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A NEW SYSTEM OF AUTOMATIC AMINO ACID ANALYSIS

PART III

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SUMMARY

The method of evaluating peaks by means of $H \times W$ integration and by means of the simple measurement of peak height were compared on chromatograms obtained with a new amino acid analyzer. The evaluation of the peaks in terms of their heights expressed in millimeters is more precise and supplies data with a low number of digits. This permits subsequent rather complex data processing by means of a small electronic desk-top computer. Full quantitation of a chromatogram, including peak evaluation and data processing, takes no more than six to seven minutes. A data processing example is reported.

INTRODUCTION

A most interesting feature of this new system of analysis of protein hydrolysates and physiological fluids, reported in the preceding papers of this series[1,2], is the possibility of evaluating the peaks by simply measuring their heights in terms of millimeters above the base line. In this way, evaluation of the chromatogram becomes much more precise, objective and easy, and the time involved in this operation, feasible by means of an ordinary ruler, is reduced to a few minutes. This is very important since, as will be shown in another paper, the hydrolysate procedure can be accelerated with the possibility of performing 6–8 chromatograms in a working day; however the time required for digitizing so many results by this method is insignificant in the furtherance of research, so that the employment of expensive and complicated automatic integrators, coupled with the photometer outputs, becomes quite unnecessary.

It should be mentioned that the measurement of the peak height can also be employed in connection with gas chromatographic analyses; but it is useful only when all the operating parameters can be reproduced exactly from experiment to experiment. Since such a condition in gas chromatography is very difficult to achieve, peak height is not a very satisfactory measurement in this kind of analysis[3,4].

With regard to ion exchange chromatography of amino acids on the automatic analyzers of the usual type, peak height measurement generally gives a degree of

precision lower than that obtained by means of the usual $H \times W$ integration method[5].

On the other hand, as has been reported in Part I of this paper[1], in this new automatic system of amino acid analysis an extremely high reproducibility of the operating parameters can be attained; consequently, in this case, the height measurement offers a much higher degree of precision than the $H \times W$ measurement usually employed in amino acid column chromatography.

With regard to the accuracy of the results obtained by the peak measurement, examples have been given in Parts I and II[1,2] for the range of amounts of solute which can be applied to the system (0–200 nmoles).

In the present paper, a comparison, with respect to precision, between the method of evaluating the peaks by $H \times W$ integration (where H is the height of the peak and W the width at half height) and the method whereby the peaks are evaluated by a simple measure of their height in millimeters, is reported.

In addition, an example of subsequent data processing by means of a desk-top electronic computer (Program 101 manufactured by Olivetti Co., Ivrea), is illustrated. The possibility of using this small and inexpensive desk-top computer for the reported program, which is rather complex, is due to the fact that the evaluation of the peaks in terms of their heights expressed in millimeters supplies data with a low number of digits by comparison with the $H \times W$ values. All the memory positions available in the Program 101 are utilized in this program, and the calculation of the numerical values of twenty amino acids, which would almost take as much time as the analysis itself with a traditional calculating machine, is performed in less than 4 min.

It is important to point out that the possibilities of error, both in measuring the peaks and printing the data to be processed on the computer keyboard, are extremely low; at the same time it has to be stressed that the full quantitation (peak evaluation and data processing) of a chromatogram takes no more than 6–7 min, giving in this way a more realistic meaning to efforts made to accelerate chromatographic procedures, which at time of writing can be performed in less than 90 min[6].

By use of the Program 101, the advantages of electronic computing can be attained in conjunction with the amino acid analyzer, at a very low cost and at any time. This is of importance because most computer centers cannot offer immediate processing services and a computer center is not always available in the neighborhood of the laboratory.

EXPERIMENTAL AND DISCUSSION

In order to compare the two methods of peak evaluation 14 determinations of tryptophan have been performed on the short column at the 200 nmole concentration level, loading the column with 0.1 ml of a solution containing 2 μmoles of tryptophan per ml in 0.1 N HCl. The equipment was set up as described in Part I for a protein hydrolysate analysis, except that the recorder chart speed has been brought up to 30 in. per h, in order to obtain peaks whose width at half height could be measured more precisely.

Each peak was evaluated in terms of height as well as in terms of area. The heights above the base line, H, were measured with a ruler and expressed in millimeters. The areas were obtained by multiplying the height value, H, by the width

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TABLE I

REPEATED DETERMINATIONS OF 200 nmoles OF TRYPTOPHAN, RECORDED AT 30 in./h CHART SPEED

Coefficient of variation of peak height values, $H = \pm 0.69$; coefficient of variation of peak width values, $W = \pm 3.02$; coefficient of variation of peak area values, $H \times W = \pm 3.00$.

No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
H (mm)	117.0	117.8	117.5	116.0	118.0	118.0	117.8	117.5	119.4	118.3	118.7	116.8	118.0	118.0
W (mm)	25.5	25.5	26.0	25.8	28.0	27.8	27.4	27.5	26.6	27.0	26.5	26.8	26.4	26.8
$H \times W$ (mm^2)	2984	3004	3055	2993	3304	3280	3228	3231	3176	3194	3146	3130	3115	3162

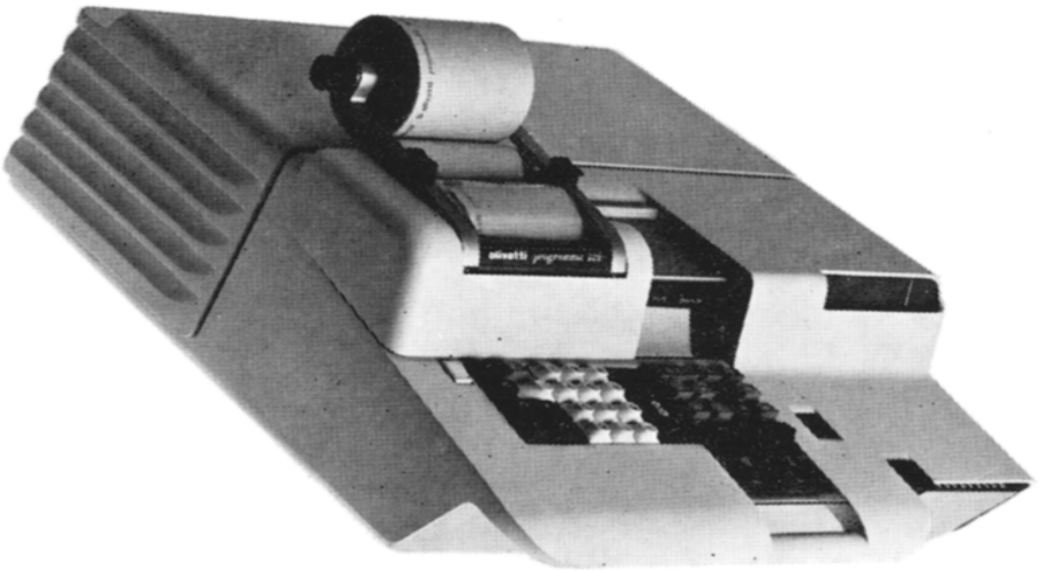

Fig. 1. The Olivetti Program 101 electronic desk-top computer.

at half height, W (measured in millimeters with a pin-point caliper); measurements were made from outer border to outer border of the line and particular care was taken to ensure that the recorder pen wrote clearly and evenly.

Table I reports the results obtained as well as the coefficients of variation of both evaluation methods. As can be seen the method of evaluating the peaks by their heights offers a much higher degree of precision than the $H \times W$ method. Considering the W values and their coefficient of variation, it can be supposed that the reproducibility of the width measurement and consequently the precision of the $H \times W$ method, is affected by the fact that peaks are not true Gaussian curves. The differences between the coefficient of variation of the W values and that of the H values is so great that it must be excluded that the low reproducibility of the W measurements can only depend upon the skill of the operator.

As an example for data processing by means of a desk-top computer, Olivetti Program 101, a chromatogram of a mixture of pure natural L-amino acids, which is used in the preparation of injectable solutions in the pharmaceutical field, was chosen. This mixture is obtained on an industrial scale by the ion exchange resin purification of chemically hydrolyzed proteins of animal origin and is peptide free[7].

From an analysis of this powder the following informations are expected:

The absolute amount of the single amino acids

The sum of the absolute amounts of the single amino acids, which should coincide with the amount that was loaded onto the columns

The percentage composition of the amino acids in the mixture, which will check its nutritional value

The amount of nitrogen contained in the amounts of each amino acid constituting the mixture

The sum of the single amounts of nitrogen

The percentage of nitrogen which must be in agreement with the experimental value obtained by means of the Kjeldahl method. This concordance confirms the

absence of polypeptides in the mixture. For the reported example the Kjeldahl result was 13.95% against 14.12% obtained by computation from the chromatogram. Merck Selenium Mixture GR for determination of nitrogen according to WIENINGER was added to the concentrated sulfuric acid for the digestion, which must be carried out for at least 7 h in order to achieve full mineralization of tryptophan.

Sample: FMH-426; 182 μg in 0.1 ml of 0.1 N HCl.
Standard chromatograms: 784 AN 755 B.
Sample chromatograms: 800 AN 800 B.

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
1 Cysteic acid	210	0	0	169.2	0	0	8.27	0
2 Aspartic acid	91.5	87.5	95.62	133.1	12.73	7.13	10.52	1.34
3 Threonine	85	44	51.76	119.1	6.16	3.45	11.75	0.72
4 Serine	92.5	59	63.78	105.1	6.70	3.75	13.32	0.89
5 Glutamic acid	74	103	139.18	147.1	20.47	11.46	9.52	1.95
6 Proline	15	12	80.00	115.1	9.21	5.16	12.17	1.12
7 Glycine	80	118.5	148.12	75.1	11.12	6.23	18.66	2.07
8 Alanine	53	75	141.50	89.1	12.61	7.06	15.72	1.98
9 Cystine	42	3.5	8.33	240.3	2.00	1.12	11.66	0.23
10 Valine	34	29	85.29	117.1	9.99	5.59	11.96	1.19
11 Methionine	120	70	58.33	149.2	8.70	4.87	9.39	0.82
12 Isoleucine	85	55.5	65.29	131.2	8.57	4.80	10.68	0.92
13 Leucine	88	131	148.86	131.2	19.53	10.94	10.68	2.09
14 Tyrosine	55.5	23.5	42.34	181.2	7.67	4.29	7.73	0.59
15 Phenylalanine	50	28.5	57.00	165.2	9.42	5.27	8.48	0.80
16 Tryptophan	62	9.5	15.32	204.2	3.13	1.75	13.72	0.43
17 Lysine	92	86	93.47	146.2	13.67	7.65	19.16	2.62
18 Istidine	83	26	31.32	155.2	4.86	2.72	27.09	1.32
19 Ammonia	53	17.5	33.01	17.0	0.56	0.31	82.30	0.46
20 Arginine	36	23.5	65.27	174.2	11.37	6.37	32.16	3.66
					178.47			25.20

Nitrogen percentage in the mixture = 14.12
A = standard chromatograms
B = sample chromatograms
C = nmoles
D = molecular weights
E = μg of amino acid
F = percent composition of the amino acids in the mixture
G = nitrogen percentage in the molecule
H = μg of nitrogen

Fig. 2. Data processing form.

Fig. 2 shows the form which summarizes the data and operations; the column legends are as follows:

A = peak heights of the amino acids on the standard chromatogram (100 nmoles of each amino acid).
B = peak heights of the amino acids on the sample chromatogram.
$C = (B/A) \times 100$ = nmoles of each amino acid.
D = molecular weights.
$E = C \times D/1000 = \mu g$.
$F = (E/\Sigma E) \times 100$ = % composition of the amino acids in the mixture.
G = % nitrogen content in a single amino acid molecule.
$H = E \times G/100 = \mu g$ of nitrogen.
Percentage of nitrogen in the mixture $= (\Sigma H/\Sigma E) \times 100$.

The computer (Fig. 1), receives the program instructions by inserting magnetic cards on which the instructions have been stored previously by means of the computer itself.

This program foresees calculations for up to 20 amino acids and employs 23 magnetic cards, divided in two groups:

(1) from 1 to 20 (one card for each amino acid in numerical order) for the single intermediate answers,

card No.

1		8		15		21	
0	S	75	S	28•5	S		S
0•00	A◊	141•50	A◊	57•00	A◊	25•20	a◊
0•00	a◊	12•61	a◊	9•42	a◊	178•47	◊
0•00	A◊	1•98	A◊	0•80	A◊	14•12	A◊
2		**9**		**16**			
87•5	S	3•5	S	9•5	S	0•00	a◊
95•62	A◊	8•33	A◊	15•32	A◊	7•13	a◊
12•73	a◊	2•00	a◊	3•13	a◊	3•45	a◊
1•34	A◊	0•23	A◊	0•43	A◊	**22**	
							S
						3•75	a◊
3		**10**		**17**		11•46	a◊
44	S	29	S	86	S	5•16	a◊
51•76	A◊	85•29	A◊	93•47	A◊	6•23	a◊
6•16	a◊	9•99	a◊	13•67	a◊	7•06	a◊
0•72	A◊	1•19	A◊	2•62	A◊	1•12	a◊
4		**11**		**18**		5•59	a◊
59	S	70	S	26	S	4•87	a◊
63•78	A◊	58•33	A◊	31•32	A◊	4•80	a◊
6•70	a◊	8•70	a◊	4•86	a◊	10•94	a◊
0•89	A◊	0•82	A◊	1•32	A◊	**23**	
							S
5		**12**		**19**		4•29	a◊
103	S	55•5	S	17•5	S	5•27	a◊
139•18	A◊	65•29	A◊	33•01	A◊	1•75	a◊
20•47	a◊	8•57	a◊	0•56	a◊	7•65	a◊
1•95	A◊	0•92	A◊	0•46	A◊	2•72	a◊
						0•31	a◊
6		**13**		**20**		6•37	a◊
12	S	131	S	23•5	S		
80•00	A◊	148•86	A◊	65•27	A◊		
9•21	a◊	19•53	a◊	11•37	a◊		
1•12	A◊	2•09	A◊	3•66	A◊		
7		**14**					
118•5	S	23•5	S				
148•12	A◊	42•34	A◊	•			
11•12	a◊	7•67	a◊				
2•07	A◊	0•59	A◊				

Fig. 3. Program print out. The figures indicated by S are printed in by the computer keyboard and correspond to the peak heights in millimeters (column *A*, Fig. 2); all the remainder are printed out by the computer itself. The cards from 1 to 20 correspond to the 20 amino acids, in numerical order, and give the intermediate answers printing out the number of nanomoles and micrograms of the amino acid and the corresponding number of μg of nitrogen (columns *B*, *E* and *H*). After inserting cards 21, 22 and 23 the computer prints out the final answers. The first 3 figures of card 21 are ΣH, ΣE and the nitrogen percentage in the mixture. The remaining figures on card 21 and all the figures on cards 22 and 23, in numerical order, correspond to the percent composition of the amino acids in the mixture and go to complete column *F*.

(2) from 21 to 23 for the final answers.

Considering the data shown in Fig. 2, it will be sufficient to introduce the values of column B after inserting the corresponding cards, one at a time from 1 to 20. The other data contained in column A, D and G do not have to be introduced, since they are constant values memorized on the cards:

Column A: variable constants. As these data correspond to the peak heights of the amino acids on the standard chromatogram, they are subject to modification; therefore, they are memorized on the card by means of a particular register which allows easy and rapid substitution, when a new standard chromatogram has to be run.

Column D: permanent constants, coded on cards.

Column G: permanent constants, coded on cards.

The data contained in column F and the final values ΣH, ΣE and % nitrogen are printed at the end of all entries after inserting the three last cards (group 2), while data contained in columns C, E and H are printed as intermediate answers, just after the B value of the corresponding amino acid has been entered. If $B = 0$ the cypher must be entered on the keyboard, as for a normal value. In Fig. 3 the print out example illustrated will clarify the operation procedure, which takes about 3.5 min.

In order to take advantage of the analytical system, which permits peak evaluation with only a few digits, the limitations of the program are as follows:

(1) The constant A must not exceed 3 digits (including 1 decimal).

(2) The constant B must generally not exceed 3 digits (including 1 decimal); only a few values in the column can have 4 digits.

(3) The constants D and G must not exceed 4 digits (including 1 or 2 decimals respectively).

(4) The decimal wheel must be in position 2 (two decimals in the results are considered sufficiently accurate).

For readers desiring full details of this program, which has been developed in cooperation with the Program 101 Department of the Olivetti Co., the card instructions are available from the author.

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