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APPLICATIONS OF A GAS–LIQUID CHROMATOGRAPHIC METHOD FOR AMINO ACID ANALYSIS

A SYSTEM FOR ANALYSIS OF NANOGRAM AMOUNTS*

ROBERT W. ZUMWALT**, KENNETH KUO*** AND CHARLES W. GEHRKE§
University of Missouri, Columbia, Mo. 65201 (U.S.A.)

(Received November 23rd, 1970)

SUMMARY

The quantitative gas–liquid chromatographic determination of the protein amino acid content of biological substances has been clearly demonstrated with analyses of corn grain, soybean oil meal, blood plasma, and human urine. The precision and accuracy of the gas–liquid chromatographic technique was found to be equal to that of classical ion-exchange chromatography, and superior in some instances.

Further, an instrumental-chromatographic system has been invented which allows the injection of 100 μl or more on a standard analytical column. This device eliminates the TFA peak during analysis of amino acids on the EGA column; results in a more stable baseline due to the decreased amount of solvent and reagents traversing the column; greatly simplifies analysis for nanogram amounts of amino acids; and should find a wide range of applications in many gas–liquid chromatographic and gas chromatography–mass spectrometry investigations. A superior mixed phase chromatographic column is reported for the separation of His, Lys, Arg, Trp and Cys.

INTRODUCTION

A recent manuscript published in this journal[1] described in detail a gas–liquid chromatographic (GLC) method for quantitative analysis of amino acids in complex biological substances. The complete sample preparation procedures were described, including removal of protein, cation- and anion-exchange cleanup, derivatization to the amino acid N-trifluoroacetyl *n*-butyl esters, GLC analysis, and comments on critical points in the method. Also, initial studies on semimicro and micro methods

* Contribution from Missouri Agricultural Experiment Station Journal Series No. 6120. Approved by the Director. Supported in part by grants from the National Aeronautics and Space Administration (NGR 26-004-011-S5), the National Science Foundation (GB 7182), and the Experiment Station Chemical Laboratories.

** National Science Foundation Predoctoral Fellow, University of Missouri-Columbia.
*** Chemist, Experiment Station Chemical Laboratories, University of Missouri-Columbia.
§ Professor and Supervisor of the Experiment Station Chemical Laboratories, University of Missouri-Columbia.

for the derivatization and GLC analysis of amino acids were reported. A general review of this subject has also been presented recently by GEHRKE *et al.*[2].

This paper presents results obtained in two areas: (a) the application of the total analytical procedure described previously[1], demonstrating the analytical potential of the complete method, and (b) the development of a GLC instrumental system that greatly simplifies the analysis for nanogram amounts of amino acids.

To demonstrate the quantitative GLC determination of the amino acid content of corn grain and soybean oil meal, hydrolysates of each were analyzed by both GLC and classical ion-exchange chromatography. The chromatograms of the corn grain and soybean meal, obtained with simultaneous operation of the EGA and OV-17 columns, are presented in Figs. 1 and 2. The data resulting from these analyses are presented in Tables I and II, and the GLC and ion-exchange methods are seen to be in close agreement.

Fig. 3 presents the chromatogram obtained from the analysis of free amino acids in blood plasma. Again, good agreement of the GLC and ion-exchange techniques was achieved, as seen in Table III.

The corn grain, soybean meal, and blood plasma samples were amenable to GLC analysis after cation-exchange cleanup, however, both cation and anion-exchange cleanup were necessary prior to the analysis of human urine. An extensive

TABLE I

AMINO ACID ANALYSIS OF CORN GRAIN

Hydrolyzed for 22 h at 110° in a closed tube with 6 *N* HCl under N_2, cation-exchange cleaned. All percentages are given in w/w%.

Amino acid	*Gas–liquid chromatography*[a]		*Average*	*Ion-exchange chromatography*[b]		*Average*
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[c]	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[d]	—	—	—	—	—	—
Total	8.070	7.980	8.025	8.279	7.748	8.014

[a] Two independent analyses, *n*-butyl stearate as internal standard.
[b] Two independent analyses, norleucine as internal standard.
[c] Partially destroyed during 6 *N* HCl hydrolysis.
[d] Destroyed during 6 *N* HCl hydrolysis.

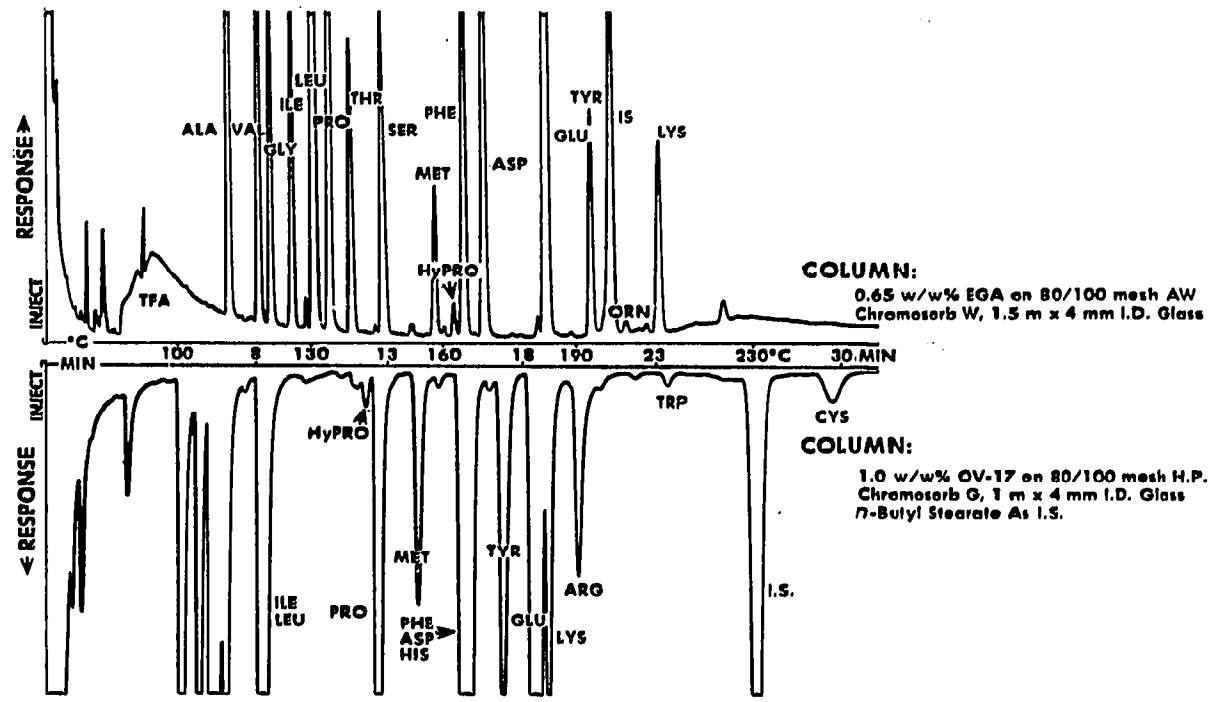

Fig. 1. GLC analysis of corn grain hydrolysate. 50 mg hydrolyzed with 6 *N* HCl, 110°, 22 h, cation-exchange cleaned. Final acylation volume, 2 ml; injected, 5 μl; *ca.* 5 μg total amino acids injected; attenuation, 8 × 10^{-10} A.F.S.; initial temperature, 70°; program rate, 6°/min; final temperature, 230°.

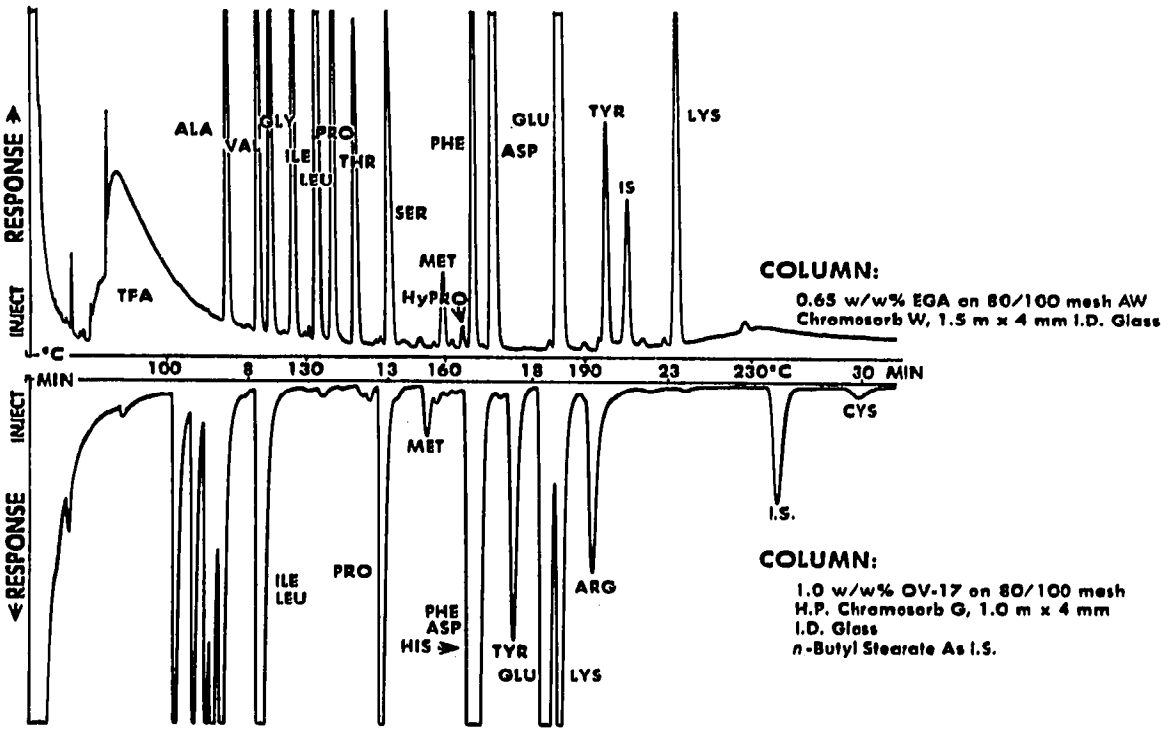

Fig. 2. GLC analysis of soybean meal hydrolysate. 25 mg hydrolyzed with 6 *N* HCl, 110°, 22 h, cation-exchange cleaned. Final acylation volume, 2 ml; injected, 5 μl; *ca.* 5 μg total amino acids injected; attenuation, 8 × 10^{-10} A.F.S.; initial temperature, 70°, program rate, 6°/min; final temperature, 230°.

study of the GLC analysis of urine was presented earlier[1], but since that study, improved chromatography now results in typical GLC analyses as seen in Fig. 4. The data obtained from the GLC and ion-exchange chromatography may vary somewhat, mainly due to the presence of small amounts of extraneous substances, with the level of these substances being dependent on the composition of the original urine sample. These interfering substances are eluted at different relative positions by GLC and ion-exchange chromatography, and thus are responsible for data variation as these substances interfere with different amino acids by GLC and ion-exchange chromatography. The differences in the data obtained by these techniques are generally small[1], but can be significant depending on the particular sample.

TABLE II

AMINO ACID ANALYSIS OF SOYBEAN MEAL

Hydrolyzed for 22 h at 110° in a closed tube with 6 *N* HCl under N_2, cation-exchange cleaned. All percentages are given in w/w%.

Amino acid	*Gas–liquid chromatography*[a]		*Average*	*Ion-exchange chromatography*[b]		*Average*
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[c]	—	—	—	—	—	—
Cystine	0.264	0.264	0.264	0.232	0.295	0.264
Histidine	1.639	1.515	1.577	1.440	1.536	1.448
Total	45.730	44.343	45.037	44.564	44.816	44.690

[a] Two independent hydrolysates, *n*-butyl stearate as internal standard.
[b] Two independent analyses, norleucine as internal standard.
[c] Destroyed during 6 *N* HCl hydrolysis.

Table IV presents the data obtained on the analysis of a urine sample containing a relatively large amount of interfering material. The ion-exchange data were obtained from a single column, 10 h analyses, with the GLC analyses requiring *ca.* 45 min to complete. As noted in Table IV, the GLC and ion-exchange results for isoleucine, threonine, serine, methionine, ornithine, lysine, and histidine were somewhat divergent. Therefore, a careful study was made of the chromatograms to determine if one technique was generally more susceptible to interferences than the other. By the classical ion-exchange technique, definite interferences were noted for isoleucine,

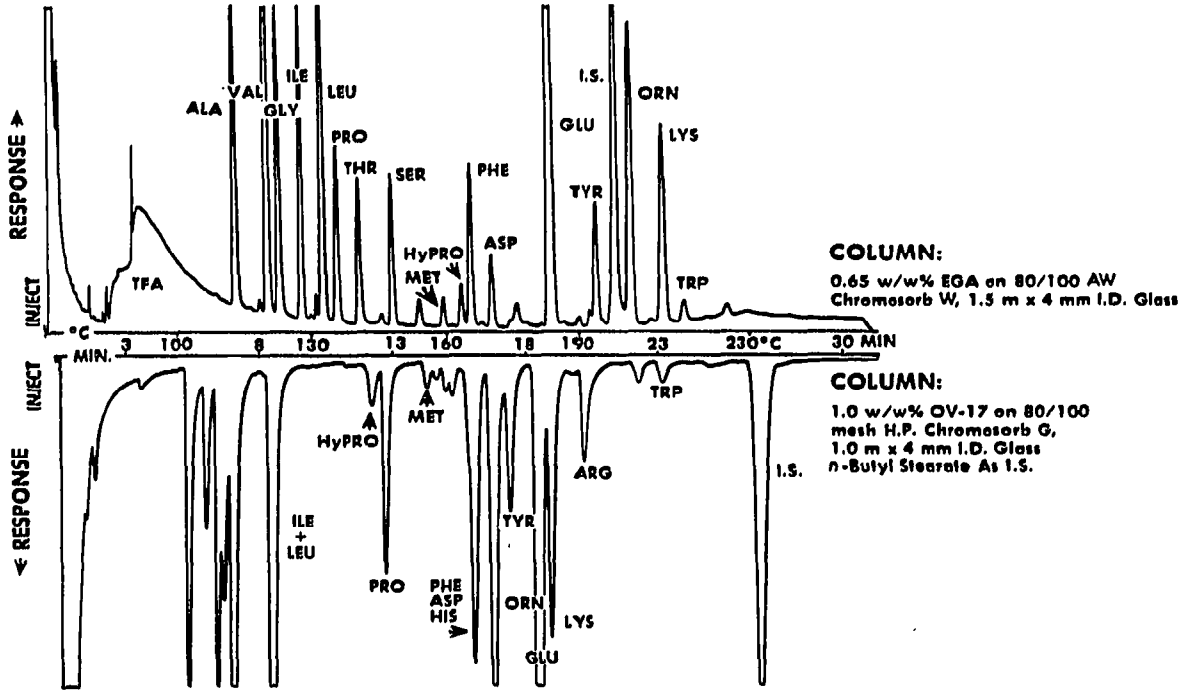

Fig. 3. GLC analysis of bovine blood plasma. 10.0 ml of plasma deproteinized with 40 ml of 1% picric acid, cation-exchange cleaned. Final acylation volume, 2 ml; injected, 5 μl; *ca.* 5 μg total amino acid injected; attenuation, 8×10^{-10} A.F.S.; initial temperature, 70°; program rate, 6°/min; final temperature 230°.

TABLE III

AMINO ACID ANALYSIS OF BOVINE BLOOD PLASMA

Blood plasma was cleaned by cation-exchange.

Amino acid	*mg/100 ml of plasma*					
	Gas–liquid chromatography[a]		*Average*	*Ion-exchange chromatography*		*Average*
Alanine	1.51	1.45	1.48	1.49	1.53	1.52
Valine	2.66	2.72	2.69	2.67	2.79	2.73
Glycine	1.38	1.44	1.42	1.41	1.53	1.47
Isoleucine	1.29	1.31	1.27	1.21	1.20	1.21
Leucine	1.81	1.83	1.82	1.79	1.81	1.80
Proline	0.91	0.94	0.93	0.91	0.95	0.93
Threonine	0.71	0.73	0.72	0.75	0.73	0.72
Serine	0.80	0.81	0.80	0.80	0.81	0.80
Methionine	0.22	0.22	0.22	0.24	0.22	0.23
Hydroxyproline	0.22	0.23	0.23	0.26	0.27	0.27
Phenylalanine	0.71	0.74	0.73	0.70	0.71	0.70
Aspartic acid[b]	0.32	0.32	0.32	0.10	0.10	0.10
Glutamic acid[b]	4.07	4.21	4.14	2.37	2.42	2.40
Tyrosine	0.63	0.61	0.62	0.59	0.60	0.59
Ornithine	1.04	1.10	1.07	1.08	1.09	1.09
Lysine	1.43	1.45	1.44	1.23	1.30	1.27
Arginine	1.38	1.40	1.39	1.44	1.47	1.46
Tryptophan	0.29	0.27	0.28	0.23	0.24	0.23
Cystine	trace	trace	trace	trace	trace	trace
Histidine	0.74	0.76	0.75	0.78	0.79	0.79
Total	22.13	22.53	22.33	20.01	20.56	20.29

[a] N-TFA *n*-butyl esters.

[b] GLC values include $AspNH_2$ and $GluNH_2$.

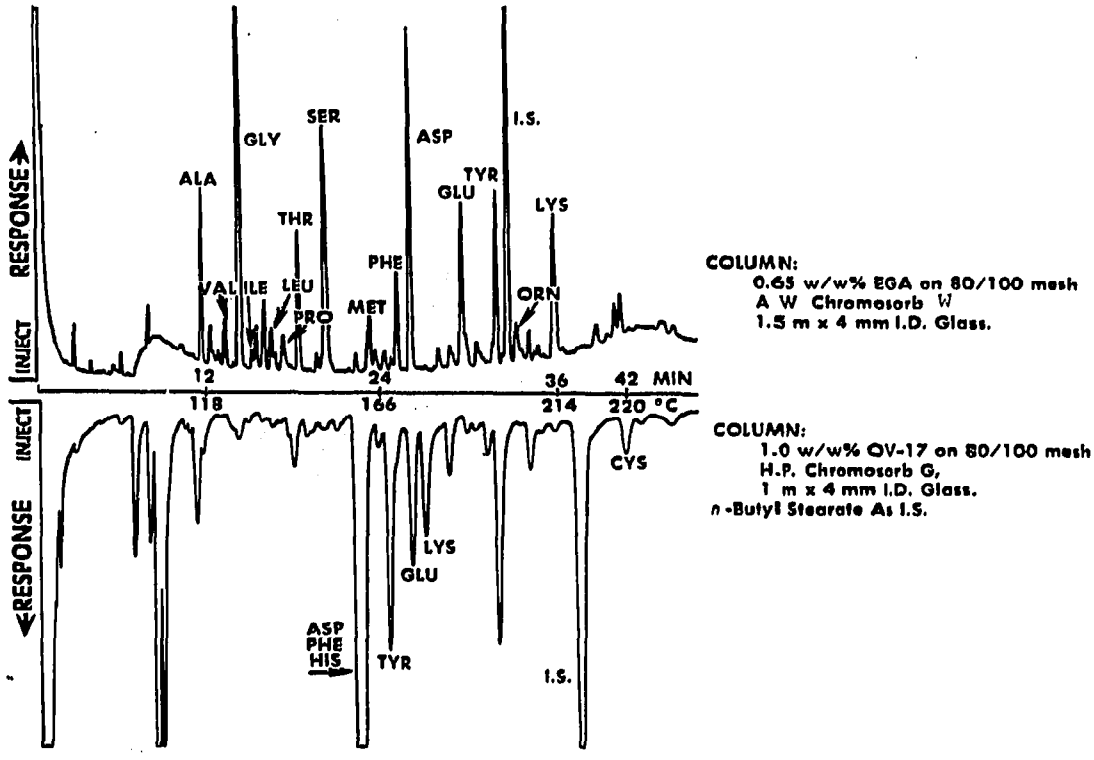

Fig. 4. GLC analysis of human urine. 4.5 ml of human urine hydrolyzed with 1 *N* HCl 110°, 2 h, final acylation volume, 2 ml; injected, 5 μl; *ca.* 2 μg of total amino acids injected; initial temperature, 70°; program rate, 4°/min; final temperature, 220°; attenuation, 8 × 10^{-10} A.F.S.

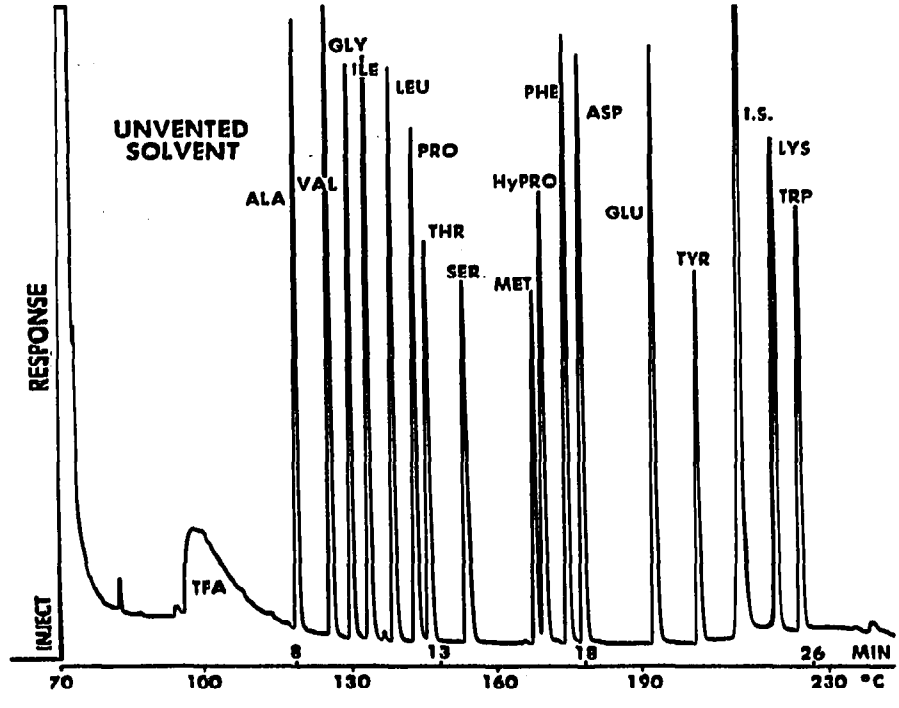

Fig. 5. GLC analysis of standard amino acid mixture. Standard mixture, 200 μg each amino acid; final acylation volume, 3 ml; injected, 15 μl; *ca.* 1.0 μg of each amino acid injected; initial temperature, 70°; injection port temperature, 180°; program rate, 6°/min; final temperature, 230°; attenuation, 32 × 10^{-10} A.F.S. Column, 0.65 w/w% EGA on 80/100 mesh AW Chromosorb W, 1.5 m × 4 mm I.D. glass. Pre-column, 1.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 4 in. × 4 mm I.D. *n*-butyl stearate as I.S.

TABLE IV

AMINO ACID ANALYSIS OF HUMAN URINE

Cleaned by cation- and anion-exchange, and hydrolyzed with 1 *N* HCl, for 2 h at 110°.

Amino acid	*mg/100 ml of urine*					
	Gas–liquid chromatography[a]		*Average*	*Ion-exchange chromatography*		*Average*
Alanine	1.512	1.458	1.485	1.480	1.460	1.470
Valine	0.313	0.300	0.307	0.360	0.280	0.320
Glycine	5.968	5.900	5.934	—	5.160	5.160
Isoleucine	0.121	0.115	0.118	0.180[e]	0.160[e]	0.170[e]
Leucine	0.297	0.278	0.288	0.360	0.240	0.300
Proline	0.390	0.439	0.415	—	0.540[g]	0.540[g]
Threonine	1.484	1.473	1.479	0.920[d]	1.340[d]	1.130[d]
Serine	3.641	3.538	3.590	3.360[e]	3.240[e]	3.300[e]
Methionine	0.895	0.911	0.903	—[e]	——[e]	[e]
Phenylalanine	0.824	0.865	0.846	0.900	0.800	0.850
Aspartic acid	3.025[f]	2.948[f]	2.987[f]	2.740	2.140	2.440
Glutamic acid	1.841[f]	1.568[f]	1.705[f]	1.180	1.080	1.130
Tyrosine	1.880	1.763	1.822	1.860	1.700	1.780
Ornithine	0.289[d]	0.291[d]	0.290[d]	0.240	0.240	0.240
Lysine	2.284[e]	2.308[e]	2.296[e]	1.080	0.920	1.000
Arginine[b]	—	—		—	—	
Tryptophan[c]	—	—		—	—	
Cystine	1.015	0.903	0.959	1.280	0.880	1.080
Histidine	3.252	2.736	2.994	4.340	5.000	4.670

[a] N-TFA *n*-butyl esters.
[b] Not recovered from anion-exchange cleanup.
[c] Destroyed during hydrolysis.
[d] Not completely resolved.
[e] Intefering peak(s).
[f] Includes $AspNH_2$ and $GluNH_2$.
[g] Manual calculation, 440 nm.

threonine, serine, and methionine, with methionine being incalculable due to the presence of two unidentified interfering peaks. The GLC analyses gave interferences at the elution positions of ornithine and lysine, with a minor interference of isoleucine. Also, a minor interference of leucine was noted with both GLC and classical ion-exchange chromatography; however, the disparity of the histidine values could not be explained. Under these analytical conditions, it was observed that GLC resulted in a better resolution of the amino acids from interfering substances than did ion-exchange chromatography.

The amount of interference observed with the two analytical methods is dependent on the level and nature of the extraneous substances, with GLC and ion-exchange chromatography exhibiting comparable levels of interferences, although a pre-analysis cation and anion-exchange cleanup of the urine sample was made.

EXPERIMENTAL

A solvent–chromatographic venting system—nanogram analysis

In 1968, GEHRKE *et al.*[3] published a monograph describing in detail a derivatization method for submicrogram amounts of amino acids which included a discussion

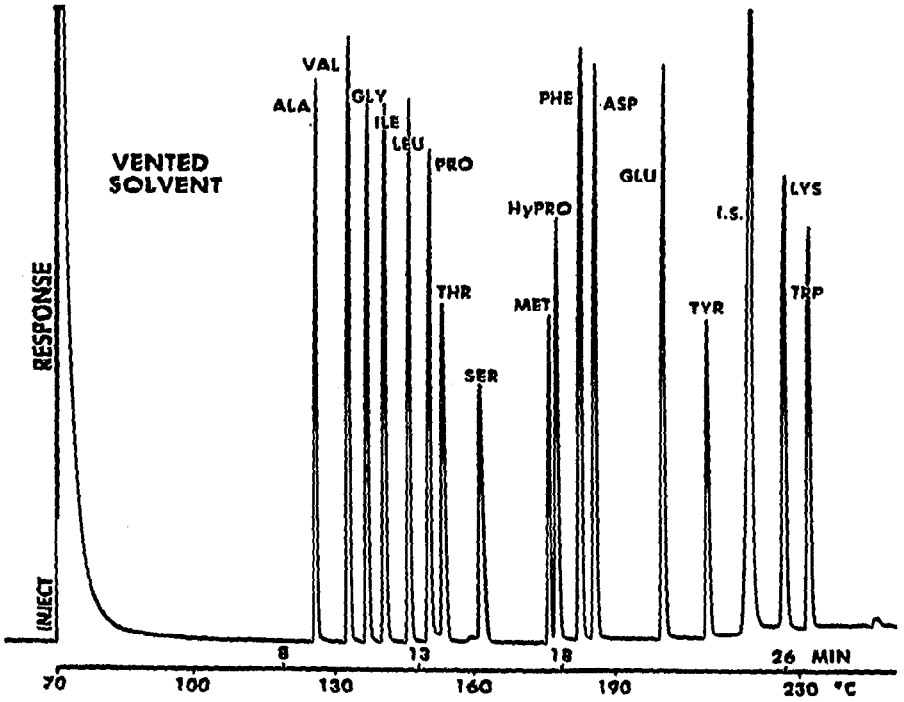

Fig. 6. GLC analysis of standard amino acid mixture. Standard mixture, 200 μg each amino acid, final acylation volume, 3 ml; injected, 15 μl; *ca.* 1.0 μg of each amino acid injected; initial temperature, 70°; injection port temperature, 180°; solvent vent time, 15 sec; program rate, 6°/min; final temperature, 230°; attenuation, 32 × 10^{-10} A.F.S. Column, 0.65 w/w% EGA on 80/100 mesh AW Chromosorb W, 2.5 m × 4 mm I.D. glass. Pre-column, 1.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 4 in. × 4 mm I.D. *n*-butyl stearate as I.S.

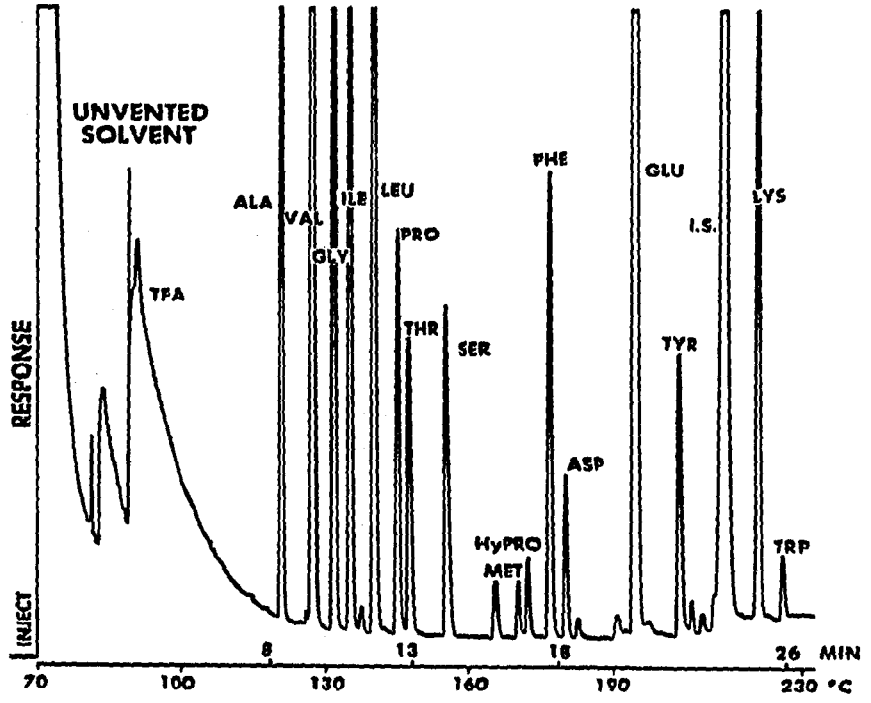

Fig. 7. GLC analysis of bovine blood plasma. 10 ml of plasma deproteinized with 40 ml of 1% picric acid; cation-exchange cleaned; final acylation volume, 2 ml; injected, 5 μl; *ca.* 5 μg total amino acid injected; initial temperature, 70°; injection port temperature, 180°; program rate, 6°/min.; final temperature, 230°; attenuation, 8 × 10^{-10} A.F.S. Column, 0.65 w/w% EGA on 80/100 mesh AW Chromosorb W, 1.5 m × 4 mm I.D. glass. Pre-column, 1.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 4 in. × 4 mm I.D. *n*-butyl stearate as I.S.

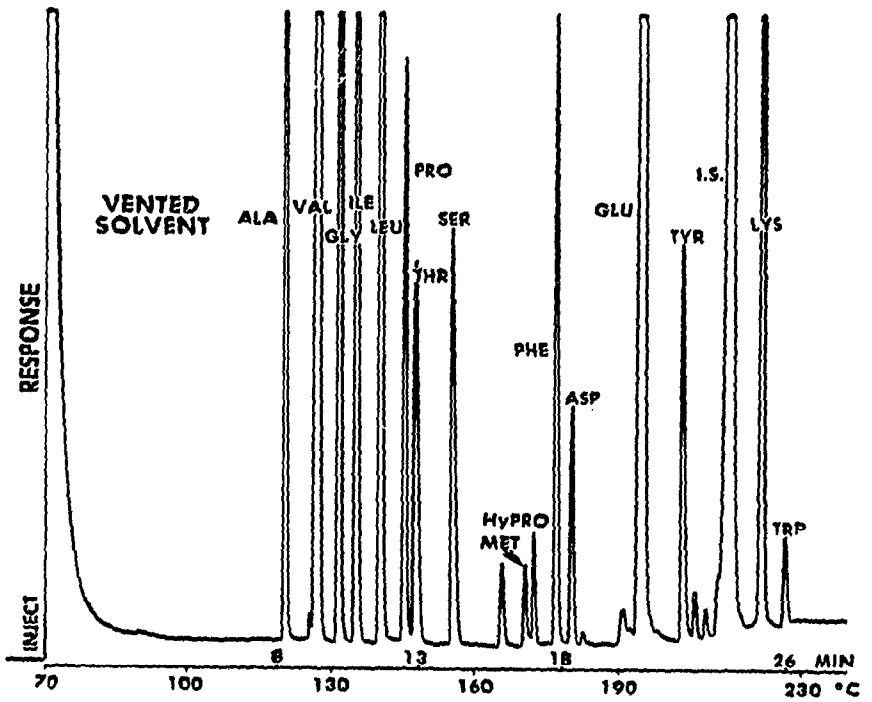

Fig. 8. GLC analysis of bovine blood plasma. 10 ml of plasma deproteinized with 40 ml of 1% picric acid; cation-exchange cleaned; final acylation volume, 2 ml; injected, 5 μl; *ca.* 5 μg total amino acid injected; initial temperature, 70°; injection port temperature, 180°; solvent vent time, 25 sec; program rate, 6°/min; final temperature, 230°; attenuation, 8×10^{-10} A.F.S. Column, 0.65 w/w% EGA on 80/100 mesh AW Chromosorb W, 1.5 m × 4 mm I.D. glass. Pre-column, 1.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 5 in. × 4 mm I.D. *n*-butyl stearate as I.S.

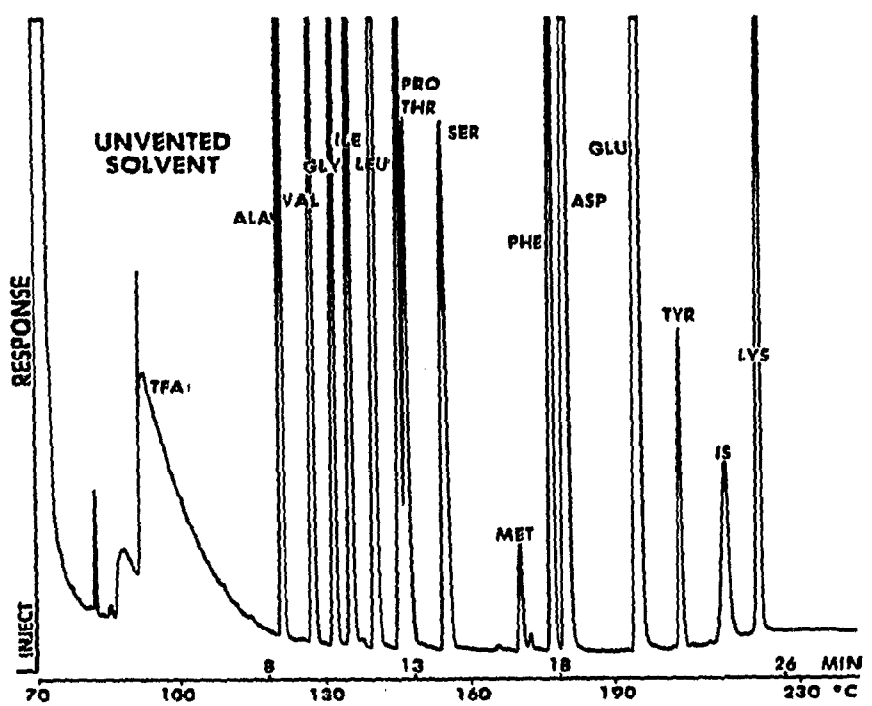

Fig. 9. GLC analysis of soybean meal hydrolysate. 25 mg of soybean meal, hydrolyzed 22 h, 110°, cation-exchange cleaned, final acylation volume, 3 ml; injected, 6 μl; *ca.* 18 μg total amino acid injected; initial temperature, 70°; injection port temperature, 180°; program rate, 6°/min; final temperature, 230°; attenuation, 32×10^{-10} A.F.S. Column, 0.65 w/w% EGA on 80/100 mesh AW Chromosorb W, 1.5m × 4 mm I.D. glass. Pre-column, 1.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 4 in. × 4 mm I.D. *n*-butyl stearate as I.S.

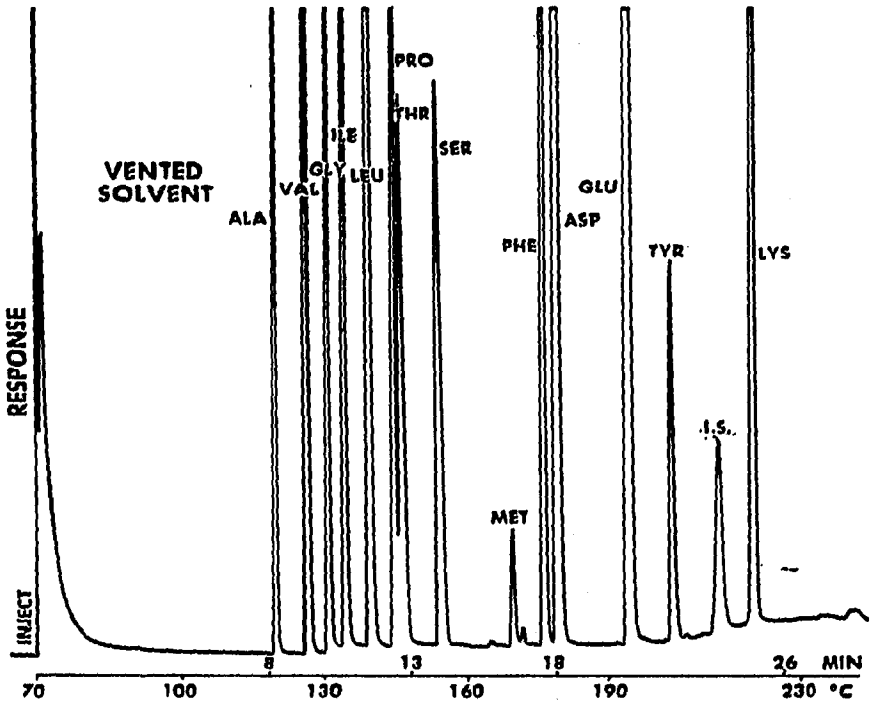

Fig. 10. GLC analysis of soybean meal hydrolysate. 25 mg of soybean meal, hydrolyzed 22 h, 110°, cation-exchange cleaned; final acylation volume, 3 ml; injected, 6 μl; *ca.* 18 μg total amino acid injected; initial temperature, 70°; injection port temperature, 180°; solvent vent time, 30 sec.; program rate, 6°/min.; final temperature, 230°; attenuation, 32 × 10^{-10} A.F.S. Column, 0.65 w/w% EGA on 80/100 mesh AW. Chromosorb W, 1.5 m × 4 mm I.D. glass. Pre-column, 1.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 4 in. × 4 mm I.D. *n*-butyl stearate as I.S.

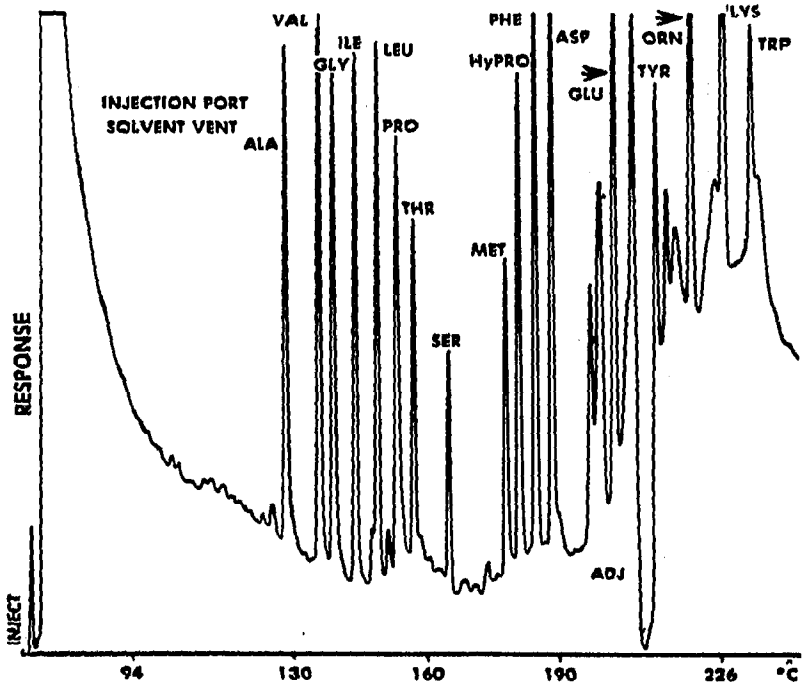

Fig. 11. Derivatization and GLC analysis of 100 ng of each amino acid. Derivatization; esterification, 150 μl of *n*-butanol with 3 *N* HCl, 100°, 30 min; acylation, 100 μl CH_2Cl_2–TFAA (2:1), 100°, 10 min; GLC analysis, sample injected, 50 μl; solvent vent time, 15 sec; injection port temperature, 180°; initial temperature, 70°; program rate, 6°/min; final temperature, 230°; attenuation, 8 × 10^{-11} A.F.S. Column, 0.65 w/w% EGA on 80/100 mesh AW Chromosorb W, 1.5 m × 4 mm I.D. glass. Pre-column, 1.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 4 in. × 4 mm.

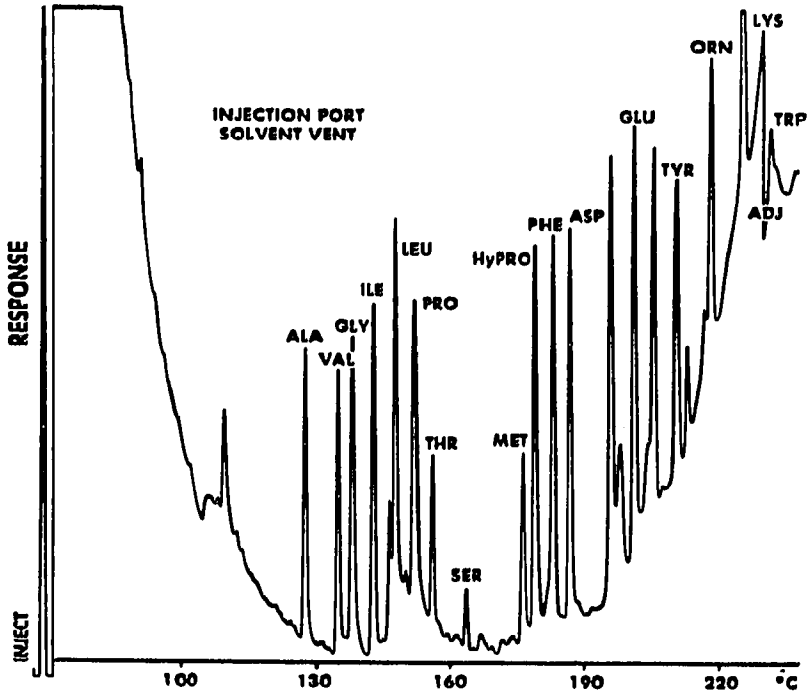

Fig. 12. Derivatization and GLC analysis of 50 ng of each amino acid. Derivatization: esterification, 100 μl of *n*-butanol with 3 *N* HCl, 100°, 30 min; acylation, 100 μl CH_2Cl_2–TFAA (2:1) 100°, 10 min; GLC analysis, sample injected, 25 μl; solvent vent time, 15 sec; injection port temperature, 180°; program rate 6°/min; final temperature, 230°; attenuation, 4×10^{-11} A.F.S. Column, 0.65 w/w% EGA on 80/100 mesh AW Chromosorb W, 1.5 m × 4 mm I.D. glass. Pre-column, 1.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 4 in. × 4 mm.

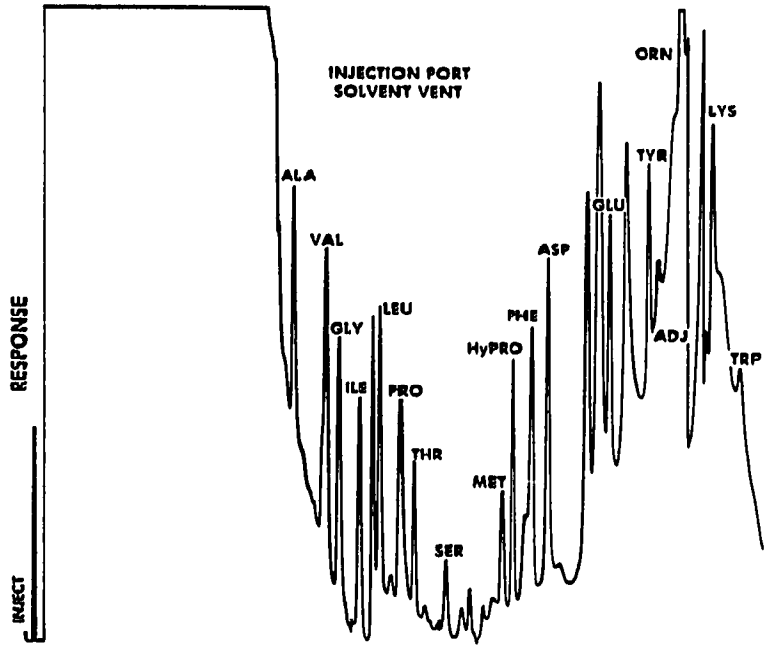

Fig. 13. Derivatization and GLC analysis of 10 ng of each amino acid. Derivatization: esterification, 50 μl *n*-butanol with 3 *N* HCl, 100°, 30 min; acylation, 50 μl CH_2Cl_2–TFAA (2:1), 100°, 10 min; GLC analysis, sample injected, 50 μl; solvent vent time, 15 sec.; injection port temperature, 180°, program rate, 6°/min; final temperature, 230°; attenuation, 32×10^{-12} A.F.S. Column, 0.65 w/w% EGA on 80/100 mesh AW Chromosorb W, 1.5 m × 4 mm I.D. glass. Pre-column, 1.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 4 in. × 4 mm.

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 (patent applied for) which allows injection of the total derivatized sample (50 to 100 μl) on a standard packed analytical column. The device prevents the large volume of solvent and acylating reagent injected 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 peak from the chromatogram. Fig. 5 presents a typical chromatogram obtained of a standard amino acid mixture at the macro level without the instrumental adaptation. Fig. 6 shows the effect of the venting device with the corresponding reduction of the solvent peak, and the absence of TFA throughout the chromatogram. Figs. 7 and 8 are similar comparisons with analysis of bovine blood plasma and soybean oil meal analyses are presented in Figs. 9 and 10.

Initial investigations of the system have shown great promise in the GLC analysis of nanogram amounts of amino acids. Fig. 11 presents the chromatogram obtained on derivatization and analysis of 100 ng of each amino acid. These initial studies were designed to evaluate the derivatization and chromatographic procedures, thus stringent precautions to exclude contaminants were not taken.

The derivatization and chromatography of samples containing 50 to 10 ng of each amino acid were also successfully conducted, with the chromatograms obtained presented in Figs. 12 and 13. Studies are currently underway to refine this technique further for the analysis of 1 ng of each amino acid taken through the complete chemistry and chromatographic methods.

CHROMATOGRAPHIC COLUMNS FOR ANALYSIS OF AMINO ACIDS AS N-TRIFLUOROACETYL *n*-BUTYL ESTER DERIVATIVES

Column I. Ethylene glycol adipate (EGA) on Chromosorb W, 0.65 w/w%

Materials

Column packing I* can be procured from Analytical Biochemistry Laboratories, P.O. Box 1097, Columbia, Mo., 65201 and Regis Chemical Company, 1101 N. Franklin Street, Chicago, Ill. 60610. Code No. 201033.

Ethylene glycol adipate, stabilized grade, (Analabs Inc., Hamden, Conn.).

Chromosorb W, 80/100 mesh, acid washed (Johns-Manville product, obtained from Applied Science, State College, Pa. or Fisher Scientific, St. Louis, Mo.).

Acetonitrile (anhydrous, "Nanograde") Mallinckrodt Chemical Works, St. Louis, Mo.

Adsorbent traps containing charcoal and molecular sieve 5A, Guild Corporation, P.O. Box 217, Bethel Park, Pa. 15102, Regis Chemical Company, and Supelco, Inc.

* The chromatographic packings described for columns I and II following, are the subject of a separate manuscript. Their use and application will be discussed in detail.

Procedure for column I

For preparation of 25 g of column packing I, 24.84 g of Chromosorb W are weighed into a 500 ml ridged round bottom flask, then anhydrous "Nanograde" acetonitrile is added until the liquid level is *ca.* ¼ in. above the Chromosorb W.

Ten milliliters of a solution containing 16.25 mg/ml of EGA in anhydrous "Nanograde" acetonitrile are then added to the flask containing the Chromosorb W. The flask is then rotated on a rotary evaporator, slowly removing the solvent at room temperature under partial vacuum for *ca.* 45 min. When the Chromosorb is still slightly damp, the vacuum is increased and the flask is immersed in a 60° water bath with continued rotation until the solvent is completely removed. At this point, no Chromosorb W packing should adhere to the inner wall of the flask during rotation.

At the end of this period, the dry, freely-flowing column packing is poured into clean, dry 1.5 m × 4 mm I.D. glass columns with gentle tapping. Dry silanized glass wool plugs are then placed in each end of the column to hold the packing in place. Prior to analytical use, the column is placed in the gas chromatograph and conditioned at 220° with a carrier flow of *ca.* 50 ml/min of pure N_2. Analyses can be made after conditioning for 1 h when 0.5 to 1 μg of each amino acid are injected. Longer conditioning times (8–24 h at 220°) are required for analyses at lower concentrations.

When not in use, the columns should be kept at 200° in the chromatograph with a carrier flow of 20–50 ml/min. If the columns must be removed from the instrument, the ends should be tightly closed during storage to exclude atmospheric moisture. The EGA columns must not be subjected at any one time to temperatures in excess of 225° for longer than 1 to 2 h.

Column II. Separation of His, Arg, Trp, and Cys (2.0 w/w% OV-17 + 1.0 w/w% OV-210 on Supelcoport)

Materials

Column packing II can be procured from Analytical BioChemistry Laboratories, P.O. Box 1097, Columbia, Mo. 65201.

OV-17 (Supelco, Inc., Bellefonte, Pa.) and OV-210 (Applied Science, State College, Pa.).

Supelcoport, 100/120 mesh (Supelco, Inc., Bellefonte, Pa.).

Prepare solutions containing: OV-17 in anhydrous "Nanograde" methylene chloride (20 mg/ml). OV-210 in "Nanograde" acetone (10 mg/ml). These solvents were obtained from Mallinckrodt Chemical Works, St. Louis, Mo.

Procedure for Column II

For preparation of 30 g of column packing II, 29.1 g of Supelcoport are weighed into a 500 ml ridged round bottom flask, then "Nanograde" acetone is added until the liquid level is *ca.* ¼ in. above the support material. Pipet 30.0 ml OV-17 solution (600 mg) and 30.0 ml of OV-210 solution (300 mg) into the flask. The solvent is removed in the manner described above for preparation of column I, and the dried column packing is placed in 1.5 m × 4 mm I.D. glass columns. The columns are then placed in the gas chromatograph, and conditioned overnight at 250° with a carrier flow of *ca.* 50 ml/min of pure N_2.

Comments

Column I, EGA on Chromosorb W, AW. Filters containing a high grade charcoal (an efficient adsorbent for hydrocarbons), and $CaSO_4$ and Linde 5A indicating molecular sieve for water should be placed in the N_2, H_2, and air lines to the gas chromatograph. The charcoal end of the filters is connected to the gas inlet side. The purity of the carrier gas is very important, especially when analyses are made at the submicrogram level.

Properly prepared columns should last *ca.* two months and give the desired separation for 17 amino acid derivatives, depending on the individual column and the types of samples injected. Signs of column degradation are: loss of the glycine–valine separation; loss of resolution in the methionine–hydroxyproline–phenylalanine region and loss of separation for the ornithine–*n*-butyl stearate pair. Also, the TFA peak will be eluted later in the chromatogram as the column deteriorates.

Column II, separation of basics and cystine. The OV-17, OV-210 mixed phase columns should last three to six months, depending on the types of samples analyzed. The first sign of column deterioration is usually a loss of quantitative elution of arginine and cystine indicated by a reduced $RWR_{a.a./I.S.}$

Note on chromatography and columns

In our previous publications[4,5], reference is made to the chromatographic separation of the amino acid derivatives. The packing composed of stabilized grade EGA and acid washed Chromosorb W (heated at 140° for 12 h) is an excellent one and gives effective separation of 17 amino acids. Column packing II, consisting of the mixed phase of OV-17 and OV-210 is a superior packing and shows highly efficient and effective separation of the basic amino acids plus cystine over that reported by us earlier. No longer is it necessary to make a computation for histidine as reported in ref. 6.

With these packings (columns I and II), as described above, one can now simultaneously analyze and separate all 20 of the protein amino acids on these two columns in 30 min with automatic electronic integration of all peaks.

In our recent studies on chromatographic separations of the amino acids, it was found that heating the Chromsorb W at 140° for 12 h was unnecessary. However, certain lots of Chromosorb W may still require a heat treatment for the removal of surface adsorbed water as described in ref. 5.

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