noted that the variation of the constants obtained with different systems is significant enough not to be ignored in calculations.

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A new accelerated fully automated system for amino acid analysis by ion-exchange chromatography

In a recent paper we reported on the development of a chromatographic system which separates the amino acids usually present in acidic protein hydrolysates within 63 min[1]. This was mainly achieved by improved column technologies and reduction of extra column contributions to band spreading in fittings and analytical system.

Since then, an improved resin has been developed for this system and precision data have been generated. Also, a final manifold for the peristaltic valve and pump has been assembled and evaluated.

Improved resin

The original resin used in the system tended to pack down during the first three runs and the top fittings of the columns had to be adjusted in order to avoid any dead space detrimental to resolution. The back pressure in the column for the separation of the acidic and neutral amino acids reached 600 p.s.i.

The new resin does not pack after loading and the back pressure in the columns has been reduced by about 50 % when resin beads of comparable size distribution are used. Its basic chemical composition remains unchanged, but the new resin has been subjected to a special hardening procedure. It shows excellent mechanical strength. The chromatographic performance of the improved resin is very similar to the original material (see Fig. 1). In order to improve the resolution of the basic amino acids, the resin bed in the short column was lengthened to 4.5 cm and the time for the whole chromatographic cycle extended to 65 min.

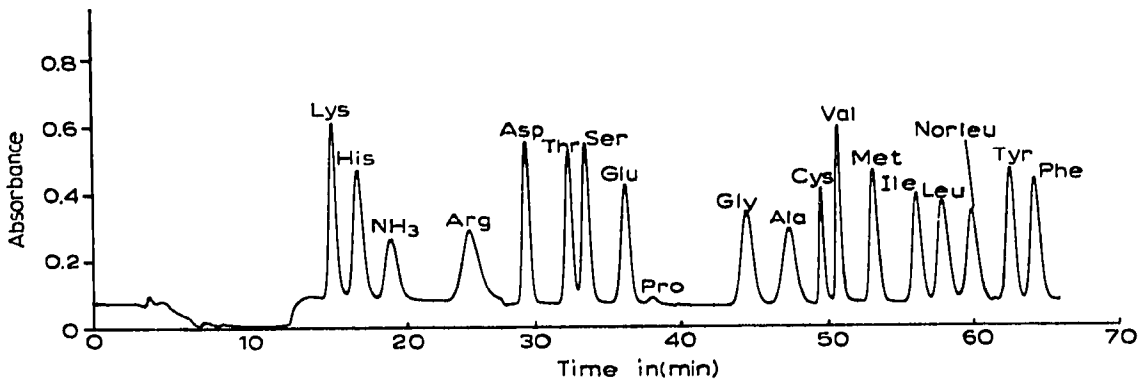

Fig. 1. Chromatogram of an amino acid standard representing one complete automatic cycle. The sample contained 0.03 μmoles of each amino acid. Absorbance read at 570 mμ.

The manifold

The complete flow diagram of the system manifold is represented in Fig. 2. The column effluents are first fed into a segmented stream of 0.2 *M* hydrazine sulfate solution. Regular segmentation with nitrogen can be provided by means of an 'air bar' or by a pressurized loop configuration in which nitrogen is pumped from a large pump tube into a small pump tube. Thus a gas bubble is released from the pressurized smaller tube whenever a roller bar of the peristaltic pump is lifted from the tubes. The peristaltic valve, the principle and function of which have been described previously[2], determines which of the two column effluents goes to the analytical system and which goes to waste. Valve positions 4 through 7 are assigned this control function. Only after passage through the valve is the column effluent combined with the ninhydrin reagent.

Buffer feed for the separation of the acidic and neutral amino acids to positive displacement pump 2 is controlled by valve positions 9, 10, 11, 13 and 14; in other words, in any valve position only one of these lines is opened. Positions 8 and 15 control the flow to the short column via positive displacement pump 1 (Fig. 2).

Another useful modification has been incorporated with the new procedure. The absorbance signal produced by the bulk of the acidic and neutral amino acids initially eluted from the short column was difficult to handle by an automatic integrator. Therefore, a segmented stream of water, instead of the column effluent, is now pumped into the analytical system until lysine, the first basic amino acid, is eluted from the column. Valve positions 16 and 17 were selected to perform this switch. Valve positions 18 to 20 control the flow of ninhydrin or wash solutions—sodium hydroxide and methylcellosolve—into the analytical system.

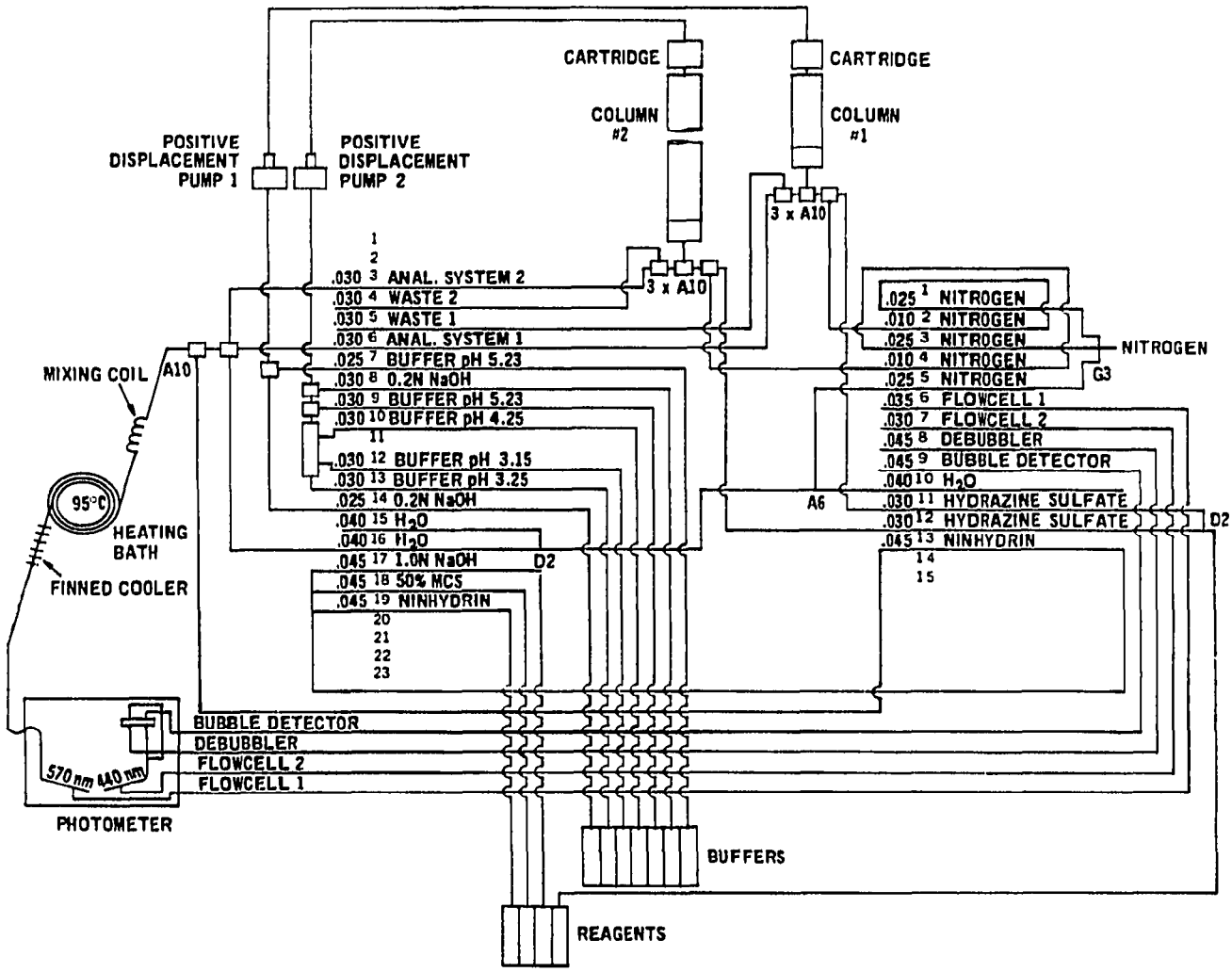

Fig. 2. Complete flow diagram of system. Manifold of peristaltic valve on the left, manifold of peristaltic pump on the right side. Internal diameter of pump tubes given in inches.

A standard heating bath coil of 12 m length and 1.6 mm I.D. is used for the development of the analytical reaction.

Precision data

The precision of the procedure was established with ten consecutive chromatograms. Samples were applied automatically and peak areas and retention times were determined with an electronic digital integrator (Table I).

For the identification of individual amino acids by means of retention time good precision of this parameter is essential. In order to establish confidence limits for a single measurement of retention time the standard deviation has to be multiplied with a factor τ which depends on the number of observations and the confidence limit desired. For ten observations and a confidence limit of 99 % (*e.g.* 99 % of all measured values will fall within this limit) the value for τ is 3.24. With the described system, when this test is applied to the most critical case, the identification of threonine and serine, the confidence limits do not overlap.

TABLE I

RT = retention time; σ_{RT} = standard deviation of retention time; C.V.(area) = coefficient of variation of peak areas; C.V.(peak height) = coefficient of variation of peak heights.

	RT (*sec*)	σ_{RT} (*sec*)	*C.V.* (*area*) (%)	*C.V.* (*peak height*) (%)
Lys	544	2.5	1.15	1.51
His	1048	3.6	0.63	1.13
Arg	1506	7.2	1.67	1.67
Asp	1763	6.2	2.51	2.07
Thr	1935	6.9	1.12	1.61
Ser	2006	7.5	1.40	1.86
Glu	2167	8.5	0.96	0.57
Gly	2680	12.2	0.60	1.98
Ala	2854	13.2	0.72	1.59
Cys	2973	7.3	1.74	4.14
Val	3047	8.6	1.93	2.00
Met	3200	9.5	1.42	3.08
Ile	3370	10.9	0.78	0.95
Leu	3481	11.8	0.59	0.64
Norleu	3601	13.5	1.46	1.98
Tyr	3757	10.7	1.44	1.71
Phe	3863	12.2	2.10	1.22

The data obtained for the retention times indicated that peak height precision should be good. The peak height precision was therefore calculated, and proved acceptable.

The system has been successfully operated for a period of several months and is now being field tested by a number of independent users.

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