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INTEGRATION, IDENTIFICATION AND CONCENTRATION MEASUREMENT OF AMINO ACIDS IN PLANT SAMPLES BY MEANS OF AN AUTOMATIC AMINO ACID ANALYZER LINKED TO A MINI-COMPUTER*

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SUMMARY

To save a significant amount of time in the processing of the data generated by an automatic amino acid analyzer, an on-line system has been developed using a mini-computer interfaced with the automatic analyzer. The program provides high flexibility with 13 parameters that can be modified at will. The system can be used with different analyzers and it can operate with limited peripherals.

The computer integration of the data is at least as precise as a manual integration performed by a qualified technician.

INTRODUCTION

Nowadays, it is almost impossible to collect and calculate manually the numerous data generated by sophisticated automatic analyzers on extracts from soil, plant or animal samples. Therefore, data-processing systems have been developed, most of which use digital integrators for peak area measurements and identification, with computer assistance for final calculations[1–5]. Electronic integrators, because of their extreme specificity, cannot be used with other equipment and become obsolete when the original analyzer is superseded. Moreover, the data acquired from integrators have to be transferred to computers through devices such as magnetic tapes, disks or other means for final calculations.

Inexpensive mini-computers can be programmed to perform in a single operation data acquisition and transformation of the raw data into a final requested print-out. Not only do they save a significant amount of time in obtaining the useful data, but they are very flexible and compatible with many analytical instruments.

* Contribution No. 121.

The above considerations led us to develop an on-line system that uses a mini-computer interfaced with one out of four TSM Amino Acid Autoanalyzers (TSM-AAA) and that can also be interfaced with other instruments. A similar mini-computer-based system for an amino acid analyzer was recently described by Fox and Wilkinson[6]. Both systems provide for a comprehensive on-line analysis of an amino acid chromatogram. However, while also using a Nova mini-computer, our system was linked to a different model and make of automatic analyzer and aimed at offering the greatest versatility towards the variations encountered by our analyses of extremely varied samples with the TSM, the adaptation of the system to different "analyzers" and its ability to cope with limited peripherals. Thus far, successful analyses from the TSM-AAA have been obtained during a period of over 2 years.

EXPERIMENTAL AND RESULTS

Instrumentation

The automatic amino acid analyzer was a TSM (Technicon Sequential Multi-Sample) Amino Acid Analyzer manufactured by Technicon, Tarrytown, N.Y., U.S.A., equipped with two columns, a short one for the basic amino acids and a long one for the acidic and neutral amino acids. It has a sampler that holds 40 cartridges in duplicate, corresponding to 40 samples, which permits the instrument to be run for several days without intervention. The TSM was set up for the analyses of physiological fluids, each of which lasted 5 h and 40 min. Absorbance readings from the two-channel spectrophotometer at 440 and 570 nm were linearized and expanded, if necessary, through a colorimeter control. Consequently, it gave a straight-line relationship between the concentration of an amino acid and its peak area on the recorder chart, which simplified the calculations.

The data-processing system was very similar to that of Fox and Wilkinson[6]. It included a Data General Nova 1210 with an 8K 16-bits words of core memory, a 12-bit analog to digital converter coupled to an eight-channel multiplexer, an ASR-33 teletype and a programmable clock to generate the sampling control signals.

Operation and programming

A standard containing 0.05 μM each of 40 pure amino acids from commercial sources was run at the beginning and after every 10 unknown samples. An amino acid not present in biological tissues, norleucine, was incorporated in all samples as a reference for calculating the specific correction factor for each unknown.

Signals collected at the output of the colorimeter control were amplified from 0–100 mV to 0–5 V, sampled with an analog multiplexer and then fed to a digital converter with a conversion time of less than 50 μsec. The digitized data were then conveyed to the computer at a high-frequency level and passed through a double filtering process in order to remove interfering signals.

Twelve minutes after the beginning of a chromatographic run, a signal triggered the computer, which started searching for the first peak. From this time on, the computer used ten parameters to determine the background and the true occurrence of a peak. These parameters were determined after a thorough analysis of the first and second derivatives of the original output of the analyzer. The search program was developed in the Assembly language of Nova computers in order to use most

effectively the capacity of the computer to act as a "filter" and than as a sophisticated peak-recognition device using criteria as defined above (Table I, parameters 1–10). The quantitative values of these criteria can be modified at will, a feature which seems to differ from the Fox and Wilkinson program[6].

TABLE I

PARAMETERS USED FOR BASELINE AND PEAK RECOGNITION (1–10) AND PEAK IDENTIFICATION (11–13)

Unless otherwise stated, the units are arbitrary.

No.	*Parameter description*	*Units*
1	Minimum duration of baseline signals for baseline recognition	15 sec
2	Maximum slope of baseline signal	±1250
3	Minimum slope for the beginning of a peak	5000
4	Maximum slope for the beginning of the top of a peak	2500
5	Maximum slope for the end of the top of a peak	−5000
6	Minimum slope for the end of a peak	−2500
7	Maximum duration of a peak between beginning and top	50 sec
8	Maximum duration of the top of a peak	13 sec
9	Maximum duration of a simili-top of a peak	16 sec
10	Maximum duration of a peak between top and end	65 sec
11	Retention time of the reference, norleucine	162 min
12	Acceptable variation of retention times of peaks in the standard	±1 min
13	Acceptable variation of retention times of peaks in unknown samples	±5 min

In addition, the acceptable variation of the retention time of the reference, norleucine, and the acceptable variation of retention time of all amino acids included in the standard as well as those from the unknown samples can be changed (Table I, 11–13).

Our program provides for the analysis of 50 amino acids in a single chromatographic run. These are defined by sequential numbers, retention times and tag stored in a reference list (Table II).

After loading of the main program "Herbin" and of the floating-point arithmetic routines, any one of four operators has access to 16 commands via the teletype. Once he is identified, an operator may choose to define three sets of data: (1) the parameters used for baseline and peak recognition and identification (Table I), (2) the reference list of amino acids (Table II) and (3) the experimental conditions for the ten forth-

TABLE II

EXAMPLE OF A REFERENCE LIST OF AMINO ACIDS

Sequential No.	*Retention time (min)*	*Tag*	*Amino acid*
1	16.1	CSTE	Cisteic acid
2	21.1	TAUR	Taurine
3	23.9	PETN	Phosphoethanolamine
4	45.4	ASPA	Aspartic acid
5	50.5	HYPR	Hydroxyproline
6	57.3	THRE	Threonine

coming chromatographic runs (Table III). For each set of data, the operator can answer a questionnaire on the teletype or feed in a paper tape obtained from previous runs. If, however, only a few parameters have to be changed, the "modify" commands allow him short-cut procedures. The last three commands enable the operator to drive the TSM-AAA operation through the computer.

TABLE III

EXPERIMENTAL CONDITIONS DEFINED BY THE OPERATOR BEFORE STARTING A SET OF CHROMATOGRAPHIC RUNS

Conditions (questionnaire)	*Answers (example)*
Correction time to channel 1 in minutes?	0.0
Correction time to channel 2 in minutes?	−0.3
Number of samples or runs?	7
Which standard, old (0) or new (N)?	N
SAMPLE No. 0 name	STAN 2762
Concentration of amino acids in μM?	0.05
Starting time for computer readings in minutes?	12
Ending time for computer readings in minutes?	325
SAMPLE No. 1 name	2766 E
Is this a new standard?	No
Concentration of norleucine (reference) in μM?	0.04
How many grams of fresh weight?	0.017
Starting time in minutes?	12
Ending time in minutes?	325
SAMPLE No. 2...	

Once a series of analyses is started, the computer stores in its memory the retention time (RTu, Table IV) of every peak at its maximum height and the peak area. At the end of the analysis the computer prints all data pertinent to the particular sample and then compares the retention time with the corresponding one (RT) in the reference list and identifies the peak (Table IV, heading and columns A and B). Retention times and peak areas are also printed for both channels (columns C, D, G and H). In the print-out of a standard, no figures appear in columns E, F, I and J. In the analysis of the unknown samples, retention times from the standard (RTs) are printed in columns E and I to help in peak identification. Amino acid concentrations are printed in columns F and J under the form chosen by the operator, in our case, in micromoles per gram of fresh weight (μmole/g F.W.).

Concentrations of amino acids are calculated according to equations defined in the Operation Manual for the Technicon TSM system[7], with the exception of a correction factor (CORR FACTOR, Table IV) based on areas and concentrations of norleucine from both standard and the unknown chromatogram. The correction factor is calculated taking into account the fresh weight, and is used for calculating concentrations of all amino acids contained in an unknown sample. The correction factor appears on the print-out and can be utilized as a further check or for corrections in case of wrong printings of some amino acids.

On succeeding chromatographic runs, a few amino acids are repeated depending on the time limits selected by the operator (parameter 13, Table I), such as THRE

TABLE IV

PRINT-OUT OF A CHROMATOGRAPHIC RUN SHOWING A FEW AMINO ACIDS CONTAINED IN A LEAF EXTRACT OF ALFALFA THAT HAD BEEN HARDENED AT 1° FOR 2 WEEKS

SAMPLE 2, 2766, E, 0.017 G.F.W., 0.040 μM NOR FROM 12.0 to 325.0 MIN, CORR FACTOR: 2.7772.

Reference list		*Channel 1 (570 nm)*				*Channel 2 (440 nm)*			
RT	*Name*	*RTu*	*Area*	*RTs*	*μM*	*RTu*	*Area*	*RTs*	*μM*
A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
15.1	CSTE	15.6	163	—	—	15.3	11	15.7	0.103
44.4	ASPA	45.6	1232	47.0	0.441	45.6	222	47.0	0.442
56.3	THRE	56.2	1107	56.6	0.480	56.1	179	56.5	0.454
59.4	SERI	56.2	1107	59.2	0.469	56.1	179	59.2	0.460
56.3	THRE	59.1	3767	56.6	1.634	59.1	615	56.5	1.554
59.4	SERI	59.1	3767	50.2	1.597	59.1	615	59.2	1.576
63.5	ASN	63.5	4494	63.0	5.794	63.4	1929	63.0	5.832
102.7	PRO	105.2	2205	—	—	105.6	13996	100.9	28.018
131.9	ARTE	132.5	184	132.2	2.171	—	—	—	—
158.1	LEUC	159.3	540	158.7	0.218	—	—	—	—
162.0	NORL	162.9	5388	162.5	2.247	162.9	881	162.4	2.049
259.8	LYSI	261.7	408	258.4	0.215	—	—	—	—
313.4	ARGI	308.6	2774	312.5	1.751	—	—	—	—

and SERI for theonine and serine in Table IV. This happens when one amino acid falls within the limits of several corresponding amino acids from the reference list. With practice and looking at the print-out of the standard, one finds at a glance which is the right one. In the example given, the upper THRE is the good one on account of its retention time, while the lower SERI must be selected for its retention time and the change in peak area.

One must look at the results obtained with channel 2 (440 nm) for the hydroxyproline and proline concentrations.

The absence of figures on the print-out signifies that peaks were too small or outside the time limits of any amino acid to be considered by the program.

Some peaks are artifact (ARTE) due to buffer changes and are identified as such on the print-out.

Precision of the program

The precision of the program was evaluated by means of the following experiment. Nine chromatograms of standards containing fourteen amino acids concentrated at each of five levels (0.02, 0.04, 0.05, 0.06 and 0.08 μM) were carried with the TSM-AAA for a total of 45 runs. Three of the fourteen amino acids were excluded from the analysis becauses errors occurred during printing of the chromatograms.

Peak areas were integrated according to two methods: (1) manually using the triangle formula and (2) through the computer. For each method of integration and each concentration level, three estimates of variability were obtained from three groups of three chromatograms each. For each group, an analysis of the variance of the observed concentrations according to a randomized block design with eleven

amino acids and three replicates (the three chromatograms) yielded estimates of error, s^2, which are free from variations due to differences between acids or to differences between chemical analyses. The inverse of the standard error, $1/s^2$, reflects the precision of the method through which the observations were obtained. The estimated standard errors are given in Table V and shown graphically on a logarithmic scale as a function of concentration level in Fig. 1.

TABLE V

ESTIMATED VARIANCES, $10^6 s^2$

*Rep**	*Method of integration*	*Concentration level (μM)*				
		0.02	*0.04*	*0.05*	*0.06*	*0.08*
1	Manual	3.283	1.722	6.128	10.618	22.184
	Computer	4.527	8.791	6.659	6.513	16.752
2	Manual	2.671	6.151	17.625	8.546	25.312
	Computer	0.443	2.819	18.284	9.864	8.944
3	Manual	1.583	2.422	8.973	28.972	92.160
	Computer	3.595	4.902	13.504	36.848	90.648

* Rep = repetition.

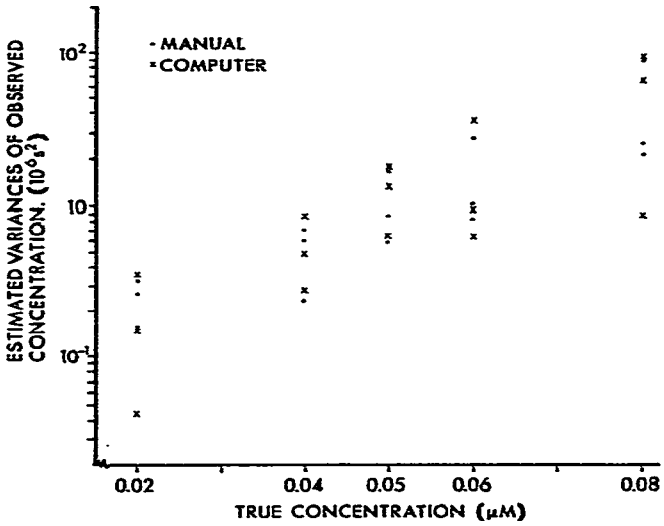

Fig. 1. Estimated variances, $10^6 s^2$, of observed concentrations as a function of true concentrations.

A logarithmic transform was used to normalize the data in Table V and a weighted regression analysis was performed on the differences between the logarithms of the standard error of the two methods of integration. It revealed that the differences between the precision of the two methods is not significantly different from zero, whatever the level of concentration may be. Hence, it appears that the computer and the manual methods have equal precision. It should be borne in mind that this experiment leads to a comparison between the precision of the computer and that of a technician who is particularly well trained and experienced. Moreover, the chromato-

grams used for this experiment lent themselves to precise manual evaluation: single peaks were clear cut, double peaks were ignored and the baseline was easily referred to.

Regression analysis of the transformed variances reveals that there is a significant (5%) increase in the variation of the observations when concentration increases.

It must be added that when the recorder is not operating normally, data from the computer are valuable because, as mentioned above, signals collected at the output of the colorimeter control are not affected by the pen recording.

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