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AUTOMATED SINGLE-COLUMN ANALYSIS OF AMINO ACIDS USING ASCORBIC ACID AS REDUCTANT FOR AIR-STABLE NINHYDRIN

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SUMMARY

The procedure for operation of a constant-temperature, single-column automated amino acid analyser in the sub-nanomole range is described. The cycle time for a complete analysis is 90 min including equilibration for next cycle. Eluting buffers can be made in the laboratory or commercially available concentrates (Pico-Buffers) can be used. A novel reducing agent, ascorbic acid, incorporated into the column buffers was used to reduce air-stable ninhydrin.

INTRODUCTION

Amino acid analysis is an essential step in protein sequencing. Our interest in sequencing proteins that are difficult to obtain in large quantities required that we increase the sensitivity of the available instrument, a Technicon TSM amino acid analyser. This instrument is a fully automated system for the analysis of amino acids by ion-exchange chromatography[1–3].

A single-column system modified from Smithies *et al.*[4] effectively doubled the sensitivity over a two-column regimen. In addition, an improved buffer system that increased the resolution of amino acids commonly encountered is described. The separation of tryptophan was improved and ammonia did not interfere with the quantitation of any amino acid. Detailed are improvements of the flow stream, and consequently the baseline, made by reducing the number of pulsed streams from the peristaltic pump and valve. Ascorbic acid was used as the reducing agent by incorporating it into the column buffers rather than adding a reducing agent either directly to the ninhydrin solution or separately to air-stable ninhydrin.

EXPERIMENTAL

Chromatographic system

A Technicon TSM amino acid analyser equipped with a proportioning pump III and a 5-mm-I.D. column was assembled as shown in the flow diagram (Fig. 1). The chromatographic column was packed with a 21.5-cm bed of C-3 Chromobeads

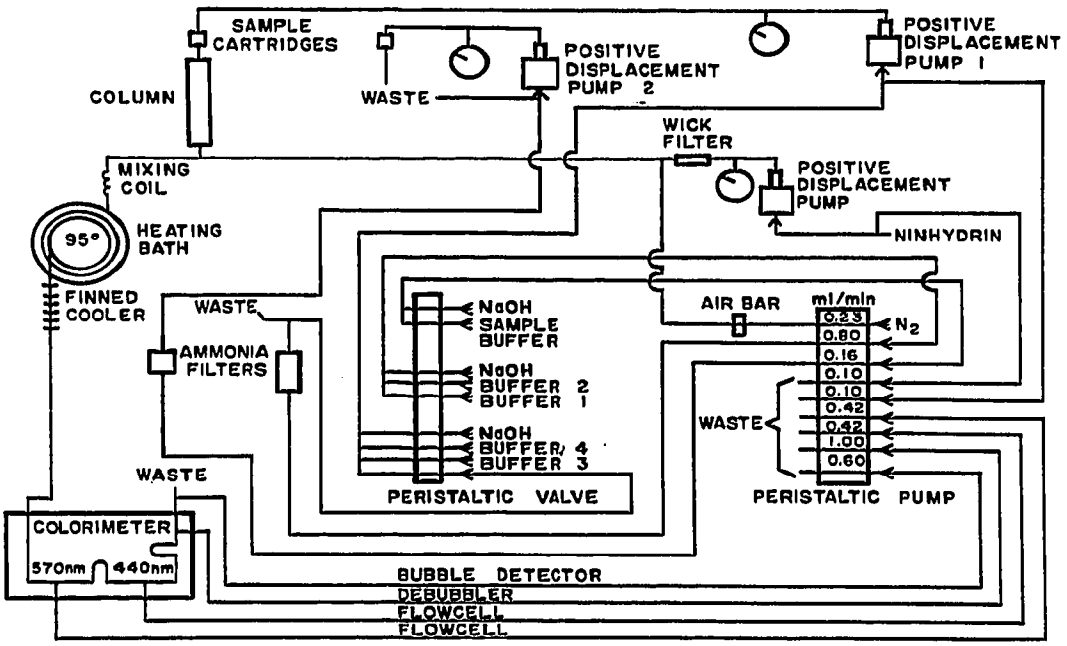

Fig. 1. Flow diagram of amino acid analyser.

(Lot No. 1-2-014-10; Technicon, Tarrytown, N.Y., U.S.A.) and operated at 60° with a flow-rate of 0.50 ml/min resulting in pressures less than 500 lb./in.^{2}. Four elution buffers were prepared from sodium citrate stock solutions or Pico-Buffer conentrates (Pierce, Rockford, Ill., U.S.A.) as described in Table I. The pH of each buffer is listed solely for reference as no final pH adjustment is necessary. The reducing agent, ascorbic acid (AnalaR; Gallard-Schlesinger, Carle Place, N.Y., U.S.A. was added to batches of 1 to 4 l (depending on consumption rate) of the column buffers to concen-

TABLE I

COLUMN BUFFERS

Buffer	*Buffer composition*			*Final conditions*			*Reductant*§ *(mM)*
	*Stock solution**		*Additive***	*Vol. (l)*	*pH****	*Na^+ (N)*	
	ml	*pH*					
1	500	2.45	240 ml methyl Cellosolve	4.0	2.98	0.25	1.5
2	400	3.35	72 g NaCl	3.0	3.60	0.68	1.5
3	400	3.85	72 g NaCl	2.0	4.0	1.02	1.3
4	400	3.55	72 g NaCl, 32 g NaOH, 30 g H_3BO_3	4.0	~9.5	0.71	2.0

* Trisodium citrate (0.67 *M*) titrated with HCl or Pico-Buffer concentrate titrated with HCl or NaOH if necessary.

** All buffers made to 1% v/v thiodiglycol and 1% v/v of 30% w/v Brij 35.

*** Final pH does not require adjustment when stock solution at specified pH is used.

§ 1 *M* ascorbic acid added to final buffer to give concentrations indicated.

trations shown in Table I. Ascorbic acid was stored frozen as a 1 *M* solution; buffers were refrigerated at all times for chemical stability and biological preservation.

Contaminating ammonia was removed from buffers 1 and 2 by passing them through a filter (0.9 × 6.5 cm) of cation-exchange resin (DC-3, Durrum, Palo Alto, Calif., U.S.A., or Rexyn 101, 40–100 mesh, Fisher, Pittsburgh, Pa., U.S.A.) before the high-pressure pump. While buffers 3 and 4 were pumped to the column, the ammonia filter was stripped with a solution of 0.5 *N* NaOH, 2% v/v ARW-7 and 0.1% w/v ethylenediaminetetraacetic acid (EDTA). The column was subsequently equilibrated with buffer 1 that had passed through the ammonia filter.

Samples were loaded on the cartridges in 0.067 *M* sodium citrate and 1% v/v thiodiglycol adjusted to pH 2.0 with HCl. The high-pressure pump ordinarily used for the second column was used to regenerate sample cartridges at 0.09 ml/min. A second ammonia filter (0.9 × 2.0 cm) and sample cartridge were stripped for 40 min and equilibrated with sample buffer for the balance of each cycle. A 90-min analytical cycle consisted of the following pumping times: 13.5 min of buffer 1; 28 min of buffer 2; 15 min of buffer 3; 10.5 min of buffer 4; 5 min of NaOH; and 18 min of buffer 1. Typical results are shown in Fig. 2.

All chemicals were reagent grade or specifically prepared for amino acid analysis by Pierce unless specified otherwise.

Analytical system

Ninhydrin solution was delivered to the bubble injector by a Milton Roy Instrument Mini Pump (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) at 1.2 ml/min through a pulse suppressor consisting of a wick filter (No. 6234, A. H. Thomas, Philadelphia, Pa., U.S.A.) that created 30–300 lb./in.^{2} backpressure on the 12-ml air chamber of a gauge (Supergauge No. 19029; U.S. Gauge, Sellersville, Pa., U.S.A.) and the column effluent was injected directly into the segmented ninhydrin stream. The ninhydrin reagent consisted of 0.7% ninhydrin, 25% methyl Cellosolve, 22% dimethyl sulfoxide (DMSO), 24% 4 *M* sodium acetate solution, and acetic acid and water to pH 6.0 at final volume. This solution was routinely made in 20-gal batches using one 40-lb. can each of methyl Cellosolve and DMSO and 18 l of buffer.

All tubing between the column and first flow cell was kept to a minimum convenient length and smallest internal diameter compatible with bubble integrity. To increase the time delay between glutamic acid monitored at 570 nm in the first flow cell and proline monitored at 440 nm in the second, the stream passed through 2 ft. of 0.062-in.-I.D. tubing between the flow cells. A pulse suppressor after each flow cell consisted of 3.3-ft. × 0.030-in.-I.D. tubing with 3.3-ft. × 0.062-in.-I.D. flexible wall tubing between the smaller I.D. tubing and the proportioning pump. The pulses throughout the system, caused by the injection of nitrogen bubbles, were suppressed by the backpressure created by 9.9-ft. × 0.030-in.-I.D. tubing after the second flow cell.

An Infotronics CRS 110A integrator (Columbia Scientific Industries, Austin, Texas, U.S.A.) determined the area of each peak. Color factors were calculated by least squares fit of a straight line to at least twelve points spanning a range from 5 to 20 nmoles. Within experimental error, the same color factors were obtained over the range of 0.5 to 50 nmoles.

RESULTS AND DISCUSSION

Chromatograms of 1.0 and 10 nmoles of 23 amino acids are shown in Fig. 2; elution times are listed in Table II. Amino acids encountered in protein sequencing are adequately resolved and free from baseline errors. Ammonia does not interfere with, and tryptophan is separated from, the other basic amino acids. The only buffer change of consequence occurs between histidine and tryptophan and the integrator has time to find a new baseline before the next peak. Histidine is eluted before the last buffer change to avoid compounds eluted by the increase in pH. The column re-equilibrates rapidly and does not suffer from shrinkage caused by most buffers of high salt concentration.

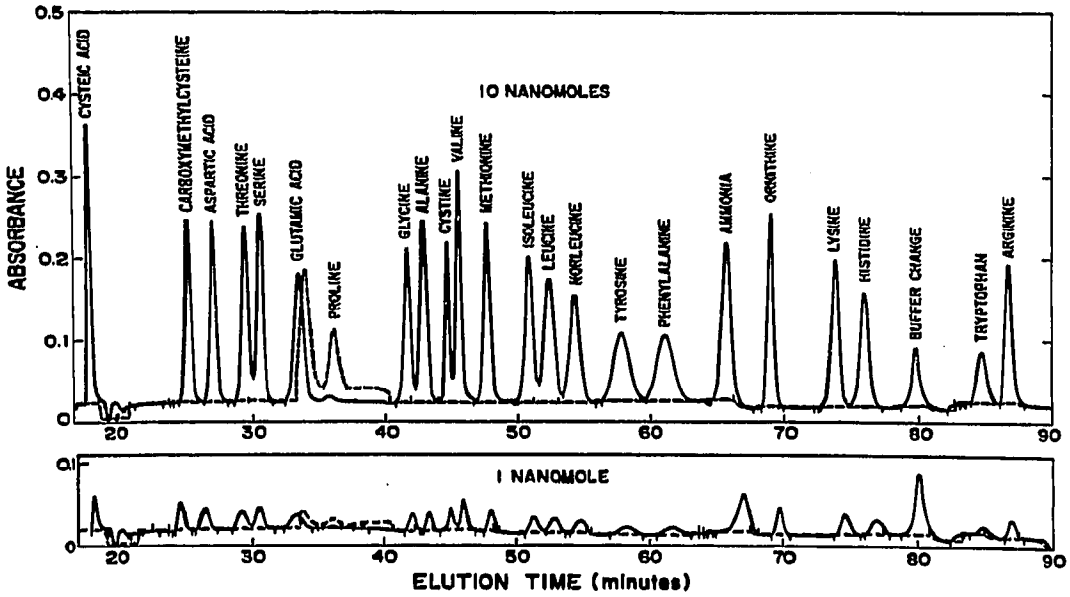

Fig. 2. Chromatograms (tracings) of 10 nmoles (upper) and 1.0 nmole (lower) of standard 23 amino acid calibration mixture. Full scale deflection of 570-nm channel set at 0.5 O.D. and of 440-nm channel set at 0.25 O.D. The 440-nm channel pen (dashed line) displays baseline except for interval encompassing proline.

Quantitative data based on least squares fit of a straight line for four samples each of 5, 10 and 20 nmoles are shown in Table III. The slope (counts/unit colorimeter expansion/mmole) represents the color factor. The standard deviation of the slope was less than 2% for all amino acids except cysteic acid and ammonia. For the most precise calculations, intercepts that are more than 2 or 3 standard deviations from the origin require a correction. Except for cysteic acid and ammonia, the correction represents 0.5 nmole or less. Levels of 0.1 nmole are easily seen and easily quantitated ($\pm$ 10%) at 0.5 nmole. Color yield is linear to at least 50 nmoles with the ascorbic acid concentrations listed in Table I but falls off with less than 0.5 m*M* ascorbic acid.

TABLE II

ELUTION TIMES

Peak	*Time (min)*	*Peak*	*Time (min)*
Glutathione (performic oxidized)	17.9	Isoleucine	51.2
Cysteic acid	18.3	N^{2}-Acetyllysine	51.8
Phosphoserine	18.5	Leucine	52.6
$O^{4'}$-Sulfotyrosine	19.3	Prolylglycine	54.5
Glutathione (reduced)	24.4	Norleucine	54.6
Methionine sulfoxides	25.2	Threonylmethionine	56.6
S-Carboxymethylcysteine	25.5	Tyrosine	58.1
4-Hydroxyproline	26.8	β-Alanine	58.2
Aspartic acid	27.3	$O^{4'}$-Acetyltyrosine	58.4
Methionine sulfone	27.8	Phenylalanine	61.5
Threonine	29.7	Aminoethanol	63.0
Asparagine	30.2	Ammonia	66.0
Glutamine	30.4	S-Aminoethylcysteine	69.1
Serine	30.8	Ornithine	69.4
Homoserine	33.2	N^{6}-Trimethyllysine	69.6
Glutamic acid	33.8	N^{6}-Dimethyllysine	73.1
Citrulline	35.0	Lysine	74.1
Proline	36.9	Homoserine lactone	74.4
Cysteine	37.9	N^{6}-Methyllysine	74.7
Glycine	42.0	Histidine	76.3
Glycylaspartic acid	42.3	N^{π}-Methylhistidine	76.7
Alanine	43.2	Buffer change	80.1
2-Aminobutyric acid	44.4	Leucyltyrosine	81.7
Cystine	45.1	Arginylglutamic acid	81.8
Glucosamine	45.5	Glycyltryptophan	82.1
Valine	46.0	Diiodotyrosine	82.8
Serylglycine	46.2	N^{G}-Dimethylarginine	83.4
Galactosamine	47.5	Tryptophan	84.9
Methionine	48.1	N^{G}-Methylarginine	86.4
Glycylglycine	48.3	Arginine	86.9
allo-Isoleucine	49.8	Lysyllysine	87.5

More than 9500 analyses have been performed using ascorbic acid as the reducing agent and 7000 with the buffer system described. As nitrogen is the only part of the analytical system that is delivered by peristalsis, biweekly replacement of the nitrogen tube is sufficient to maintain constant color yield and baseline levels over long periods of time even at full scale expansion of 0.125 O.D.

The conditions described here increase the buffering capacity, sharpen peaks and reduce the time required for re-equilibration in contrast to the standard Pico-Buffers. The additional buffering capacity of the first buffer is especially useful when sample pH cannot conveniently be adjusted because of small volume or large number of samples. Although this 90-min cycle time requires four buffers, an instrument capable of handling three buffers can operate with a 100-min cycle by replacing buffers 2 and 3 with Pico-Buffer B at standard concentration and pH, eluting ammonia just before the pH rise. Histidine, lysine, tryptophan and arginine are eluted in that order after the buffer change. However, a number of contaminants such as diiodotyrosine interfere with the quantitation of histidine. Alternatively, buffer 4 may be eliminated

TABLE III

COLOR_FACTORS*

Amino acid	*Slope ± S.D.***	*Intercept ± S.D.*	*Residual variance*
Cysteic acid	35.10 ± 1.27	56.90 ± 16.84	756.0
Carboxymethylcysteine	41.67 ± 0.30	0.65 ± 3.95	41.6
Aspartic acid	46.50 ± 0.31	− 1.74 ± 4.10	44.9
Threonine	46.70 ± 0.21	− 0.69 ± 2.83	21.4
Serine	47.26 ± 0.23	5.04 ± 3.02	24.3
Glutamic acid	48.34 ± 0.36	− 8.39 ± 4.70	59.0
Proline	20.63 ± 0.39	− 8.33 ± 5.21	72.4
Glycine	46.44 ± 0.24	−15.08 ± 3.22	27.7
Alanine	47.37 ± 0.27	−11.09 ± 3.61	34.8
Cystine	27.16 ± 0.30	13.55 ± 3.95	41.6
Valine	47.53 ± 0.49	21.67 ± 6.45	110.9
Methionine	47.59 ± 0.56	− 1.98 ± 7.35	143.9
Isoleucine	48.82 ± 0.32	− 2.01 ± 4.25	48.2
Leucine	47.96 ± 0.29	−10.46 ± 3.85	39.6
Norleucine	47.06 ± 0.37	−20.27 ± 4.91	64.2
Tyrosine	47.66 ± 0.41	−24.55 ± 5.40	77.9
Phenylalanine	46.47 ± 0.53	− 0.17 ± 6.97	129.4
Ammonia	36.94 ± 1.96	247.21 ± 25.90	1788.3
Ornithine	48.85 ± 0.28	8.16 ± 3.73	37.1
Lysine	49.24 ± 0.29	2.22 ± 3.82	39.0
Histidine	45.33 ± 0.36	1.53 ± 4.80	61.5
Tryptophan	22.38 ± 0.22	−22.54 ± 2.96	23.3
Arginine	43.94 ± 0.32	−14.61 ± 4.26	48.3

* Data based on four runs each at 5, 10 and 20 nmoles.
** S.D. = Standard deviation.

(retaining buffers 1, 2 and 3) by eluting all the basic amino acids with buffer 3 in about 110 min. This leads to considerable peak spreading but avoids problems with histidine quantitation. The use of Pico-Buffer C in place of buffers 3 and 4 lengthens the cycle time to about 150 min because of the additional time required for re-equilibration. These buffer systems, ascorbic acid and air-stable ninhydrin have been used successfully on a Beckman 120 analyser. The buffers are also compatible with the standard TSM manifold using hydrazine as the reductant for air-stable ninhydrin as described by Eveleigh *et al.*[1], although the sensitivity is halved.

Some DMSO in the ninhydrin solution is necessary to avoid the formation of precipitate in the analytical system. Methyl Cellosolve is beneficial to the flow properties of a bubbled system and is less expensive than DMSO. Current cost per chromatogram of the ninhydrin reagent is $0.23, column buffers cost $0.11 and other expendibles cost $0.09.

NOTE ADDED AFTER ACCEPTANCE

Since this paper was submitted, in this laboratory HCl has been replaced by methanesulfonic acid (MSA) (see ref. 5) as the agent for hydrolysis of proteins and peptides with the consequent recovery of tryptophan. Many samples can be hydrolyzed simultaneously in a desiccator (Smithies *et al.*[4]) in the following manner. Dry

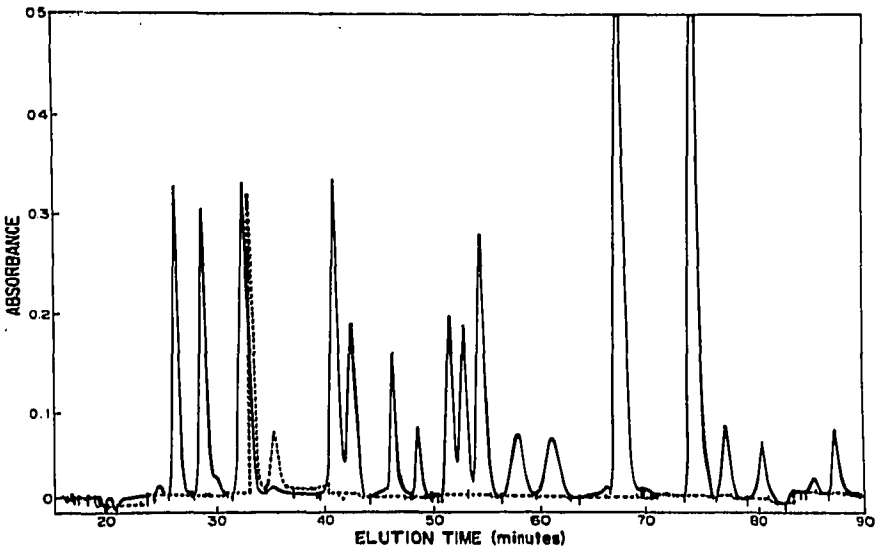

Fig. 3. Chromatogram of cytochrome *c* hydrolysate. The elution pattern of the amino acids is the same as in Fig. 2.

samples in new, unwashed Kinble disposable 10 × 75 mm culture tubes (No. 73000; Owens-Illinois, Toledo, Ohio, U.S.A.) *in vacuo* over NaOH to remove all HCl. Add 20 μl 4 *N* MSA containing 0.2% 3-(2-aminoethyl)indole (Pierce, Rockford, Ill., U.S.A.) to each tube and 9 ml 8 *N* H_2SO_4 to bottom of desiccator; evacuate, flush

TABLE IV

COMPOSITION OF CYTOCHROME *c* AFTER MSA HYDROLYSIS

Cysteine was excluded from the calculations because its absence indicates that thioether linkages to heme were not cleaved.

Amino acid	*Nanomoles*	*Mole %*	
		Observed	*Expected*
Aspartic acid	14.6	8.0	7.8
Threonine	16.4	9.0	9.8
Glutamic acid	20.8	11.4	11.8
Proline	8.1	4.5	3.9
Glycine	22.6	12.4	11.8
Alanine	11.1	6.1	5.9
Valine	6.7	3.7	2.9
Methionine	3.4	1.8	2.0
Isoleucine	9.9	5.4	5.9
Leucine	10.9	6.0	5.9
Norleucine	18.9		
Tyrosine	7.0	3.8	3.9
Phenylalanine	6.6	3.6	3.9
Lysine	32.6	17.9	18.6
Histidine	5.0	2.7	2.9
Tryptophan	2.7	1.5	1.0
Arginine	4.1	2.2	2.0

and clamp in the usual manner. Hydrolyze in autoclave for 18 h at 19 lb./in.^{2} (125°). Dilute each tube with 9 vol. 0.148 *M* trisodium citrate (with 2% thiodiglycol) and load directly on sample cartridges.

Fig. 3 shows a routine amino acid analysis of horse heart cytochrome *c* hydrolyzed with MSA. The calculated composition is listed in Table IV. The agreement between the theoretical and the observed composition for this sample (including tryptophan) is considerable, especially considering that no duplicates were used, no time series run, and less than 2 nmoles of protein were required for the analysis.

NOTE ADDED IN PROOF

Since this paper was submitted for publication, comparable results using other sulfonic acids for hydrolysis of proteins have been reported[6].

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