DETERMINATION OF ERRORS IN A DENSITOMETRIC ANALYSIS OF GLYCINE ON PAPER CHROMATOGRAMS

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In an investigation on the sequence of amino acids in peptides, it became necessary to make quantitative analyses on the hydrolyzates of simple peptides. The primary object of this investigation was to distinguish between dinitrophenyl-glycyl-glycine (DNP-gly-gly) and DNP-gly-gly-gly. Taking DNP-gly as unity, it was only necessary to obtain the number of glycine units bound to it with a precision of $\pm$ 20 %.

In the method to be described a recording densitometer without filters was used. On account of this circumstance, it was expected that the errors would be large and experiments were carried out to determine their magnitude. However if a densitometer is used in combination with an electronic area integrator, the method is probably the most rapid quantitative analysis of amino acids on paper chromatograms available. In the present work, only the maximum error under fixed experimental conditions, using manual area measurements, has been established. The method therefore provides a practical means of establishing the sequence of amino acids in peptides when only very small quantities are available, and where the maximum error under fixed experimental conditions would be within the limits necessary to give an unambiguous answer.

Direct photometric evaluation of spots on paper chromatograms has been made by numerous investigators[1-34] in the past. According to INGLE AND MINSHALL[24], the most precise method of evaluation is by reflectance on dry paper. These authors also recommend transmittance through dry paper, while they discard both reflectance on and transmittance through paper made transparent by impregnation. Although maximum spot density methods[1,3,6,31] involve much less work, their limitations have been discussed by several authors[3,15,27].

The procedures employing maximum spot density times area[1,6,27] have some advantages, but are in general less accurate than the procedure of total scanning and curve integration, which is the method used here. However, the latter involves a considerable amount of manual work if no electronic integrator is available[38].

EXPERIMENTAL

Apparatus

An electronic densitometer, Model 525 of Photovolt Corporation*, consisting of:
    (1) a transmission density unit, Model 52-C, for chromatography;
    (2) a photometer, Model 501-A; and

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(3) a Varicord variable response recorder, Model 42-A. This recorder is equipped with a variable response selector switch with twelve positions. In position 1 it works as a conventional millivolt recorder. All other positions introduce curvature, with position 6 closely approximating the logarithm of the input.

Application and development of spots

Three solutions of chromatographically pure glycine were prepared: 0.10 mg/ml; 0.50 mg/ml and 1.00 mg/ml. For the first two solutions, 0.5 to 1.5 mg of glycine were weighed out to the nearest 0.02 mg, while for the most concentrated solution 5 mg was weighed out in the same way. Each sample was dissolved in an appropriate amount of 30 % isopropyl alcohol in distilled water to yield solutions of the above concentrations. The estimated errors for these solutions are:

0.10 mg/ml	3.7 %
0.50 mg/ml	2.1 %
1.00 mg/ml	0.61 %

These solutions were stored in glass-stoppered 1 ml micro-Erlenmeyer flasks and kept at below 0°.

An automatic pipette[35] was calibrated by weighing water to the nearest 0.02 mg and was found to deliver 4.400 ± 0.026 μl. at 25°. The statistical confidence limit is 95 %. This calibrated pipette was used throughout the series of experiments.

Three separate sheets of chromatographic paper (Schleicher & Schüll No. 2043 b) were spotted with eight equidistant spots of 4.400 ± 0.026 μl each, from the three glycine solutions. Each spot was formed by allowing the pipette to empty by capillarity. The chromatograms were developed in a descending manner for 24 hours with *n*-butanol–acetic acid–water, in the proportion of 4:1:5 and were then dried overnight (10 h) in an air current at 45°. The sheets were then cut along the direction of solvent flow into 3.8 cm wide strips, in such a way that the spots occurred centrally along the strips. Each paper strip was supported vertically and sprayed on both sides with a solution of ninhydrin (1 g of ninhydrin, 90 g of *n*-butanol and 10 g of phenol) until translucent, then immediately heated for 4 min in an oven saturated with water vapour, at $110 \pm 10°$.

Densitometry

The strips were left for 3 hours between sheets of clean filter paper and then passed through the photometer, using response No. 1 on the variable response recorder, and a slit aperture of 1 × 25 mm. The instrument was first zeroed on a blank area in the proximity of the spots. Each spot was passed ten times through the photometer and the area under each curve was measured ten times each with a manual planimeter.

The values are given in Table I and are the averages of about 800 area measurements. Some of the spots were also recorded at response setting No. 5, and these are given in Table II. It had been ascertained previously that ten photometric curves, with a total of one hundred area measurements, ten for each curve, would result in an error of 2.0 % with a 95 % confidence limit. It must be stated though, that these experiments were carried out with response setting No. 5 on the Varicord and then applied to response setting No. 1.

TABLE I

RESULTS OF MEASUREMENTS OF AREAS UNDER THE CURVES TRACED BY THE VARIABLE RESPONSE RECORDER, SETTING NO. I

	0.10 mg/ml Glycine (7 spots, 10 curves, each 0.44 μg/spot)	0.50 mg/ml Glycine (7 spots, 10 curves, each 2.20 μg/spot)	1.00 mg/ml Glycine 10 spots, 5 curves, each 4.40 μg/spot)
No. of curves measured	70	83	50
Total No. of areas measured	700	830	500
$\bar{x}$(+)	4.98 cm²	15.29 cm²	19.47 cm²
$s\bar{x}$	0.19	0.43	0.31
s	0.51	1.22	0.69
s'	0.47	1.14	0.62
δ	0.29	0.85	0.25
At 95 % confidence limit the error is	± 0.39 cm² or 7.8 %	± 0.86 cm² or 5.6 %	± 0.62 cm² or 3.2 %

+ $\bar{x}$ = arithmetic mean. $s\bar{x}$ = mean error of mean (67% confidence limit). s = standard error. s' = root mean square error. δ = mean deviation.

TABLE II

RESULTS OF MEASUREMENTS OF AREAS UNDER THE CURVES TRACED BY THE VARIABLE RESPONSE RECORDER SETTING NO. 5

	One spot of 0.10 mg/ml glycine	One spot of 0.50 mg/ml glycine	One spot of 1.00 mg/ml glycine
No. of curves measured	10	10	10
Total No. of areas measured	100	100	100
$\bar{x}$ (cm²)	1.93	5.96	10.82
$s\bar{x}$(+)	0.03	0.05	0.14
s	0.09	0.14	0.46
s'	0.09	0.14	0.43
δ	0.08	0.11	0.34
At 95 % confidence limit the error is	± 0.06 cm² or 3.1 %	± 0.09 cm² or 1.5 %	± 0.29 cm² or 2.7 %

+ 67 % Confidence limit.

TABLE III

AREA MEASUREMENTS ON A SPOT OF 0.10 μg/ml GLYCINE AT VARIOUS TIME INTERVALS, RECORDER RESPONSE SETTING NO. I

	0 h	3 h	10 h
No. of curves measured	9	10	10
Total No. of areas measured	90	100	100
$\bar{x}$ (cm)²	4.23	4.39	4.19
$s\bar{x}$(+)	0.04	0.05	0.03
s	0.13	0.16	0.11
s'	0.12	0.15	0.11
δ	0.08	0.13	0.08

+ 67 % Confidence limit.

Determination of colour stability

To determine the variation of colour intensity of the spots after being sprayed with ninhydrin, one spot was passed through the photometer immediately after spraying and drying; the measurement was repeated after 3 hours and again after 10 hours. The results are given in Table III.

Determination of sensitivity to glycine concentration

Two chromatograms were spotted according to Table IV and were treated in the same way experimentally as the first three chromatograms. The results are given in Table V. All results have been plotted on the graph in Fig. 1.
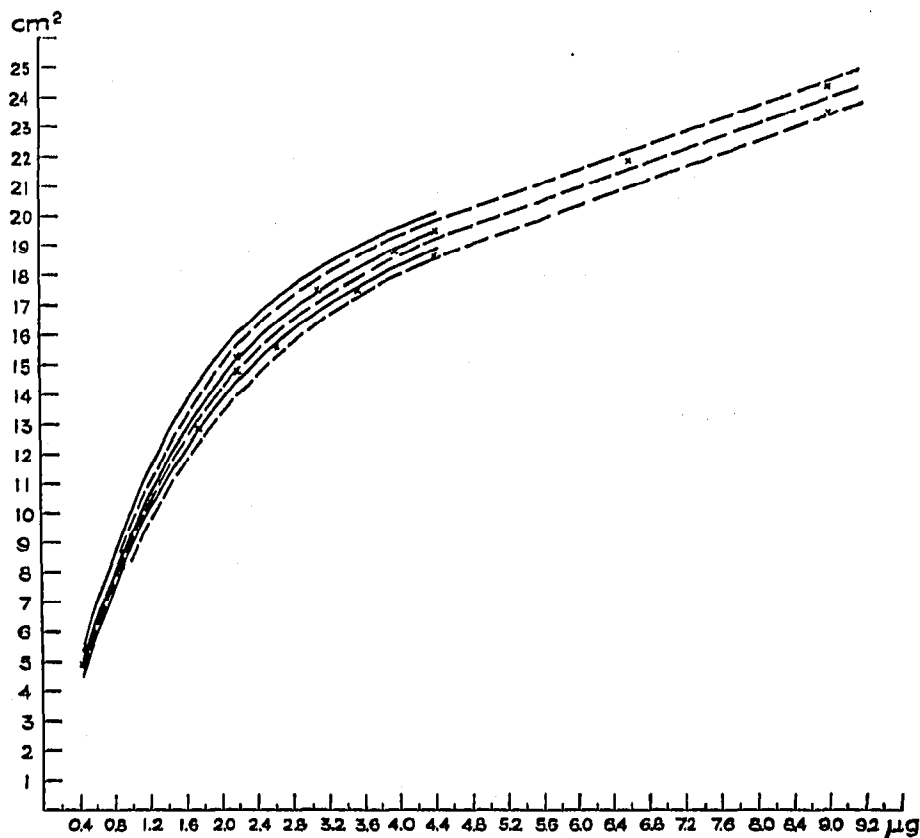

Fig. 1. Full line: Results of Table I. Dashed line: corrected curve for multiple spotting. See explanation in Discussion.

DISCUSSION AND RESULTS

The method can be made more precise if desired. For instance, the solutions of glycine can be prepared with much greater accuracy and the ninhydrin reaction can be made more uniform in the different spots by spraying the whole sheet of the chromatogram before cutting it into strips. Adherence to the experimental precautions given in the excellent work of HANES *et al.*[30], would undoubtedly result in smaller errors.

The method described in this paper was conducted in such a way, apart from not using filters with the photometer*, as to obtain the largest possible error and compare this with the 20 % limit previously given.

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* There were none available in Argentina.

TABLE IV

GLYCINE CONCENTRATIONS EMPLOYED IN DETERMINATION OF SENSITIVITY OF THE METHOD

Spot No.	No. of spottings with glycine solution of:			Amount of glycine spotted	
	0.10 mg/ml	0.50 mg/ml	1.00 mg/ml	μg	nmols. (10^{-9} mol)
1	2	—	—	0.88 ± 0.04	5.86 ± 0.54
2	3	—	—	1.32 ± 0.06	17.6 ± 0.80
3	4	—	—	1.76 ± 0.08	23.4 ± 1.1
4	5	—	—	2.20 ± 0.09	29.3 ± 1.2
5	1	1	—	2.64 ± 0.08	35.2 ± 1.1
6	2	1	—	3.08 ± 0.09	41.0 ± 1.2
7	3	1	—	3.52 ± 0.10	46.9 ± 1.4
8	4	1	—	3.96 ± 0.12	52.7 ± 1.6
9	—	2	—	4.40 ± 0.12	58.6 ± 1.6
10	—	1	1	6.60 ± 0.11	87.0 ± 1.5
11	—	2	1	8.80 ± 0.17	117.2 ± 2.3
12	—	—	2	8.80 ± 0.11	117.2 ± 1.5

TABLE V

AREA MEASUREMENTS OF VARIOUS GLYCINE CONCENTRATIONS AS IN TABLE IV

Spot No.	Recorder response setting No. 1. Mean area (95% confidence limit) cm^2	Recorder response setting No. 5. Mean area (95% confidence limit) cm^2
1	8.28 ± 0.17	—
2	8.62 ± 0.17*	—
3	12.90 ± 0.34	—
4	14.80 ± 0.35	—
5	15.62 ± 0.29	—
6	17.49 ± 0.32	—
7	17.49 ± 0.49	7.34 ± 0.25
8	18.79 ± 0.24	—
9	18.63 ± 0.50	9.03 ± 0.18
10	21.85 ± 0.66	8.08 ± 0.21
11	24.31 ± 0.43	10.45 ± 0.17
12	23.42 ± 0.48	—

* Discarded.

The following sources of error contribute to the final result:
(a) weighing;
(b) volumetric: making up the solutions and pipetting;
(c) drying the chromatogram (volatilization of amino acids);
(d) the ninhydrin reaction (variability of colour response);
(e) variability of colour intensity with time;
(f) photometric;
(g) graphical: area measurement and proper base line.

Errors "a" and "b" can be calculated to a fair degree of accuracy; errors "c" and "e" can be supposed to have the same value for the test substance as for the unknown and are consequently not evaluated; errors "f" and "g" have been limited

to 2 % on the basis of experimental observation, as stated above. The remaining error "d" can be estimated by the difference between the overall error and the sum of the estimated errors. The values assigned to these are given in Table VI.

TABLE VI

CONTRIBUTION OF TYPES OF ERROR TO THE TOTAL ERROR

Kind of error	% Error for solutions of glycine of concentration		
	0.10 mg/ml	0.50 mg/ml	1.00 mg/ml
a	3.7	2.1	0.61
b	0.78	0.83	0.72
f + g	2.0	2.0	2.0
Total:	6.48	4.93	3.33

For the principal curve in Fig. 1, the per cent errors given in Table VII have been found:

TABLE VII

OVERALL ERRORS FOR VARIOUS GLYCINE CONCENTRATIONS

Area, cm^2	Concentration, μg	Error, %
4.89	0.44 ± 0.05	11.3
15.29	2.20 ± 0.24	10.9
19.47	4.40 ± 0.56	12.7

From this it can be seen that if an unknown gave an area of 15.29 cm^2, the central full curve of Fig. 1 will give 2.20 μg of glycine, while the error limit curves will indicate an uncertainty range of 0.48 μg. Therefore, this area corresponds to 2.20 ± 0.24 μg glycine with a confidence limit of 95 %. The per cent error is 10.9 %. This means that for one nanomole of glycine, a quantity of 0.89 to 1.11 nanomoles will be found. This is sufficient accuracy for establishing that one instead of two nanomoles of glycine are bound to one nanomole of DNP-glycine.

By taking the difference between the overall error of 10.9 % and the estimated error of 6.5 % for the 0.10 mg/ml glycine solution, error "d" is calculated at 4.4 %.

The results of Table I have been plotted as already stated in the graph of Fig. 1. The central full line represents the most probable value of the area at the three experimental points. The upper and lower full lines represent the mean error of this value, with a confidence limit of 95 %. All experimental points should fall into the area determined by these last two curves. The points representing the data of Table V fall only into the lower half of this area and some are even below.

This can be explained by the fact that for the principal curve only one spotting was made, while for the points recorded in Table V a number of spottings were made at each spot on the paper. Consequently, a loss of glycine resulted.

If the whole curve is shifted parallel to itself by one half of the mean error of the mean value ($s_{\bar{x}}/2$), as represented by the broken lines and is extrapolated with a

straight line from the point corresponding to 4.40 μg to the point at 8.80 μg, all experimental points fall within the error limits, well distributed in the upper and lower regions.

Evidently, the full curve will be valid even if various spottings are made in practical work; if conditions are more stringent, the overall error will be smaller.

TABLE VIII

OVERALL ERRORS FOR "WORKING CURVE"

Area, cm³	Concentration, µg	Error, %
8.28	0.88 ± 0.08	10.0
12.90	1.72 ± 0.18	10.5
14.80	2.20 ± 0.22	10.0
15.62	2.46 ± 0.24	9.9
17.49	3.28 ± 0.36	10.9
18.63	4.18 ± 0.48	10.9
21.85	6.96 ± 0.56	8.0

The per cent errors found for the broken line curve are given in Table VIII, and in this "working" curve the maximum error is 10.9 %.

SUMMARY

A densitometric method, without the use of filters, for the determination of glycine after reaction with ninhydrin, is described and the overall error of the method is evaluated. The object of the study was to distinguish between DNP-Gly-Gly and DNP-Gly-Gly-Gly. The total spot density was determined by scanning through dry paper with a photometer and measurement of the area under the curve. The maximum error committed was 10.9 %, with a confidence limit of 95 %, which is about half the error admissible.

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