Quantitative Amino Acid Analysis by Gas–Liquid Chromatography

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The quantitative amino acid analysis of biological substances by gas–liquid chromatography has been clearly demonstrated with both the *N*-trifluoroacetyl *n*-butyl and TMS derivatives of the 20 protein amino acids. Derivatization of the amino acids to the *n*-butyl esters has been shortened, allowing direct formation in 15 min. A rapid method for the hydrolysis of proteins was developed (145° C, 4-hr) which compares favorably with the widely accepted 110° C, 22-hr procedure. Analyses are given for ribonuclease, bovine serum albumin, and amino acid standard mixtures. Analyses of blood plasma, human urine, soybean meal, and corn grain are presented. Ion-exchange cleanup methods are described. Analyses of submicrogram amounts of amino acids are presented, incorporating an injection port–solvent vent device invented by the authors. This device excludes the solvent and reagents from traversing the chromatographic column. Analyses conducted on water extracts of the Apollo 11 and 12 Lunar fines resulted in none of the protein amino acids being observed at levels greater than 4 ppb of each. Chromatograms are presented showing 5 ng of each of the 17 amino acids taken through the entire analytical procedure. A single step method for derivatizing all 20 amino acids to their TMS derivatives was developed, and their resolution on a single column is shown. Both the *N*-TFA *n*-butyl ester and the TMS methods possess certain inherent advantages. The glc methods are sensitive, precise, rapid, and accurate.

The development of analytical systems for quantitative analysis of amino acids has been the subject of intense interest and study in many areas of science during the past 15 years. The classical investigations of Moore *et al.* (1958), Moore and Stein (1951), Hamilton (1958, 1963), Hamilton *et al.* (1960), Piez and Morris (1960), and others have developed ion-exchange chromatography into a refined technique for the determination of amino acids. Chromatographic techniques have clearly become established as the preferred method for amino acid analysis.

More recently, gas–liquid chromatography (glc) has attained great sophistication due to the inherent speed, sensitivity, and versatility of this technique. Glc methods now encompass a wide range of analytical applications; examples include methods for carbohydrates and related polyhydroxy compounds (Sweeley *et al.*, 1963), steroids (VandenHeuvel and Horning, 1962), pesticides (Aue and Moseman, 1970; Beroza and Bowman, 1965; Bowman and Beroza, 1965), lipids, metabolites, and drugs. The analysis of amino acids by glc techniques has been advanced through extensive studies involving numerous derivatization methods and chromatographic systems. Reviews of derivatization techniques, glc separations, and applications have been presented by Weinstein (1966), McBride and Klingman (1968), and Blau (1968). The *N*-trifluoroacetyl *n*-butyl esters and trimethylsilyl derivatives of the amino acids have emerged as the derivatives of choice for quantitative glc analysis.

The essential criteria that the derivatives must meet are the following: simple in its formation; quantitative in yield, 95–100%; little or no interaction with the substrate and/or support; sufficiently volatile; no losses on evaporative concentration; and good resolution in chromatography.

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Zomzely *et al.* (1962) investigated the *N*-trifluoroacetyl (*N*-TFA) *n*-butyl esters as possible derivatives. Lamkin and Gehrke (1965) reported that the most suitable derivative with respect to volatility and chromatography for the gas–liquid chromatographic analysis of the natural protein amino acids is the *N*-TFA *n*-butyl ester. The experimental conditions for quantitative derivation and chromatographic requirements for separation were detailed by Gehrke and Stalling (1967). This was followed by a recent monograph by Gehrke *et al.* (1968b) which presents: macro, semimicro, and micro methods; reagents; sample preparation; instrumental and chromatographic requirements; and sample ion-exchange cleanup of the protein amino acids as their *N*-TFA *n*-butyl esters.

Stalling *et al.* (1968) introduced a new silylating reagent, bis(trimethylsilyl)trifluoroacetamide. This compound is volatile and a powerful reagent for the derivatization of amino acids. Gehrke and coworkers demonstrated that reproducible derivatives could be obtained for all 20 protein amino acids. Recently, Gehrke *et al.* (1969) published a comprehensive method for the 20 protein amino acids as the trimethylsilyl (TMS) derivatives using BSTFA as the silylating reagent.

Gehrke *et al.* (1968a) reported on a dual column EGA-OV-17 chromatographic system for the quantitative determination of all 20 of the protein amino acids as their *N*-TFA *n*-butyl esters.

DIRECT ESTERIFICATION

A refinement in the derivatization procedure for forming the *N*-TFA *n*-butyl esters was reported by Roach and Gehrke (1969) which eliminated the interesterification step and allowed reproducible conversion of amino acids to the *n*-butyl esters directly. Table I presents the data obtained from

Table I. The Relative Molar Response of Amino Acids as a Function of Esterification Time[a]

Amino acid	$RMR_{amino\ acid/glu.}$,[b] min				
	15	20	30	60	150
Alanine	0.54	0.51	0.51	0.53	0.54
Valine	0.71	0.70	0.72	0.71	0.72
Glycine	0.43	0.42	0.42	0.42	0.43
Isoleucine	0.70	0.78	0.80	0.84	0.84
Leucine	0.83	0.81	0.81	0.82	0.82
Proline	0.73	0.71	0.71	0.70	0.71
Threonine	0.64	0.61	0.63	0.63	0.63
Serine	0.55	0.53	0.52	0.53	0.52
Methionine	0.54	0.50	0.52	0.51	0.52
Hydroxyproline	0.76	0.74	0.74	0.74	0.75
Phenylalanine	1.15	1.13	1.13	1.11	1.12
Aspartic acid	0.92	0.91	0.91	0.90	0.91
Glutamic acid	1.00	1.00	1.00	1.00	1.00
Tyrosine	0.95	0.96	0.97	0.95	0.87
Lysine	0.86	0.84	0.85	0.84	0.84
Histidine	0.63	0.63	0.64	0.63	0.61
Arginine	0.62	0.64	0.63	0.62	0.59
Tryptophan	0.97	0.88	0.77	0.74	0.63
Cystine	0.88	0.92	0.92	0.91	0.90

[a] Direct esterification in *n*-butanol 3 *N* in HCl at 100° C. RMR of glutamic acid assigned a value of 1. [b] Each value an average of two determinations.

Table II. Amino Acid Analysis of Ribonuclease as a Function of Hydrolysis Time

Amino acid	Hydrolysis time (hr[a]), and w/w%[b]					
	9.5	26	35	48	81	116
Alanine	7.06	7.12	7.35	7.46	7.57	7.51
Valine	4.33	6.43	6.90	7.28	7.56	7.52
Glycine	1.60	1.65	1.66	1.66	1.69	1.68
Isoleucine	0.81	1.50	1.69	1.99	2.39	2.59
Leucine	1.83	1.91	1.96	1.99	2.02	2.01
Proline	2.78	3.09	3.12	3.14	3.19	3.17
Threonine	6.91	7.35	7.71	7.72	7.58	7.27
Serine	9.91	10.04	9.96	9.80	9.20	8.32
Methionine	3.47	3.39	3.36	3.44	3.17	2.93
Phenylalanine	2.59	3.05	3.17	3.22	3.25	3.29
Aspartic acid	13.23	13.39	13.74	13.97	13.96	13.99
Glutamic acid	11.13	12.08	12.43	12.04	12.49	12.33
Tyrosine	5.74	6.56	6.63	6.59	6.26	6.06
Lysine	8.88	10.15	10.44	10.59	10.61	10.75

[a] Hydrolyzed in a closed tube with 6 *N* HCl for the specified time at 110° ± 1° C. EGA column only. *n*-Butyl stearate as internal standard. Ribonuclease, Type 1-A, Sigma Chemical Co. Glc analyses. [b] Each value represents an average of two determinations at the macro level (10 mg total protein + 15 ml 6 *N* HCl).

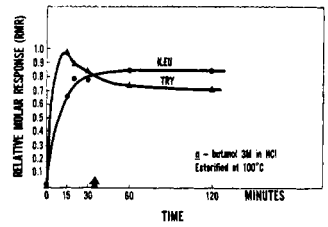


Figure 1. RMR as a function of time

an esterification study with *n*-butanol·3 *N* HCl at 100° C for 15 to 150 min.

These data show that excellent results can be achieved with an esterification time of 15 min for all of the amino acids except isoleucine. Note that the maximum RMR (relative molar response) value for tryptophan was obtained with a 15-min direct esterification. The maximum RMR for isoleucine was obtained after esterification for *ca.* 1 hr, but further studies showed little change in RMR values resulted after 45 min. Direct esterification of isoleucine at 100° C for 15 min yielded an average RMR value of about 85% of the maximum value. Since the w/w% recovery depends not only upon the RMR of the experimental sample but also upon the RMR for the calibration mixture taken through the complete method, a recovery of 100% can be achieved when both the sample and the calibration mixture are esterified for 15 min under *exactly* the same reaction conditions.

The maximum RMR value for tryptophan was obtained on direct esterification for 15 min at 100° C. After reaching a maximum, the RMR value decreased with time, due to the decomposition of tryptophan in the acidic *n*-butanol solution. From a time-derivatization study, an average of about 85% of the maximum RMR for tryptophan was obtained with a 35-min esterification. An average recovery of greater than 95% was obtained, however, when the sample and calibration mixture were reacted under the same experimental conditions (100° C for 35 min). However, samples should be esterified directly at 100° C for 15 min to obtain the most accurate tryptophan values, as seen in the derivatization time presented in Figure 1. It is well known that tryptophan undergoes decomposition during acidic hydrolysis; thus analysis for tryptophan in acidic hydrolyzates is useless. For samples which contain no tryptophan, direct esterifica-

Table III. Amino Acid Analysis of Ribonuclease as a Function of Hydrolysis Time

Amino acid	Hydrolysis time (hr[a]), and w/w%[b]						
	2	4	5	6	7	8.5	9
Alanine	7.30	7.28	7.24	7.37	7.40	7.54	7.45
Valine	6.25	7.15	7.16	7.31	7.41	7.54	7.55
Glycine	1.69	1.71	1.66	1.72	1.67	1.71	1.71
Isoleucine	1.40	1.94	2.07	2.23	2.26	2.53	2.55
Leucine	1.99	1.98	1.98	2.04	2.00	2.02	2.07
Proline	3.18	3.19	3.15	3.16	3.14	3.15	3.16
Threonine	7.73	7.62	7.52	7.36	7.11	7.18	7.17
Serine	10.25	9.66	9.40	8.82	8.52	8.15	8.01
Methionine	3.54	3.36	3.29	3.11	3.06	2.77	2.24
Phenylalanine	3.16	3.26	3.22	3.29	3.22	3.42	3.38
Aspartic acid	13.83	13.60	13.66	13.76	13.60	13.81	13.97
Glutamic acid	11.79	11.88	11.97	12.00	11.75	12.05	12.29
Tyrosine	6.57	6.80	7.00	7.26	7.29	6.17	6.47
Lysine	10.29	10.37	10.45	10.42	10.10	10.27	10.65

[a] Hydrolyzed in a closed tube with 6 *N* HCl for the specified time at 145° ± 2° C. EGA column only. *n*-Butyl stearate as internal standard. Ribonuclease, Type 1-A, Sigma Chemical Co. Glc analyses. [b] Each value represents an average of two determinations at the macro level (10 mg total protein + 15 ml 6 *N* HCl).

Table IV. Amino Acid Analysis of Bovine Serum Albumin as a Function of Hydrolysis Time

Amino acid	Hydrolysis time (hr[a]) and w/w% 2	4	6	8	9	24[b]
Alanine	4.95	5.00	5.14	4.83	4.91	4.76
Valine	4.15	4.46	5.10	4.79	4.89	4.56
Glycine	2.05	2.25	2.11	2.02	1.97	1.97
Isoleucine	1.81	2.12	2.27	2.16	2.22	2.04
Leucine	11.45	11.91	12.30	11.91	11.70	11.42
Proline	4.72	4.23	4.34	4.73	3.84	3.76
Threonine	4.97	5.07	5.05	4.91	4.93	4.97
Serine	3.64	3.73	3.47	3.08	3.40	3.55
Methionine	0.74	0.81	0.68	0.72	0.72	0.70
Phenylalanine	6.50	6.88	6.83	6.53	6.45	6.59
Aspartic acid	9.43	9.49	9.62	9.42	9.18	9.02
Glutamic acid	15.82	15.79	16.13	15.91	15.56	15.49
Tyrosine	5.57	5.92	5.34	5.38	5.15	5.53
Lysine	11.51	11.88	12.01	11.73	11.76	11.56
Histidine	4.00	4.43	4.28	4.16	4.06	4.09
Arginine	5.84	6.10	6.00	5.73	5.87	5.83
Tryptophan	...	...	...	...	...	...
Half-cystine	5.93	6.08	5.84	5.96	5.51	5.86

[a] Hydrolyzed with 6 *N* HCl for the specified time at 145° ± 2° C in a closed tube, with norleucine as internal standard. Analyzed by classical ion-exchange. [b] Hydrolyzed with 6 *N* HCl for 24 hr at 110° ± 1° C in a closed tube, norleucine as internal standard. Analyzed by classical ion-exchange.

Table V. Recovery of Amino Acids from a Standard Mixture[a]

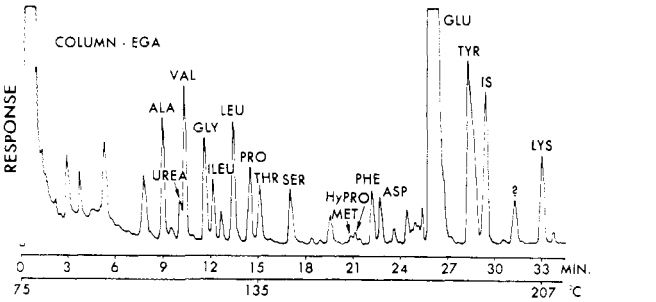
Amino acid	Hydrolysis time (hr), and recovery, % 2	4	6	7	8	9
Alanine	100.6	101.8	96.9	95.6	95.5	93.1
Valine	101.2	100.9	94.1	92.9	91.8	89.4
Glycine	100.6	99.7	95.5	92.9	90.3	87.7
Isoleucine	101.8	102.9	95.8	92.3	89.9	87.6
Leucine	101.2	98.8	94.9	92.8	91.5	89.2
Proline	99.4	94.6	89.9	88.7	89.9	88.9
Threonine	98.6	93.2	90.4	86.3	84.9	84.6
Serine	99.3	90.5	87.6	84.7	81.8	78.8
Methionine	98.1	90.6	79.2	73.6	71.7	75.5
Hydroxyproline	97.4	94.7	89.5	85.5	84.2	83.8
Phenylalanine	102.3	97.7	95.5	94.1	93.2	90.9
Aspartic acid	101.9	98.9	97.6	96.3	95.5	95.4
Glutamic acid	101.6	100.7	100.6	99.7	99.4	97.2
Tyrosine	102.6	100.0	98.7	94.2	93.5	90.9
Lysine	100.0	97.4	94.8	93.4	89.5	92.1
Arginine	95.2	88.1	85.7	76.2	77.1	65.2
Histidine	101.1	97.5	97.4	97.3	97.3	88.4
Cystine	102.8	97.1	100.0	85.7	77.1	74.3

[a] 0.4 mg of each amino acid + 10 ml 6 *N* HCl heated for the specified time at 145° ± 2° C. [b] Each value an average of two independent analyses. Analyzed by gas–liquid chromatography.

Table VI. Selected Amino Acids from the Amino Acid Analysis of Ribonuclease

Amino acid	w/w%, and hydrolysis temperature 110° C, 24 hr	145° C, 4 hr	Literature value[a]
Valine	7.54[b]	7.55[b]	7.49
Isoleucine	2.54[b]	2.59[b]	2.67
Threonine	8.00[c]	8.00[c]	8.90
Serine	10.70[c]	11.05[c]	11.40
Methionine	3.54[c]	3.83[c]	4.00
Phenylalanine	3.30[b]	3.40[b]	3.51

[a] Hirs *et al.* (1954). [b] Values obtained by drawing a tangent to the maximum in a plot of yield of amino acid *vs.* time of hydrolysis to obtain the maximum values. [c] Values obtained by extrapolation to zero time a plot of yield of amino acid *vs.* time of hydrolysis.



HUMAN BLOOD PLASMA N-TFA n-BUTYL ESTERS. SAMPLE: ca. 6 ml OF PLASMA IN FINAL VOLUME OF 2.5 ml. 5 µl INJECTED (ca. 3.5 µg TOTAL AMINO ACIDS). VARIAN AEROGRAPH 2100.

EGA COLUMN: 0.325 w/w% ON 80/100 MESH a.w. HEAT TREATED CHROMOSORB G 1.5 m x 4 mm I.D. GLASS; INITIAL TEMP. 75 °C, 4 °C/MIN. n - BUTYL STEARATE AS IS.

Figure 2. Glc chromatogram of human blood plasma not cleaned by cation-exchange

tion for 35 min at 100° C is recommended to assure quantitative esterification of isoleucine.

An esterification time of 35 min is recommended for samples which are to be analyzed for all of the amino acids, including isoleucine and tryptophan. However, only 15 min are required if a calibration standard is taken through the same procedure.

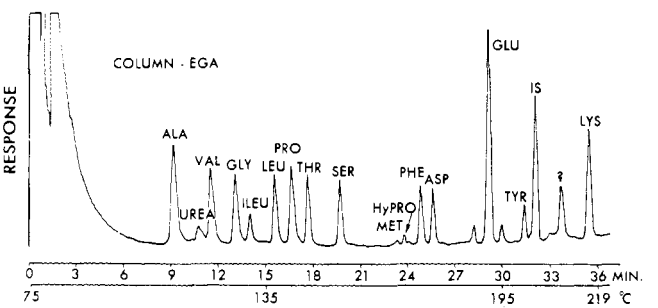
In quantitative work, a reference calibration mixture is analyzed under exactly the same experimental conditions as are the samples. Thus correction is made for any breakdown of tryptophan and the response values of all of the other amino acids are placed on the same basis.

Using the direct esterification method, recoveries of amino acids from a mixture averaged 99.7% with an average relative standard deviation of 0.92%.

This direct esterification method allows a rapid, precise, and accurate derivatization of the protein amino acids to their *N*-TFA *n*-butyl esters with a minimum of transfers and sample handling. A complete glc analysis of the protein amino acids, including derivatization and chromatography, can be completed in less than 1 hr.

HYDROLYSIS OF PROTEINS

The particular method used for the hydrolysis of proteins prior to an amino acid analysis is of considerable importance since some amino acids are preferentially destroyed in part and the hydrolysis of others is incomplete. In view of the high precision attained in the gas–liquid chromatographic analysis of amino acid mixtures, the nature of the hydrolytic



HUMAN BLOOD PLASMA N-TFA n-BUTYL ESTERS. SAMPLE: ca. 6 ml OF PLASMA IN FINAL VOLUME OF 2.5 ml. 5 µl INJECTED (ca. 3.5 µg TOTAL AMINO ACIDS). VARIAN AEROGRAPH 2100.

EGA COLUMN: 0.325 w/w% ON 80/100 MESH a.w. HEAT TREATED CHROMOSORB G. 1.5 m x 4 mm I.D. GLASS; INITIAL TEMP. 75 °C, 4 °C. MIN. n-BUTYL STEARATE AS IS.

Figure 3. Glc chromatogram of human blood plasma cleaned by cation-exchange

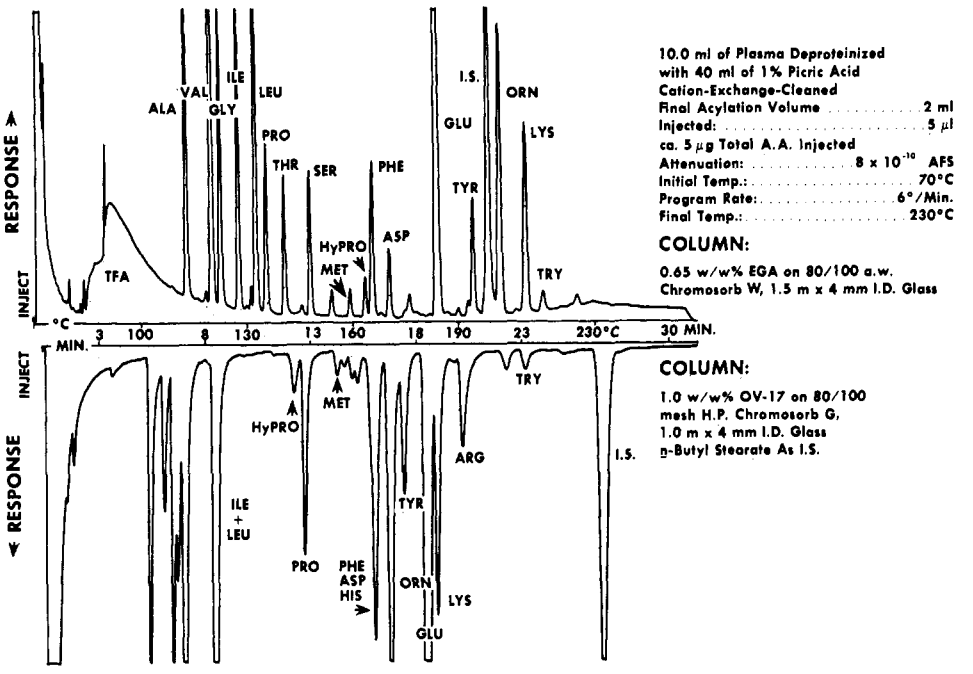


Figure 4. Glc analysis of bovine blood plasma

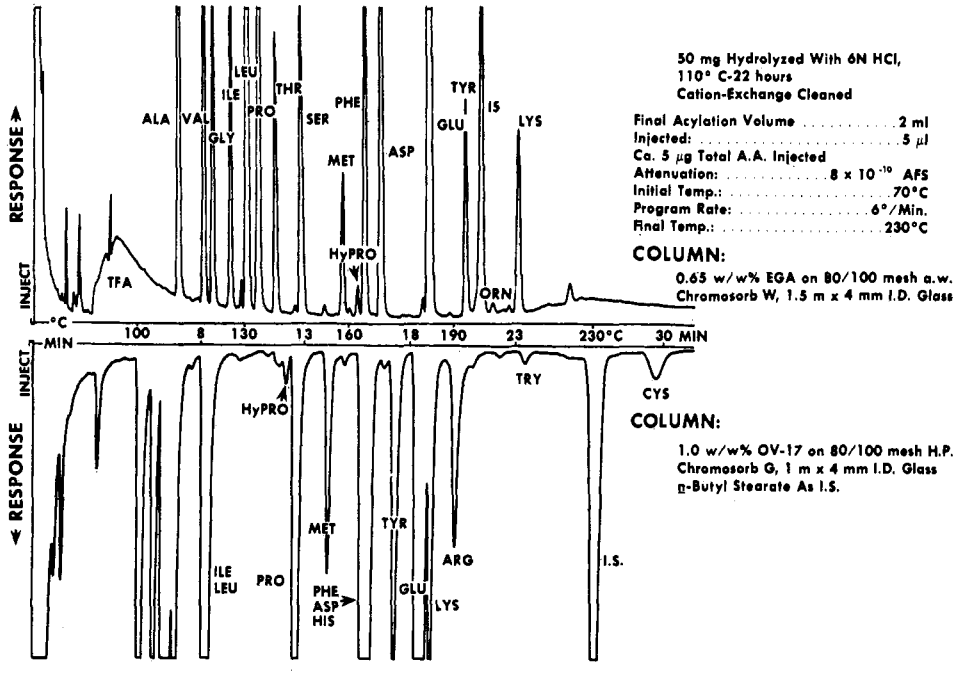


Figure 5. Glc analysis of corn grain hydrolyzate

conditions plays an increasingly important role and can be easily evaluated. Roach and Gehrke (1970) conducted a thorough study of hydrolysis conditions designed to facilitate the rapid analysis of amino acids by glc. Table II presents the data obtained from a hydrolysis time study of ribonuclease, using 6 *N* HCl at 110° C for 9.5 to 116 hr. Table III presents similar data obtained from hydrolysis at 145° C for 2 to 9 hr. Good agreement of these data at hydrolysis times of 26 hr at 110° C and 4 hr at 145° C was noted. Table IV presents a comparison of hydrolysis conditions with bovine serum albumin. Hydrolysis at 145° C for 4 hr is seen to compare favorably with hydrolysis at 110° C for 24 hr. Table V presents recovery data for a standard mixture of the protein amino acids when subjected to the above hydrolysis conditions. It was observed, as reported by others, that some amino acids are prone to hydrolytic losses, *i.e.*, proline, threonine, serine, methionine, hydroxyproline, and arginine. Losses of up to 12% were noted for treatment with 6 *N* HCl at 145° C for 4 hr. This is not inconsistent with literature reports. Table VI compares the yields of selected "problem" amino acids from ribonuclease. It should be noted that the 145° C, 4-hr hydrolysis compares favorably with the standard 110° C, 24-hr method and the reported literature values.

Of special interest is a comparison of the data obtained for ribonuclease and bovine serum albumin for the 4-hr hydrolysis at 145° C ± 2° C with the 24-hr hydrolysis at 110° C ± 1° C. In almost every case a higher recovery was obtained for the 145° C ± 2° C hydrolysis. With this

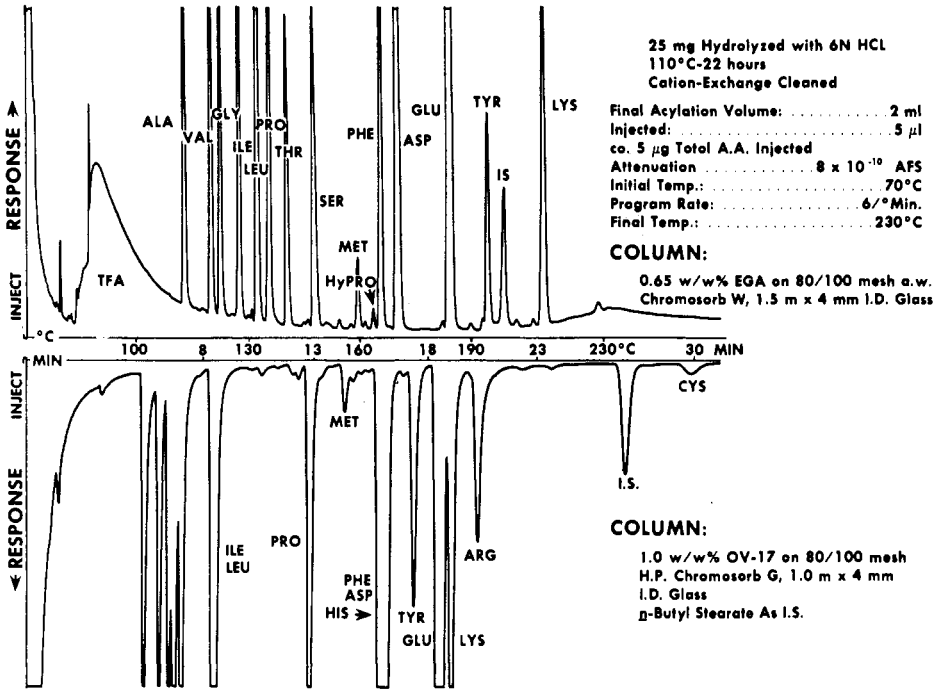


Figure 6. Glc analysis of soybean meal hydrolyzate

method a protein can be essentially completely hydrolyzed in 4 hr with a minimum of decomposition of the amino acids.

GLC ANALYSIS OF AMINO ACIDS IN BIOLOGICAL SUBSTANCES

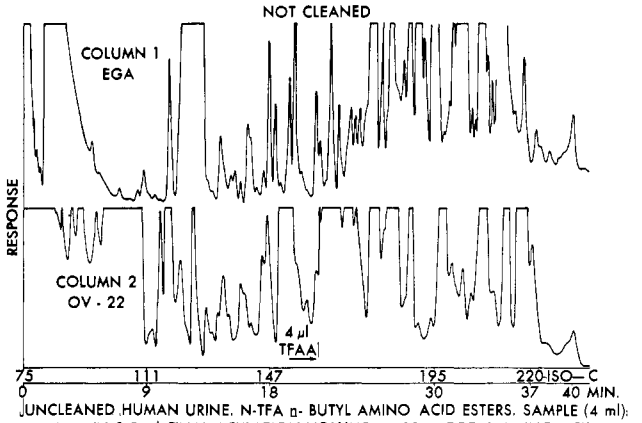
The development of a glc method for the quantitative analysis of amino acids in complex biological substances, specifically blood plasma and urine, has been extensively studied by Zumwalt *et al.* (1970). The amino acids present in these physiological fluids were quantitatively isolated by ion-exchange methods and retained on the ion-exchange resin, while the substances which interfere with the glc analysis passed through the column and were discarded. The amino acids were then eluted from the column, derivatized to their *N*-TFA *n*-butyl esters, and analyzed by glc.

The free amino acids in blood plasma were analyzed with and without prior cleanup by cation-exchange. The chromatogram obtained from the uncleaned plasma is shown in Figure 2. Figure 3 presents the plasma analysis after removal of interfering materials by cation-exchange. Plasma analyses were conducted at both a macro level (*ca.* 4 mg of total amino acids) and a micro level (*ca.* 200 µg of total amino acids), and the results were compared to the data obtained from the classical ion-exchange technique. These data are presented in Table VII. A complete analysis of free amino acids in bovine blood plasma is presented in Figure 4, with

Table VII. Amino Acid Analysis of Human Blood Plasma

	mg per 100 ml of Plasma								
	Gas–liquid chromatography[a,f]				Ion-exchange chromatography		Average		
Amino acid	Macro		Micro				Macro	Micro	Ion-exchange
Alanine	3.12	3.12	2.92	2.97	3.45	3.39	3.12	2.95	3.42
Valine	3.24	3.48	3.21	3.51	3.25	3.24	3.36	3.36	3.25
Glycine	2.58	2.72	2.54	2.88	2.68	2.69	2.65	2.71	2.69
Isoleucine[b]	1.41	1.38	1.67	1.55	1.19	1.24	1.40	1.61	1.22
Leucine	2.49	2.68	2.65	2.15	2.73	2.56	2.59	2.40	2.65
Proline[c]	2.69	2.63	2.99	2.84	2.92	2.10	2.66	2.92	2.51
Threonine	2.07	2.02	2.20	2.22	1.50	1.60	2.05	2.21	1.55
Serine	1.80	1.76	1.74	1.81	1.42	1.46	1.78	1.78	1.44
Methionine	0.61	0.62	0.75	0.71	0.37	0.32	0.62	0.73	0.35
Phenylalanine	0.92	0.92	0.97	0.86	0.98	0.95	0.92	0.92	0.97
Aspartic acid[d]	1.29	1.30	1.29	1.23	0.70	0.66	1.30[d]	1.26	0.68
Glutamic acid[d]	3.35	3.34	3.22	3.30	2.12	2.08	3.35[d]	3.26	2.10
Tyrosine	0.98	0.98	0.94	0.99	0.93	0.86	0.98	0.97	0.90
Ornithine	2.01	2.05	2.30	2.19	1.74	1.72	2.03	2.25	1.73
Lysine	3.43	3.60	3.38	3.21	3.62	3.60	3.52	3.30	3.61
Arginine	1.20	1.32	1.26	1.17	1.58	1.61	1.27	1.22	1.60
Histidine[e]	1.01	1.32	1.19	1.09	1.93	1.93	1.17	1.14	1.93[e]
Tryptophan[e]	0.63	0.57	0.66	0.69			0.60	0.68	
Cystine	0.45	0.43	0.49	0.54	T	T	0.44	0.52	T
							35.81	36.19	32.10

[a] Cleaned by cation-exchange prior to derivatization. [b] Glc values include alloisoleucine. [c] The lack of ion-exchange precision is due to poor separation of Pro and Cit. [d] Glc values include $AspNH_2$ and $GluNH_2$. [e] His and Trp not separated by ion-exchange. [f] *N*-TFA *n*-butyl esters.



UNCLEANED HUMAN URINE. N-TFA n- BUTYL AMINO ACID ESTERS. SAMPLE (4 ml): ca. 4 mg IN 2.5 ml FINAL ACYLATION VOLUME ca. 13 µg TOTAL AMINO ACIDS INJECTED (8 µl). COLUMN 1: 0.65 w/w% EGA ON 80/100 MESH DRIED (140°C - 12 HRS.) a. w. CHROM. W, 1.5 m x 4 mm I.D. GLASS. COLUMN 2: 1.5 w/w% OV - 22 ON 80/100 mesh H.P. CHROM. G, 1.0 m x 4 mm I.D. GLASS. INITIAL TEMP. 75°C, 4°C/MIN. TO 220°C.

Figure 7. Glc chromatogram of human urine

simultaneous operation of the EGA and OV-17 chromatographic columns. The cation-exchange cleanup procedure has been applied to corn grain and soybean meal hydrolyzates, with the resultant chromatograms presented in Figures 5 and 6. Excellent agreement of the glc and classical ion-exchange data was obtained as seen in Tables VIII and IX. The recovery of amino acids from the cation-exchange cleanup procedure was found to be quantitative with the data presented in Table X.

Initial glc analyses of uncleaned human urine revealed that an efficient method of removing extraneous substances was essential. A chromatogram of the *N*-TFA *n*-butyl derivatives of uncleaned human urine is shown in Figure 7.

Aliquots of a stock urine solution, cleaned by both cation and anion-exchange procedures, were analyzed by glc and the classical ion-exchange techniques. A typical glc chromatogram of cleaned urine is shown in Figure 8. Also, classical ion-exchange analyses were made on aliquots of unhydrolyzed stock solution and on aliquots subjected to a mild hydrolysis. The data from these analyses are presented in Table XI and the results are seen to be in good agreement.

To establish the recovery of the amino acids from the anion-exchange procedure, a thorough evaluation of the technique was conducted. All the protein amino acids were quantitatively recovered, with arginine the only exception. These recovery data are presented in Table XII.

Table VIII. Amino Acid Analysis of Corn Grain[a]

	w/w %					
	Gas–liquid chromatography[b]			Ion-exchange chromatography[c]		
Amino acid			Avg			Avg
Alanine	0.650	0.656	0.653	0.628	0.602	0.615
Valine	0.415	0.437	0.426	0.467	0.431	0.449
Glycine	0.328	0.333	0.331	0.329	0.322	0.326
Isoleucine	0.284	0.300	0.292	0.277	0.279	0.278
Leucine	0.967	0.965	0.966	0.969	0.937	0.953
Proline	0.890	0.916	0.903	0.952	0.824	0.888
Threonine	0.330	0.338	0.334	0.328	0.314	0.321
Serine	0.476	0.482	0.479	0.468	0.431	0.449
Methionine	0.190	0.186	0.188	0.178	0.189	0.184
Hydroxyproline	0.041	0.027	0.034	trace	trace	trace
Phenylalanine	0.391	0.399	0.395	0.384	0.394	0.389
Aspartic acid	0.516	0.522	0.519	0.540	0.513	0.527
Glutamic acid	1.409	1.389	1.399	1.549	1.413	1.481
Tyrosine[d]	0.235	0.137	0.186	0.229	0.153	0.191
Ornithine	trace	trace	trace	0.004	0.006	0.005
Lysine	0.278	0.236	0.257	0.245	0.225	0.235
Arginine	0.355	0.376	0.363	0.377	0.346	0.362
Cystine	0.044	0.044	0.044	0.061	0.057	0.059
Histidine	0.357	0.339	0.348	0.305	0.296	0.301
Tryptophan[e]						
Total	8.070	7.980	8.025	8.279	7.748	8.014

[a] Hydrolyzed for 22 hr at 110° C in a closed tube with 6 *N* HCl under N_2, cation-exchange cleaned. [b] Two independent analyses, *n*-butyl stearate as internal standard. [c] Two independent analyses, norleucine as internal standard. [d] Partially destroyed during 6 *N* HCl hydrolysis. [e] Destroyed during 6 *N* HCl hydrolysis.

Table IX. Amino Acid Analysis of Soybean Meal[a]

	w/w %					
	Gas–liquid chromatography[b]			Ion-exchange chromatography[c]		
Amino acid			Avg			Avg
Alanine	2.181	2.159	2.170	2.096	2.111	2.104
Valine	2.348	2.304	2.327	2.368	2.497	2.433
Glycine	2.002	2.047	2.025	1.944	1.963	1.954
Isoleucine	2.127	2.092	2.110	2.088	2.196	2.142
Leucine	3.453	3.442	3.448	3.496	3.618	3.557
Proline	2.816	2.903	2.860	2.728	2.554	2.641
Threonine	1.850	1.885	1.868	1.856	1.822	1.839
Serine	2.739	2.829	2.785	2.752	2.616	2.684
Methionine	0.438	0.512	0.475	0.352	0.386	0.369
Hydroxyproline	0.093	0.099	0.096	trace	trace	trace
Phenylalanine	2.338	2.331	2.335	2.320	2.504	2.412
Aspartic acid	5.323	5.141	5.232	5.312	5.160	5.236
Glutamic acid	8.180	7.815	8.050	8.220	7.879	8.050
Tyrosine	1.384	1.288	1.336	1.312	1.426	1.369
Ornithine	trace	trace	trace	0.040	0.027	0.034
Lysine	3.080	2.384	2.957	2.816	2.788	2.802
Arginine	3.476	2.883	3.180	3.192	3.438	3.315
Tryptophan	*d*					
Cystine	0.264	0.264	0.264	0.232	0.295	0.264
Histidine	1.639	1.515	1.577	1.440	1.536	1.488
Total	45.730	44.343	45.037	44.564	44.816	44.690

[a] Hydrolyzed for 22 hr at 110° C in a closed tube with 6 *N* HCl under N_2, cation-exchange cleaned. [b] Two independent hydrolyzates, *n*-butyl stearate as internal standard. [c] Two independent analyses, norleucine as internal standard. [d] Destroyed during 6 *N* HCl hydrolysis.

Table X. Recovery of Amino Acids from Cation-Exchange Cleanup

Amino acid	mg of Amino acids Added	Found[a,b,c,d]			Avg	Recovery, %	RSD, %[e]
Alanine	0.500	0.501	0.503	0.494	0.499	99.8	0.67
Valine	0.500	0.495	0.498	0.494	0.496	99.1	0.30
Glycine	0.500	0.499	0.505	0.500	0.501	100.0	0.46
Isoleucine	0.500	0.513	0.508	0.502	0.508	101.5	0.78
Leucine	0.500	0.512	0.512	0.512	0.512	102.4	0.00
Proline	0.500	0.504	0.503	0.491	0.499	99.8	1.02
Threonine	0.500	0.493	0.505	0.500	0.499	99.8	0.85
Serine	0.500	0.496	0.508	0.508	0.504	100.7	0.98
Methionine	0.500	0.494	0.506	0.486	0.495	99.0	1.42
Hydroxyproline	0.500	0.507	0.507	0.494	0.503	100.4	1.06
Phenylalanine	0.500	0.500	0.499	0.496	0.498	99.6	0.30
Aspartic acid	0.500	0.493	0.494	0.487	0.491	98.3	0.54
Glutamic acid	0.500	0.505	0.499	0.488	0.497	99.5	1.22
Tyrosine	0.500	0.496	0.501	0.496	0.498	99.5	0.41
Ornithine	0.500	0.496	0.504	0.490	0.497	99.3	0.99
Lysine	0.500	0.485	0.505	0.495	0.495	99.0	1.41
Arginine	0.500	0.500	0.537	0.505	0.514	102.7	2.84
Tryptophan	0.500	0.440	0.442	0.405	0.429	85.8	2.94
Cystine	0.500	0.508	0.489	0.541	0.513	102.5	3.72
Histidine	0.500	0.483	0.478	0.497	0.486	97.2	1.39
					Average	99.3	1.17

[a] Analyses by gas–liquid chromatography as the *N*-TFA *n*-butyl esters. [b] Each value represents an independent sample. [c] Samples: 0.5 mg of each amino acid in 5 ml of 0.1 *N* HCl, added to 20 ml of 1% picric acid. [d] Column: 1.5 × 5 cm bed of Amberlite CG-120 hydrogen form, 100/120 mesh. [e] Relative standard deviation, %.

ANALYSIS OF NANOGRAM AMOUNTS OF AMINO ACIDS

Gehrke *et al.* (1968b) reported in detail a derivatization method for submicrogram amounts of amino acids, which included a discussion of some critical points in the derivatization and glc analysis. A major obstacle associated with the glc analysis of extremely small quantities of biological samples or samples containing very low concentrations of amino acids has been the limited sample volume that could be injected. A solvent-venting system has now been developed and a patent applied for (University of Missouri) an instrumental device which allows injection of the total derivatized sample (50 to 100 μl) on a standard packed analytical column. This device prevents the large volume of solvent and acylating reagent from traversing the EGA column, while allowing the essentially quantitative transport of amino acids present in the sample to the detector. Of particular importance in the analysis of nanogram amounts of amino acids is that this instrumental system eliminates the interfering TFA peaks from the EGA chromatogram. Figure 9 presents a typical chromatogram obtained of a standard amino acid mixture

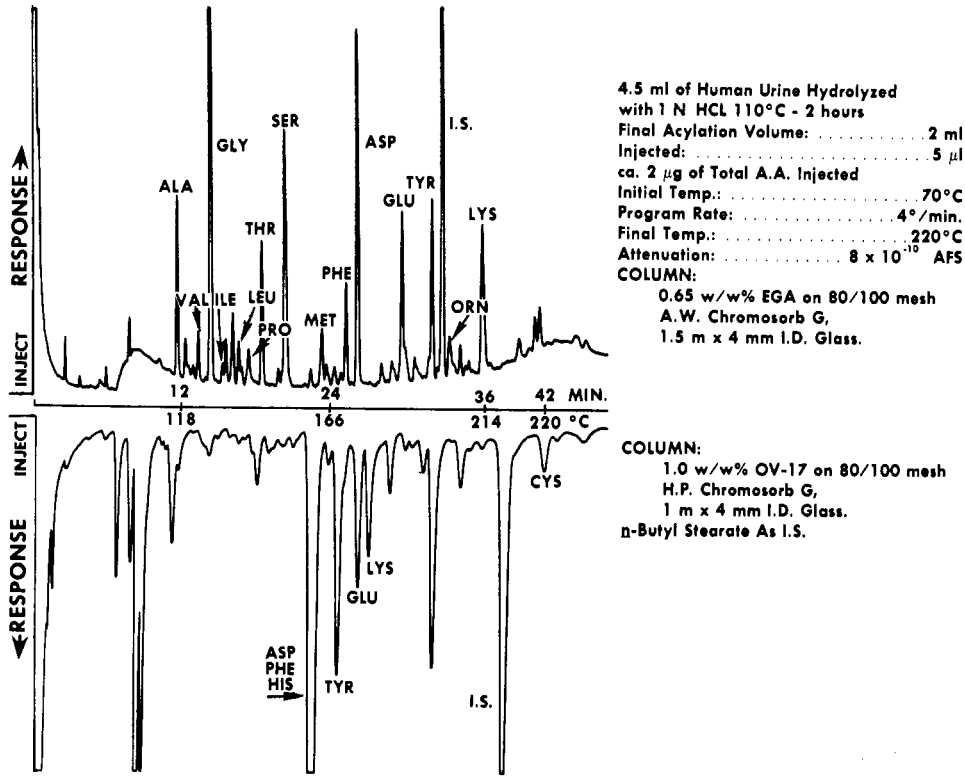


Figure 8. Glc analysis of human urine

Table XI. Amino Acid Analysis of Human Urine[a]

Amino acid	mg per 100 ml of Urine: Gas–liquid chromatography[b]			Ion-exchange chromatography[d] Nonhydrolyzed		Ion-exchange chromatography[d] Hydrolyzed[e]		Average: Glc	Average: Ion-exchange	RSD,[c] %: Glc	RSD,[c] %: Ion-exchange
Alanine	2.82	2.64	2.78	2.39	2.75	2.52	2.58	2.75	2.56	2.79	5.00
Valine	0.66	0.67	0.59	0.60	0.60	0.78	0.75	0.64	0.68	5.40	12.40
Glycine	16.52	16.58	16.64	17.18	17.19	17.78	17.53	16.58	17.42	0.03	1.40
Isoleucine	T[f]	0.18	0.24	0.23	0.28	0.24	0.22	0.21	0.24		9.20
Leucine	0.78	0.68	0.82		0.70	0.77	0.76	0.76	0.74	7.70	4.10
Proline	T	T	T	T	T	T	T	T	T		
Threonine	3.02	2.98	3.08	2.64	2.88	3.07	2.51	3.03	2.78	1.40	7.70
Serine	5.56	5.62	5.60	5.39	5.28	5.28	5.43	5.59	5.35	0.40	1.80
Methionine	0.20	0.47	0.50	0.41	0.34	0.31		0.39	0.35		
Hydroxyproline	0.00	T	T	0.00	0.00	0.00	0.00	T	0.00		
Cysteine	0.00	0.00	T	T	T	T	T	T	T		
Phenylalanine	1.63	1.71	1.66	1.68	1.81	1.92	1.77	1.67	1.80	3.70	4.70
Aspartic acid	5.88	5.74	5.62	0.52	0.71	5.00	5.30	5.74	5.15	1.40	7.20
Glutamic acid	10.31	10.20	10.16	0.68	0.72	10.15	9.69	10.22	9.92	0.60	3.60
Tyrosine	3.68	3.80	3.76	3.41	3.45	3.31	3.44	3.75	3.40	0.13	1.20
Lysine	3.98	4.20	4.12			3.92	4.50	4.10	4.21	2.20	4.60
Arginine	0.00[i]										
Histidine	26.51	25.46	27.30	27.91	26.65	29.11		26.42	27.89	0.78	3.70
Tryptophan	5.62	5.49	5.68	*g*	*g*	*g*	*g*	5.60	*g*	1.40	
Cystine	0.08	0.13	0.09	0.11	0.14			0.10	0.13		
								87.76	86.91	2.15[h]	5.12[h]

[a] Cleaned by cation and anion-exchange prior to analysis. [b] Each an independent analysis. [c] Relative standard deviation, %. [d] Each an independent analysis, norleucine as internal standard. [e] Hydrolyzed 2 hr at 110° C under N_2 in 1 *N* HCl. [f] Trace. [g] Not resolved from histidine. [h] Average RSD, %. Note conversion of $AspNH_2$ and $GluNH_2$ to Asp and Glu on hydrolysis. [i] Not recovered from anion-exchange cleanup.

Table XII. Recovery of Amino Acids from Anion-Exchange Cleanup

Amino acid	mg of Amino acids: Added	Found[a,b,c]			Avg	Recovery, %	RSD, %
Alanine	0.891	0.917	0.911	0.913	0.914	102.6	1.07
Valine	1.171	1.210	1.203	1.191	1.201	102.6	0.80
Glycine	0.751	0.738	0.746	0.741	0.742	98.9	0.55
Isoleucine	1.312	1.281	1.326	1.423	1.343	102.4	5.40
Leucine	1.312	1.278	1.285	1.293	1.285	97.9	0.58
Proline	1.151	1.126	1.113	1.213	1.151	100.0	4.72
Threonine	1.191	1.190	1.241	1.238	1.223	102.7	2.34
Serine	1.051	1.091	1.073	1.071	1.078	102.6	1.02
Methionine	1.492	1.436	1.451	1.479	1.455	97.5	1.50
Hydroxyproline	1.312	1.343	1.351	1.336	1.343	102.4	0.56
Phenylalanine	1.652	1.692	1.691	1.621	1.668	101.0	2.44
Aspartic acid	1.331	1.374	1.362	1.371	1.369	102.8	0.46
Glutamic acid	1.470	1.491	1.498	1.499	1.496	101.8	0.29
Tyrosine	1.612	1.642	1.651	1.648	1.647	102.2	0.28
Lysine	1.462	1.497	1.492	1.501	1.497	102.4	0.30
Arginine	1.742	0.000	0.000	0.000		000.0	
Tryptophan	2.042	2.091	2.084	2.080	2.085	102.1	0.27
Histidine	1.552	1.501	1.496	1.510	1.502	96.8	0.47
Cystine	2.403	2.381	2.380	2.369	2.377	98.9	0.28
	26.900				27.140	Avg 101.0	Avg 1.29

[a] Analyses by glc as the *N*-TFA *n*-butyl esters. [b] Ornithine as internal standard. [c] Each value represents an independent analysis.

at the macro level without the instrumental adaptation. Figure 10 shows the effect of the devised system, with the corresponding reduction of the solvent peak and the absence of TFA throughout the chromatogram.

The injection port–solvent vent chromatographic device can be obtained from the Analytical BioChemistry Laboratories, Inc., P.O. Box 1097, Columbia, Mo., 65201.

Figures 11 and 12 are similar comparisons with analysis of bovine blood plasma and soybean oil meal analyses presented in Figures 13 and 14.

Initial investigations of the system have shown great promise in the glc analysis of nanogram amounts of amino acids. Figure 15 presents the chromatogram obtained on derivatization and analysis of 100 ng of each amino acids. These initial studies were designed to evaluate the derivatization and chromatographic procedures; thus stringent precautions to exclude contaminants were not taken.

The derivatization and analysis of samples containing 50 and 5 ng of each amino acid were also successfully conducted, with the chromatograms obtained presented in Figures 16 and 17. Studies are currently underway to further refine this technique to the 1-ng level.

TRIMETHYLSILYL DERIVATIVES OF AMINO ACIDS

Since the introduction of the trimethylsilyl (TMS) derivatives of the amino acids by Rühlmann and Giesecke (1961), efforts have been made by several groups of researchers in attempts to use these derivatives for the quantitative gas–liquid chromatographic analysis of the 20 protein amino acids. The primary interest in the TMS derivative is the

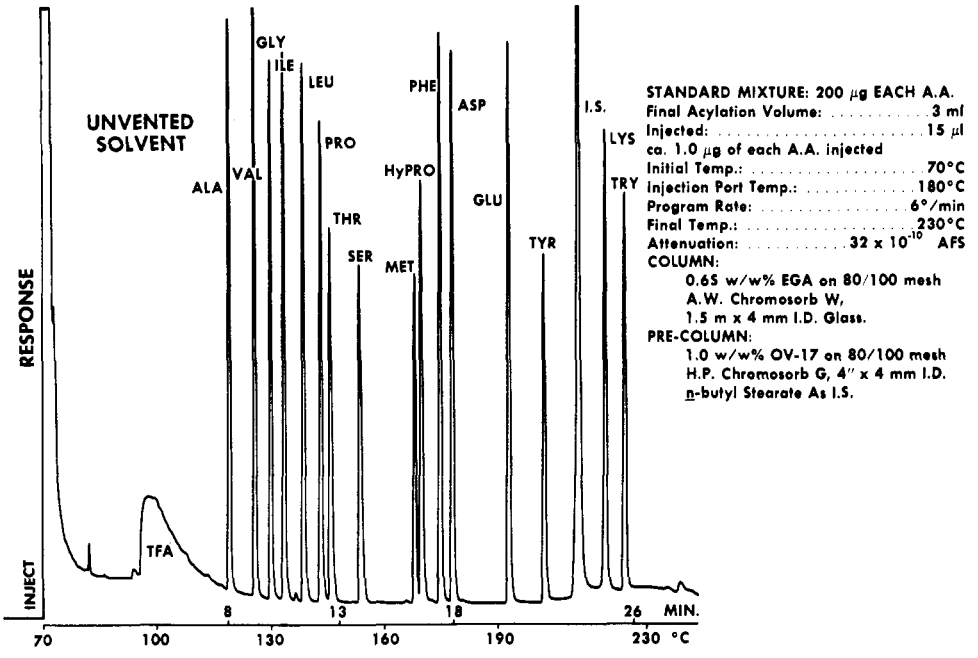


Figure 9. Glc analysis of standard amino acid mixture

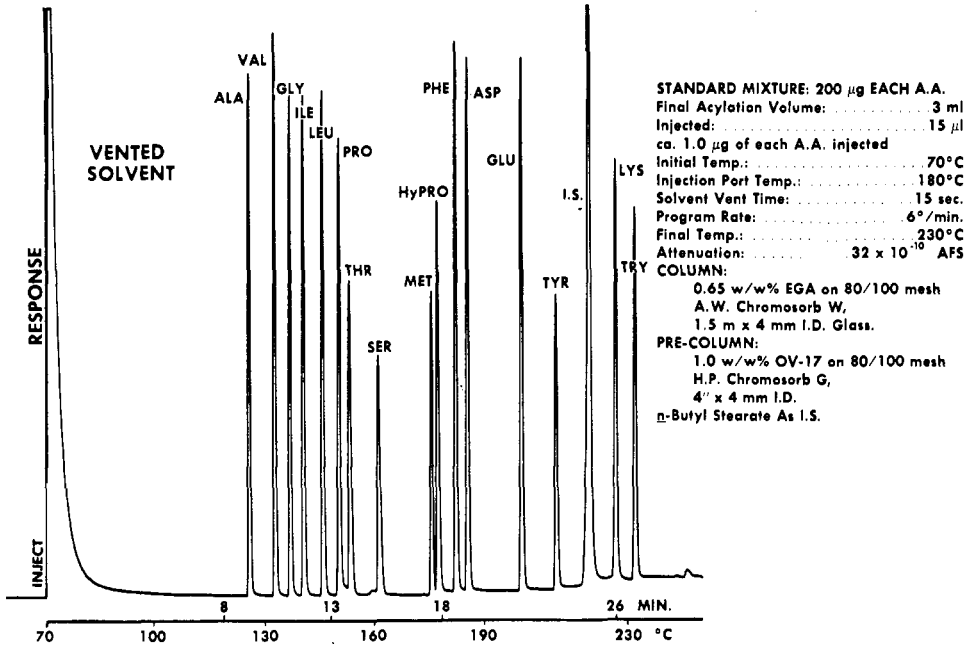


Figure 10. Glc analysis of standard amino acid mixture

one-step derivatization procedure, whereas almost all other derivatives are formed by two or more reaction steps. Rühlmann and Giesecke used hexamethyldisilizane and trimethylchlorosilane to obtain derivatives for most of the protein amino acids. Smith and Sheppard (1965) and Mason and Smith (1967) made a study of the optimum silylation conditions and concluded that trimethylsilyldiethylamine with some kind of catalyst was the best silylation reagent. Trimethylsilyldimethylamine recently has been claimed to be more volatile and was recommended for these reasons (Supelco, Inc., 1967). *N*-Trimethylsilyl *N*-methylacetamide has been recommended by Birkofer and Donike (1967). Klebe *et al.* (1966) used bis(trimethylsilyl)acetamide (BSA) to obtain sharp single peaks for all the protein amino acids except arginine, which showed indications of decomposition on the column. However they were unable to separate the derivatives of glycine and alanine from the bis(trimethylsilyl)acetamide on an SE-30 column. The introduction of bis(trimethylsilyl)trifluoroacetamide (BSTFA) by Stalling *et al.* (1968) has solved the problem of separation of the trimethylsilyl derivatives of glycine and alanine from the reagents and reactions products. Then Gehrke *et al.* (1969) published a comprehensive method for the glc analysis of all 20 protein amino acids as their trimethylsilyl derivatives using BSTFA as the silylating agent, but they reported that problems still existed in the analysis of biological fluids such as urine.

More recently investigations by Gehrke and Leimer (1971)

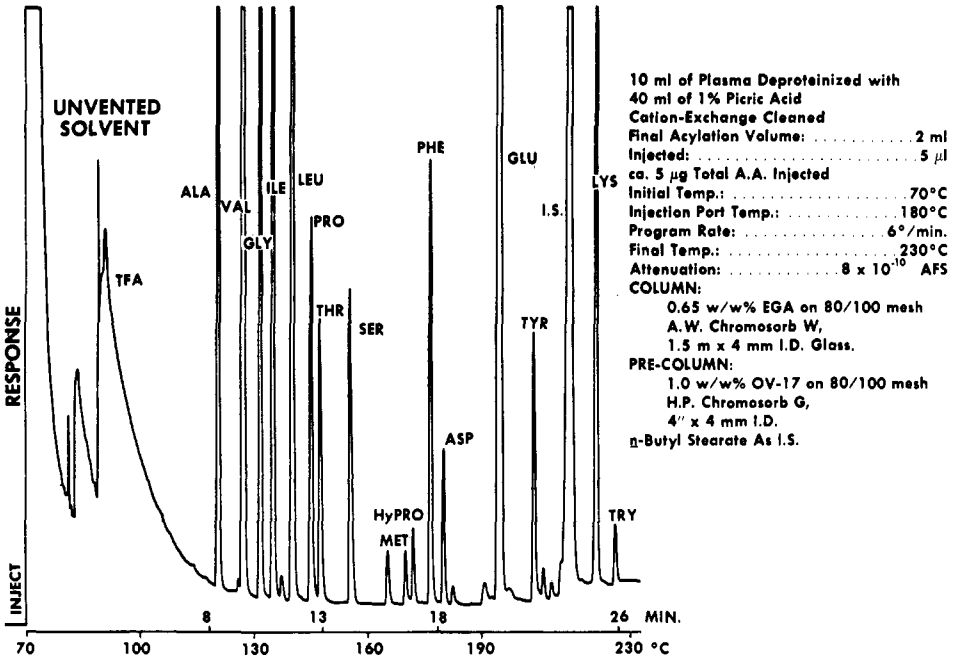


Figure 11. Glc analysis of bovine blood plasma

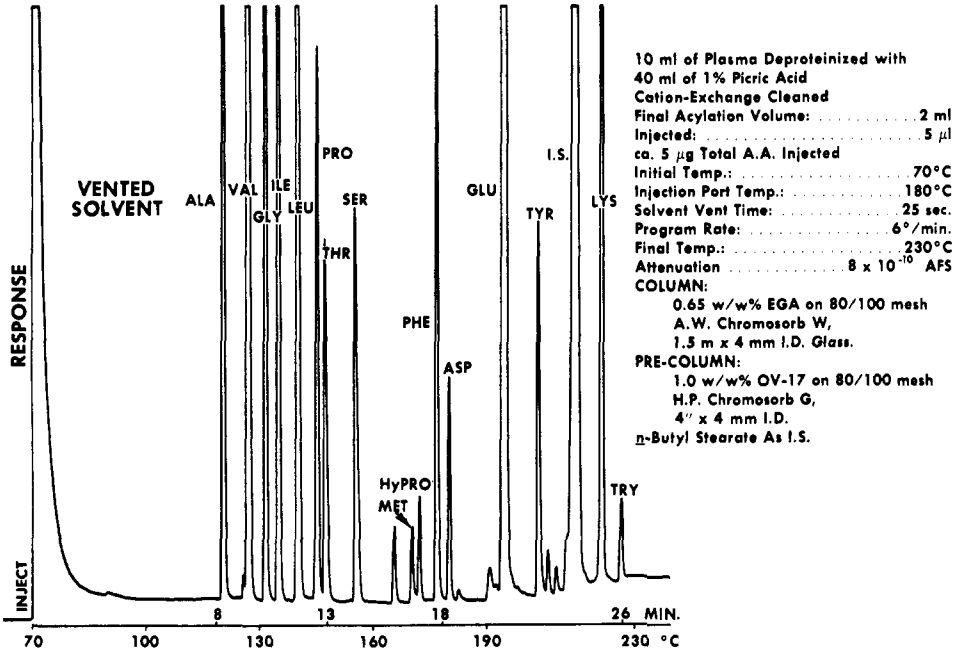


Figure 12. Glc analysis of bovine blood plasma

and Leimer (1971) have led to refinements in both derivatization and chromatography of the TMS derivatives of the 20 protein amino acids. The chromatogram obtained on silylation of a standard mixture of amino acids at 150° C for 2.5 hr is presented in Figure 18. All 20 of the amino acids can be reproducibly derivatized under these conditions. The optimum derivatization and glc separation conditions are presented. The chromatographic resolution of the *N*-trimethylsilyl TMS esters of the 20 protein amino acids can be achieved on a 10 w/w% OV-11 on 100/120 mesh Supelcoport column, 6 m × 2 mm i.d. Phenanthrene, fluorene, and decanoic acid are suitable internal standards, as they are completely separated from the 20 protein amino acids. The best reaction conditions for the quantitative silylation of the 20 amino acids were investigated. Seventeen of the amino acids can be reproducibly converted in a closed reaction tube to the TMS derivatives in 15 min at 150° C. However, for glycine, arginine, and glutamic acid, 2.5 hr at 150° C are necessary for reproducible derivatization. It is recommended that silylation for 2.5 hr at 150° C be used for all 20 of the protein amino acids. A significant advantage is that the TMS derivatives can be formed in a single step with no transfers, evaporations, or reagent additions during their formation. In later studies it has been shown that good separation can be achieved with a 2 m × 1.6 mm glass column containing 10 w/w% OV-11 on 100/120 mesh Supelcoport.

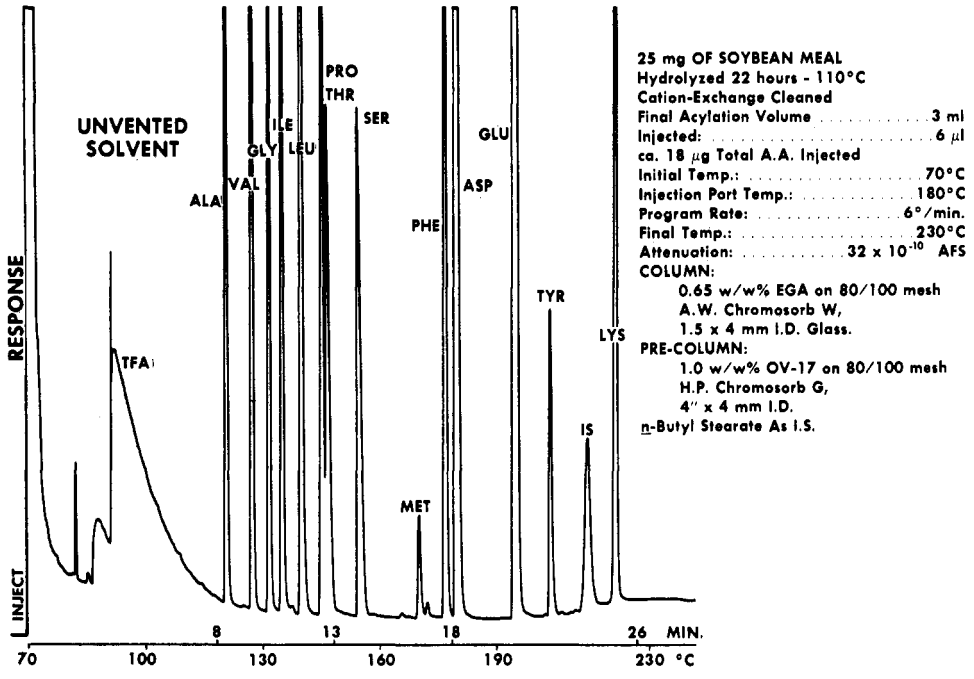


Figure 13. Glc analysis of soybean meal hydrolyzate

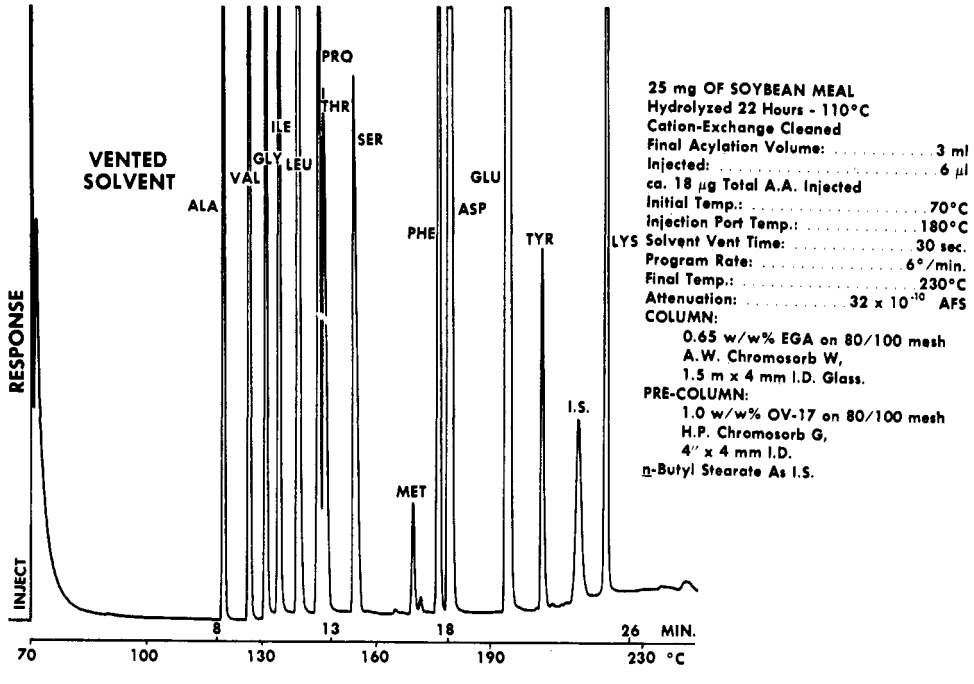


Figure 14. Glc analysis of soybean meal hydrolyzate

SUMMARY AND CONCLUSIONS

The quantitative amino acid analysis of biological substances by gas–liquid chromatography has been clearly demonstrated. Both the *N*-trifluoroacetyl *n*-butyl esters and the *N*-TMS–TMS derivatives of the 20 protein amino acids have been reproducibly formed and analyzed by glc.

The *N*-TFA *n*-butyl ester derivatives have been studied extensively, and quantitative glc analyses have been performed on a wide range of samples which include proteins, feed grains, blood plasma, urine, and others. Concurrently quantitative cation-exchange procedures developed for the removal of interfering substances have proven to be extremely useful for the analysis of materials such as soybean oil meal, corn grain, blood plasma, and urine. While the soybean meal, corn grain, and plasma samples were sufficiently free of interfering materials after cation-exchange cleanup, both cation and anion-exchange cleanup of urine was necessary prior to glc analysis. The developed anion-exchange technique resulted in the quantitative recovery of all the amino acids, with arginine the only exception.

The derivatization procedure has also been refined, in that the interesterification step has been eliminated, allowing the direct conversion of the amino acids to their *n*-butyl esters in 15 to 35 min. Use of the direct esterification method

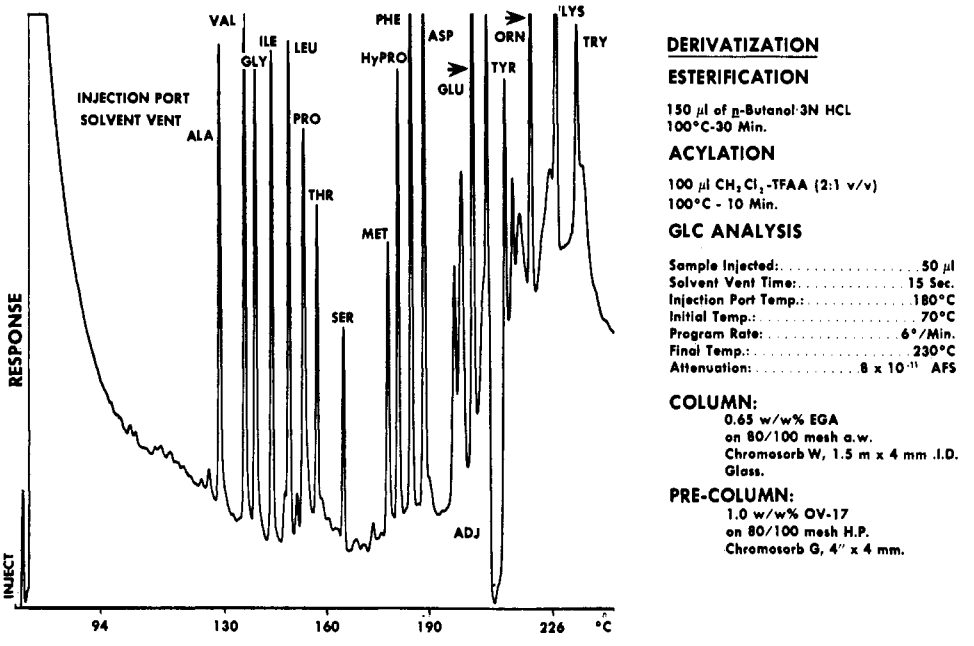


Figure 15. Derivatization and glc analysis of 100 ng of each amino acid

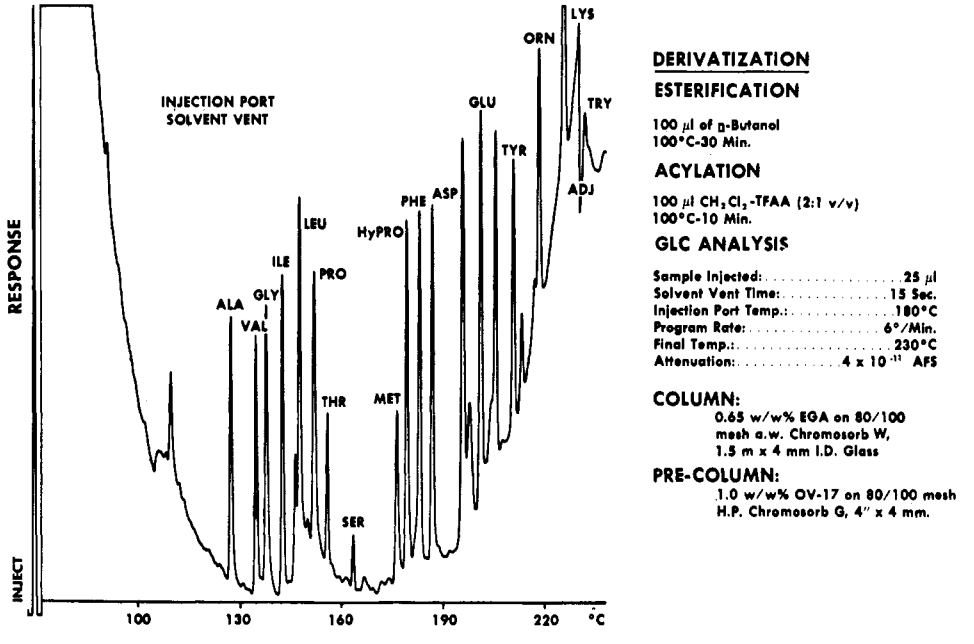


Figure 16. Derivatization and glc analysis of 50 ng of each amino acid

resulted in recoveries from mixtures of above 99% with an average relative standard deviation of less than 1%, using a reference calibration mixture. This direct esterification method allows the precise, accurate, and rapid derivatization of the protein amino acids to their *N*-TFA *n*-butyl esters with a minimum of transfers and sample handling. To further reduce the time required for the amino acid analysis of proteins, investigations were conducted to develop a rapid protein hydrolysis procedure by studying the effect of temperature on the hydrolysis of proteins in aqueous 6 *N* HCl. Essentially equivalent hydrolysis of ribonuclease was attained at 145° C ± 2° C for 4 hr as for the widely used 110° C ± 2° C for 26 hr. The results were verified with bovine serum albumin, and a standard amino acid mixture was subjected to these conditions to substantiate the fact that essentially no difference in amino acid decomposition was observed at 145° C, 4-hr and 110° C, 26-hr. With this method a protein can be completely hydrolyzed in 4 hr with a minimum of decomposition of the amino acids.

Further sophistication of the glc technique has made possible the analysis of nanogram amounts of amino acids. Studies have previously shown that quantitative data can be obtained from blood plasma containing *ca.* 200 µg of total amino acids. These studies have shown that 5 ng or less of amino acids can be taken through the derivatization procedure and analyzed by glc. The invention of an instrumental chromatography device system that allows injection of 100 µl or more of derivatized sample has greatly simplified the glc analysis of nanogram amounts of amino acids. This system should be of considerable value for glc analysis at the

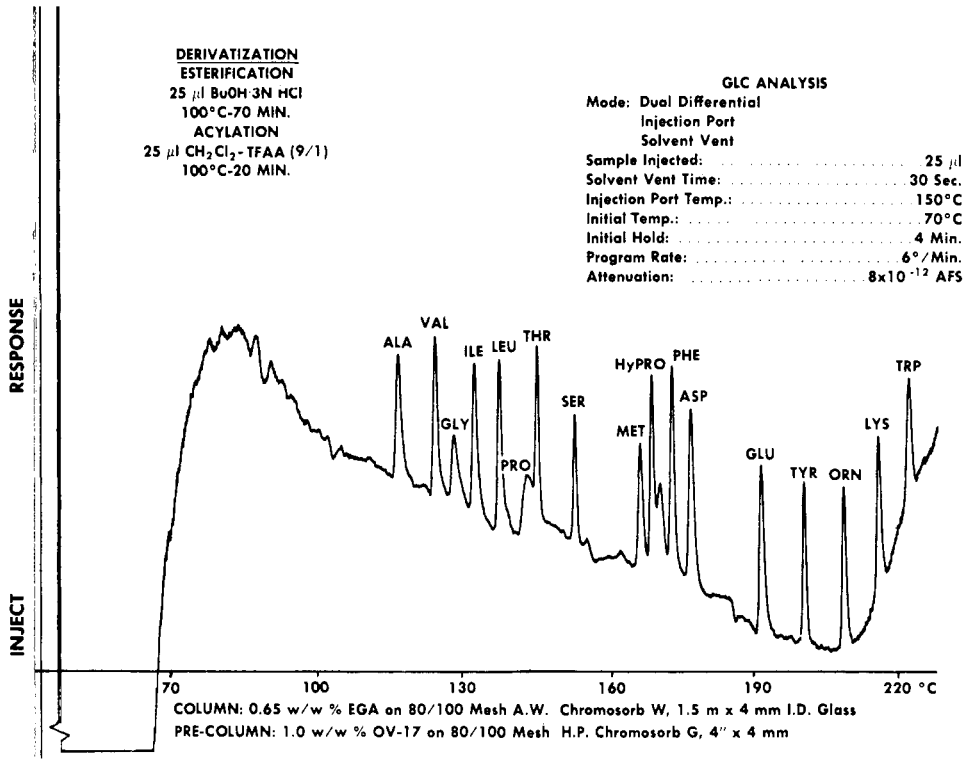
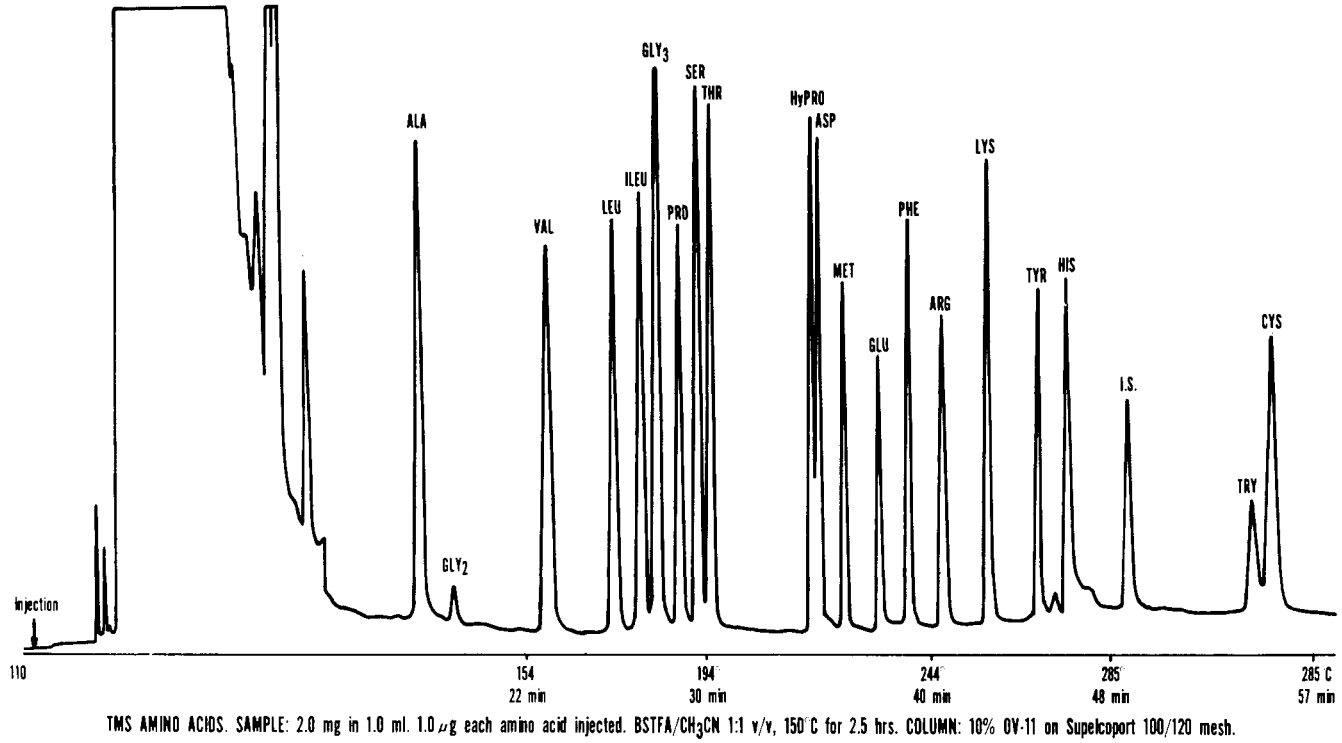


Figure 17. Derivatization and glc analysis of 5 ng of each amino acid



TMS AMINO ACIDS. SAMPLE: 2.0 mg in 1.0 ml. 1.0 µg each amino acid injected. BSTFA/CH_3CN 1:1 v/v, 150°C for 2.5 hrs. COLUMN: 10% OV-11 on Supelcoport 100/120 mesh. 6m x 2mm I.D. CONDITIONS: Injector 275 C, Detector 300 C, Initial Temperature 110°C, 2°C/min for 22 min, 5°C/min to 285°C, Carrier Gas N_2 20ml/min. I.S. Phenanthrene

Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) obtained from Regis Chemical Company, Chicago, Illinois.

Figure 18. Chromatogram of TMS protein amino acids

microgram and submicrogram levels, and for gc–ms studies.

Analyses conducted on water extracts of the Apollo's 11 and 12 Lunar fines resulted in none of the protein amino acids being observed at levels greater than 4 ppb of each.

The primary interest in the trimethylsilyl derivatives of amino acids has resulted from the fact that the derivatization could be accomplished in a one-step procedure, whereas other derivatives are formed by two or more reaction steps. Our investigations have resulted in achieving a single set of reaction conditions which reproducibly form the TMS derivatives of the 20 protein amino acids, (150° C, 2.5-hr) and subsequent separation and quantitative analysis of these derivatives by glc (10% OV-11 on 100/120 mesh Supelcoport, both 2 m and 6 m × 2 mm i.d. glass). Both the *N*-TFA *n*-butyl ester and

trimethylsilyl derivatives of the amino acids possess certain inherent advantages at this point in regard to the derivatization procedure and glc analysis. More information is available on the *N*-TFA *n*-butyl esters than for the TMS derivatives, due to the fact that the *N*-TFA *n*-butyl ester derivatives have been studied intensively for a longer period of time. However, the TMS derivatives are now emerging as a complementary procedure which should be used widely.

Reference is made to our latest manuscript on chromatographic columns and separations for the glc of amino acids as the *N*-trifluoroacetyl *n*-butyl ester derivatives (Gehrke *et al.*, 1971). In particular, a mixed phase packing is presented which separates his, asp, and phe.

In summary, gas–liquid chromatography has proven to be a precise, sensitive, accurate, and rapid method for the analysis of amino acids, and offers a versatile analytical tool for biochemists, nutritionists, medical scientists, bacteriologists, and investigators in many other areas.

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