filter paper, too high amino acid concentrations and discolorations of the filter paper by organic solvents.

The method was applied to the analysis of silk fibroin and values were obtained which agreed well with those determined by previous workers.

ACKNOWLEDGMENT.

This paper is published with the approval of the Council for Scientific and Industrial Research.

The authors wish to thank the Director of the Veterinary Research Institute Onderstepoort and Dr. R. A. Alexander of the same Institute for their interest in this work.

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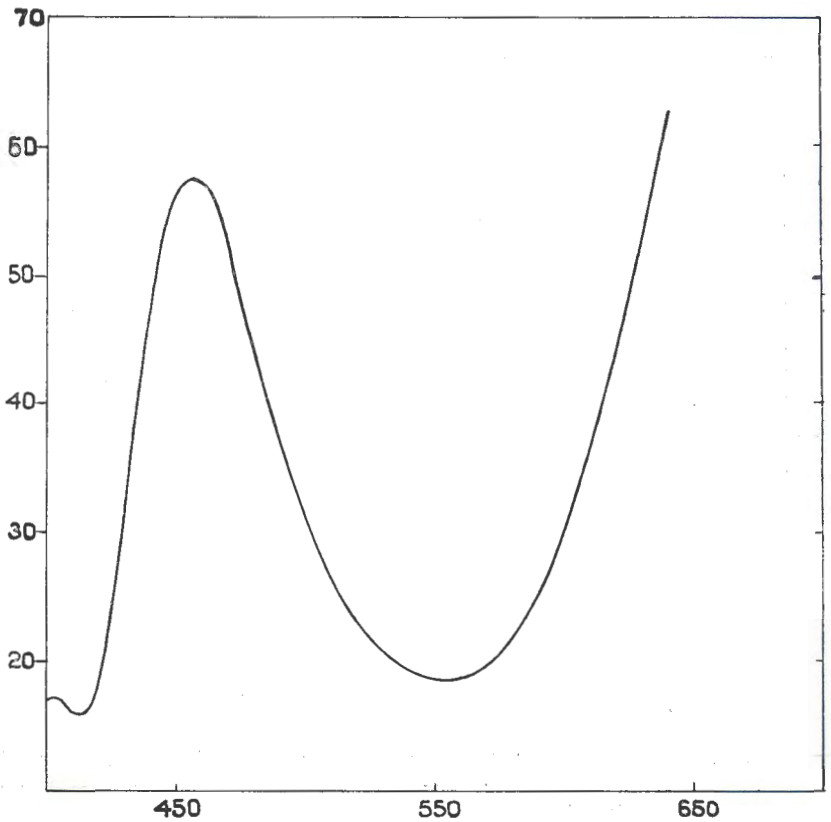


FIG. 2.—Spectral transmission curve of alanine spot.

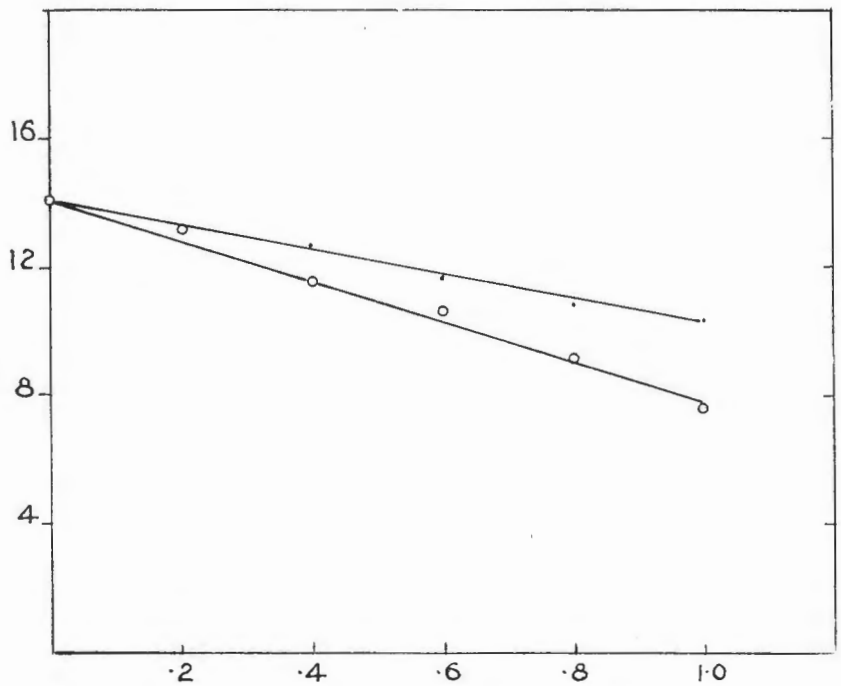


FIG. 3.—Dilution factor-galvanometer deflection curve for a case showing a linear relationship. Circles are readings obtained from the standard amino acid and dots from the amino acid to be analysed.

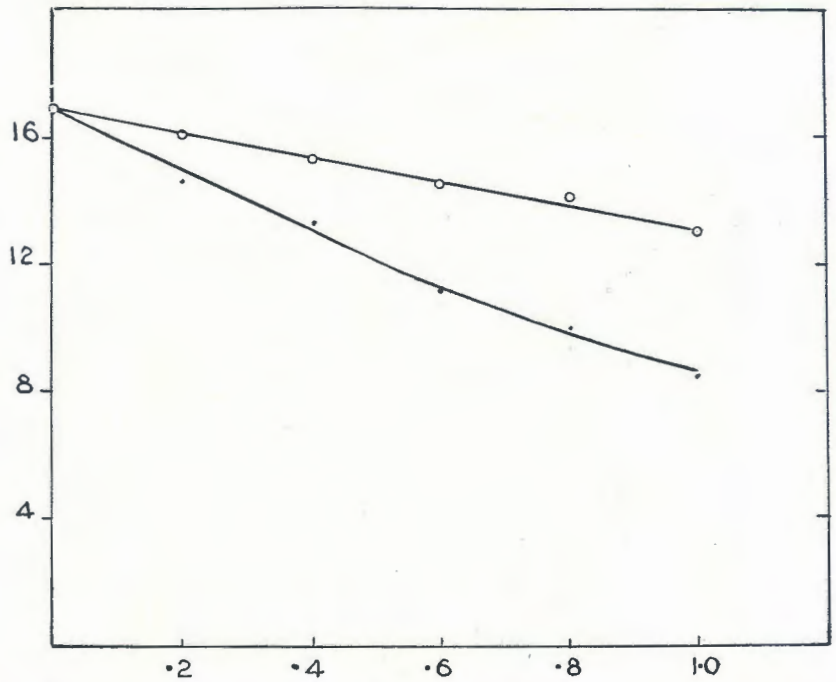


FIG. 4.—Dilution factor-galvanometer deflection curve for a partly linear and partly non-linear relationship. Circles arel standard amino acid readings.

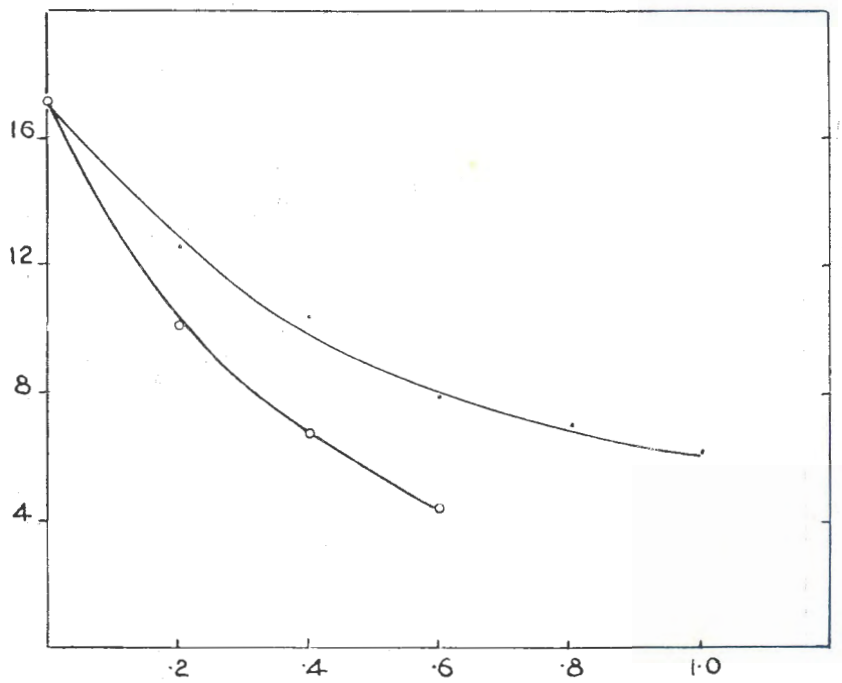


FIG. 5.—Dilution factor-galvanometer deflection curve for a non-linear relationship. Circles are readings from standard amino acid.