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Short Communications

A Spot Area Method for Quantitative Determination of Amino Acids on Two Dimensional Paper Chromatograms

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Since Fisher *et al.*[1] showed the logarithmic relationship between concentration and spot area of amino acids on one dimensional paper chromatograms, this fact has been successfully applied by several authors for quantitativ determination of amino acids (*cf.* Block [2]). As only a few amino acids can be completely separated by one dimensional paper chromatography the use of this method has, however, so far been limited.

The present investigation has shown that a logarithmic relationship between concentration and spot area is valid also for two dimensional paper chromatograms. The main difficulty in obtaining accurate quantitative results from spot areas from two dimensional paper chromatograms depends on the fact that the spots often appear irregular as to shape. This difficulty has largely been overcome by dividing the amino acids into two groups as described later in this paper.

For numerical calculations it was found practical to use the following expression:

$$^{10}\log \frac{100 \cdot C}{M} = A \cdot F_p \qquad (1)$$

where C = spot concentration of amino acid in μg; M = mol. weight of amino acid; A = spot area in cm^2; F_p = molar spot area constant.

It appears that F_p within experimental errors is *constant for each amino acid examined within the investigated range of spot concentrations* (10—50 μg). Furthermore F_p is dependent on the solvent and the filter paper used.

Method. For the preparation of two dimensional amino acid paper chromatograms a method by Boissonnas [3] has been adopted in a slightly modified form. The principle of this method is that the amino acids are parted into two groups, each being chromatographed with special solvents. Consequently two paper chromatograms must be prepared for the quantitative determination of all the amino acids.

Group A. Isoleucine, leucine, methionine, phenylalanine, valine and tyrosine.

Group B. Alanine, α-aminobutyric acid, arginine, aspartic acid, glutamic acid, glycine, histidine, lysine, serine, taurine and threonine.

Paper. It is important that the filter paper used gives spots with distinct edges and no sliding. For *group A* Whatman No. 1 was found excellent and has therefore been used. For *group B* the following papers were tested: Whatman No 1 (used by Boissonnas [3]), Whatman No. 4 and Munktell No. 20. The best spots were obtained on Whatman No. 4, which has been used. The size of each paper was adjusted to about 40 × 40 cm^2 before use.

Application of spots. Fisher *et al.*[1] applied the amino acid solution to the paper in *one* drop. The present investigator has found that if the amino acid solution is applied *drop by drop* to the paper no logarithmical relationship between concentration and spot area is obtained on one dimensional chromatograms but a linear relationship of the type:

$$C = k_1 \cdot A + k_2 *$$

where C is spot concentration, A spot area, k_1 and k_2 are constants. However, on two di-

* Guilty for most amino acids between certain limits of spot concentration.

mensional paper chromatograms no such difference can be detected and formula (1) seems to be valid for both methods of application. When the drop by drop method is used, the best results are obtained if not more than 4 drops (each of *ca.* 5 μl) are applied. It is important to dry the paper carefully after each drop has been applied. The recommended spot concentration of each amino acid is 10—30 μg.

Solvents. For *Group A:*

Ketone liquid (first direction), tert. butanol: methylethyl ketone: water, 2 : 2 : 1 (v : v : v).

Methanol liquid (second direction), tert. butanol: methanol: water, 4 : 5 : 1, (v : v : v).

For *group B:*

Propanol liquid (first direction), *n*-propanol : water, 7 : 3, (v : v).

Phenol liquid (second direction), phenol : water, 7 : 3, (v : v).

All chemicals used have been of C.P. quality. Especially important is the purity of the *n*-propanol. Sometimes it was found necessary to distil it before use. The solvents can only be used 1—2 times.

Chromatographic technique. The method with ascending solvents has exclusively been used at a temperature of 20 ± 1° C. After application of the amino acid solution the filter paper has been folded to a cylinder and the edges of the paper have been kept together with adhesive tape. To obtain a straight solvent front it is recommendable to leave a distance of about one centimeter open between the edges of the paper. After 18 ± 2 hours in the first solvent the paper has been unfolded and carefully dried. After turning the paper at right angles a new cylinder has been made in the same way. After 18 ± 2 hours in the second solvent the paper has been unfolded and again carefully dried. It is advisable to use a stream of warm air (*e.g.* from an ordinary hair drier) to dry the papers, especially when the phenol liquid has been used. The order of the solvents is important for good results.

Colour development of spots. The dried chromatograms have been sprayed with a solution of ninhydrine (0.2 % in 96 % ethanol) and the spots have been developed at 100° C during *exactly* 5 minutes.

Estimation of spot areas. The edges of the spots have been marked with a pencil and redrawn on a homogeneous paper. These new spots have been cut out and their areas have been determined by weighing.

Results. The following F_p values have been found for the 17 investigated amino acids:

	F_p	Investigated range (μg)
Group A		
Isoleucine	0.226	12—37
Leucine	0.230	10—30
Methionine	0.203	7—42
Phenylalanine	0.197	13—39
Tyrosine	0.192	6—45
Valine	0.206	18—53
Group B		
Alanine	0.166	14—47
α-Aminobutyric acid	0.154	16—62
Arginine	0.215	13—92
Aspartic acid	0.176	14—51
Glutamic acid	0.205	10—44
Glycine	0.209	15—58
Histidine	0.140	8—44
Lysine	0.180	15—83
Serine	0.222	9—44
Taurine	0.262	17—53
Threonine	0.170	13—73

The mean error in F_p is about ± 2 %.

As different observers interpret the spot edges differently (*cf.* Fisher *et al.*[1]) it follows that F_p is a "personal constant". Consequently it is recommended that each investigator determines his own value of the molar spot area constant before this method is employed for quantitative determinations.

The accuracy of this spot area method has been thoroughly investigated and the error range is given in the following table.

Number of determinations	error range
1	5—10 %
2	2— 5 »
3	0— 3 »

The estimation of spot areas, which causes the largest error in the results, might be made more accurate by a photographic technique, but this possibility has not been tested.

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