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THE GAS–LIQUID CHROMATOGRAPHY OF AMINO ACIDS*

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SUMMARY

An excellent separation of the N-TFA *n*-butyl esters of seventeen of the protein amino acids was achieved with acid washed Chromosorb W as the support material (80/100 mesh, dried at 140° for 12 h).

In these experiments, the liquid phase, stabilized EGA, was used at loadings of from 0.325 w/w % to 0.65 w/w % on both dried and non-dried acid washed Chromosorb W. The best separation was obtained with Chromosorb W dried for 12 h at 140°. Cysteine and methionine were not well resolved on any of the columns prepared with the non-dried acid washed Chromosorb W. A slightly poorer separation was obtained with the non-dried Chromosorb W, and there was a little skewing of the threonine and serine peaks on the 0.325 w/w % EGA columns. This was apparently due to an interaction of the threonine and serine derivatives with the water adsorbed on the surfaces of the Chromosorb W support material. At heavier loadings, of *ca.* 0.65 w/w %, these interactions were reduced, apparently due to the thicker layer of stationary phase.

Different mesh sizes of the dried support phase, 60/80, 80/100, and 100/120 were found to give quite similar results. Resolution improved only slightly with smaller particle size. Thus, mesh size in this range contributed only slightly to the elution properties of the column for the amino acid derivatives.

INTRODUCTION

In 1968, GEHRKE *et al.*[1] reported on a dual column EGA OV-17 chromatographic system for the quantitative determination of all twenty of the protein amino acids as their N-trifluoroacetyl (N-TFA) *n*-butyl esters. Earlier a general review by GEHRKE AND STALLING[2] reported the experimental conditions required for quantitatively derivatizing the protein amino acids as their N-TFA *n*-butyl esters. Further,

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a recent monograph by GEHRKE *et al.*[3] details macro, semimicro, and micro methods, reagents, sample preparation, instrumental and chromatographic requirements, and sample ion-exchange cleanup for the quantitative gas–liquid chromatographic (GLC) analysis of the amino acids in biological substances.

The dual column chromatographic system reported by GEHRKE *et al.*[1] uses stabilized grade ethylene glycol adipate (EGA) and OV-17 as the stationary phases. Sixteen of the protein amino acids were chromatographed on a 1.5 m glass U-shaped column containing 0.325 w/w % EGA coated on acid washed (A.W.) Chromosorb G, 80/100 mesh, which had been heat treated at 550° ± 50° for 15 h prior to coating with the EGA. It was found necessary to thermally condition the Chromosorb G to achieve satisfactory resolution of sixteen of the protein amino acids. For the analysis of arginine, histidine, tryptophan, and cystine, a 1 m column containing 1.5 w/w % OV-17 coated on high performance 80/100 mesh Chromosorb G was used.

The removal of water adsorbed on the surface of the Chromosorb G particles led to improved resolution, but the heating process had to be controlled carefully to prevent surface changes which affected the resolution of the derivatives. Heating the support material at too high a temperature resulted in poor separation of the amino acid derivatives. Also, the Chromosorb G, once heat treated, had to be stored under anhydrous conditions. Failure to rigorously control and maintain all of the correct thermal treatment parameters resulted in unsatisfactory resolution of the amino acids, in particular methionine/hydroxyproline and hydroxyproline/phenylalanine. In view of the number of laboratories which use this analytical method, and the considerable effort necessary to closely control the heat treatment, a simpler procedure was highly desirable. As an outcome of studies directed towards this goal, this paper reports on an evaluation of A.W. Chromosorb W of various mesh sizes as a support material for the separation of the N-TFA *n*-butyl esters of the amino acids. Also, various loadings of the liquid phase, EGA, were evaluated.

EXPERIMENTAL

Apparatus

Two gas chromatographs were used in this study. One, a Varian Aerograph Model 2100 gas chromatograph with a four column oven bath, was equipped with two dual flame ionization detectors, two dual channel electrometers, a linear temperature programmer, and a Varian Model 20 dual pen recorder. The second instrument, a Packard Instruments Co. Model 7300 dual column gas chromatograph, was equipped with hydrogen flame detectors and a Honeywell Electronik 16 strip chart recorder. A digital readout integrator (Infotronics, Model CRS 104) was used for determining peak areas.

Solvents were removed under partial vacuum using an all-Teflon rotary evaporator obtained from California Laboratory Equipment Company (Calif. Lab. Model C rotary evaporator). A Calab 'cold-finger' condenser containing dry ice in ethylene glycol monomethyl ether was placed between the evaporator and the vacuum pump to prevent volatile compounds from reaching the pump.

Reagents

All of the amino acids used in this study were obtained from Mann Research

Laboratories, Inc. or Nutritional Biochemicals Corporation, and were chromatographically pure.

Methanol and butanol were 'Baker Analyzed' reagents. The trifluoroacetic anhydride obtained from Distillation Products Industries was an 'Eastman Grade' chemical. Acetonitrile was a 'Baker Analyzed' reagent of 'Nanograde' purity. Anhydrous HCl, 99.0% minimum purity, was obtained from Matheson Company.

The methanol, butanol, and methylene chloride were redistilled from an all-glass system and protected from atmospheric moisture by storing in all-glass inverted top bottles. The methanol was first refluxed over magnesium turnings, and the methylene chloride and butanol over calcium chloride before distillation. The anhydrous HCl gas was passed through a H_2SO_4 drying tower before bubbling through the butanol or methanol.

Columns

The stabilized grade of EGA was obtained from Analabs, Inc., Hamden, Conn. The support materials, 60/80, 80/100, and 100/120 mesh A.W. Chromosorb W, were obtained from Supelco, Inc., Bellefonte, Pa. Columns were prepared using both the 60/80, and the 80/100 mesh A.W. Chromosorb W just as received from the bottle and after both had been dried in an oven for 12 h at 140°.

Column preparation

The column packing was prepared by weighing a small quantity, *ca.* 10 g, of the desired A.W. Chromosorb W into a fluted round bottom flask and covering with acetonitrile, the solvent used for dissolving the stationary phase. The EGA was then weighed into a 50 ml erlenmeyer flask, dissolved in acetonitrile, then added to the flask containing the Chromosorb W. The flask was placed in a 60° water bath while the acetonitrile was removed slowly with a rotary evaporator under partial vacuum. The chromatographic U-shaped glass (1.5 m × 4 mm I.D.) columns were prepared by adding the coated support slowly with gentle tapping. Dry glass wool plugs were placed in the ends of the column to prevent loss of the packing.

RESULTS AND DISCUSSION

GEHRKE *et al.*[1] reported an improved separation of the N-TFA *n*-butyl ester amino acid derivatives when the support material, Chromosorb G, was heated at 550° ± 50° for 15 h. These authors used a chromatographic column of 0.325 w/w % of stabilized grade EGA coated on 80/100 mesh, A.W., heat treated Chromosorb G for the separation of the protein amino acids, with the exception of arginine, tryptophan, histidine, and cystine. These four amino acids were chromatographed on a 1.5 w/w % OV-17 column which was prepared by coating the OV-17 on high performance, 80/100 mesh, Chromosorb G. This dual column system is illustrated in Fig. 1.

The thermal pretreatment of Chromosorb G gave an improved separation and reproducible elution of sixteen amino acid derivatives on the EGA column. This method of preparation of the chromatographic support has been used successfully at the University of Missouri for the past two years. However, a careful control of heating conditions was essential in order to avoid difficulties in the separation of cysteine

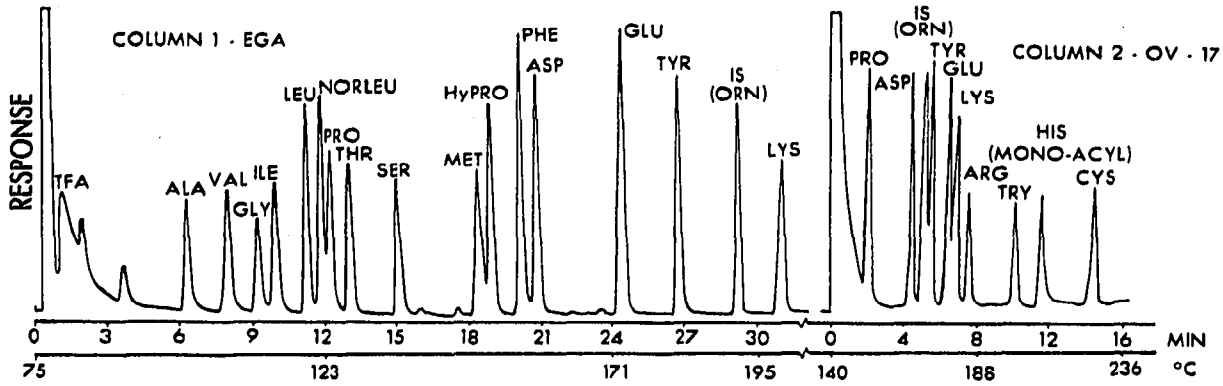
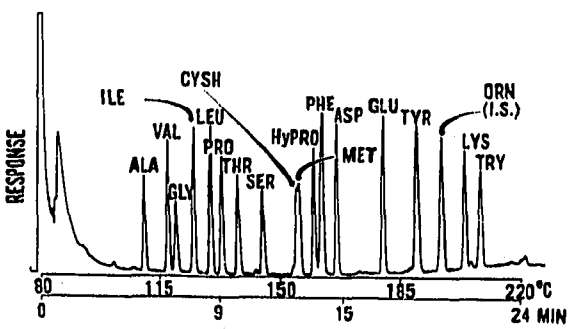


Fig. 1. GLC of equimolar standard amino acid mixture. Amino acid N-TFA *n*-butyl esters. Sample: 15 mg in 5.0 ml, 15 μg total amino acids injected (5 μl). Varian Aerograph 2100. EGA column: 0.325 w/w % on 80/100 mesh A.W. heat treated Chromosorb G, 1.5 m × 4 mm I.D. glass; initial temperature 75°, 4°/min. OV-17 column: 1.5 w/w % on 80/100 mesh, high performance Chromosorb G, 1.0 m × 4 mm I.D. glass; initial temperature 140°, 6°/min, 3 μl (9 μg total) of sample injected followed by 7 μl *n*-butanol.

from methionine, or in the separation of methionine from hydroxyproline; thus, the decision was reached to evaluate other support materials for EGA.

The first support material investigated was 80/100 mesh A.W. unheated Chromosorb G. Separation problems were noted with this support material. Then, unheated, 80/100 mesh, A.W. Chromosorb W was tried as the support material for the EGA substrate. Methionine was eluted with cysteine when this support material was used, as seen in Fig. 2. This chromatogram was obtained from a column that had been conditioned for 24 h. A slightly better separation was noted after conditioning for 48 h at 220° (Fig. 3).

The improved separation that was noted with longer conditioning prompted a study of drying time and temperature of Chromosorb W and its effect on resolution.



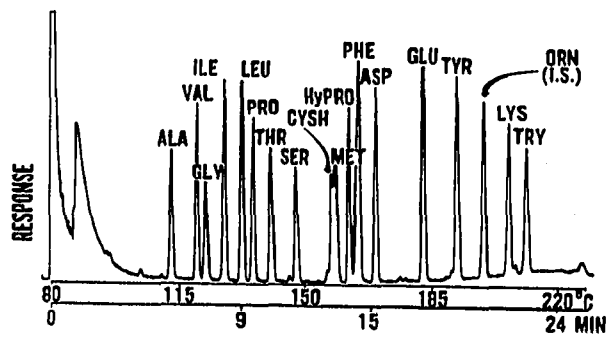


Fig. 2. GLC of equimolar standard amino acid solution with Chromosorb W, non-dried, EGA 0.65 w/w %. N-TFA *n*-butyl esters. Sample: 8 mg in 4 ml, 4 μg total amino acids injected (2 μl). Closed tube acylation, 150°, 5 min. TFAA/CH_2Cl_2, 1:1 v/v. Column: 0.65 w/w % EGA on 80/100 mesh A.W. Chromosorb W, 1.5 m × 4 mm I.D. glass, conditioned 24 h at 220°; initial temperature 80°, 6°/min.

Fig. 3. GLC of equimolar standard amino acid solution with Chromosorb W, non-dried, EGA 0.65 w/w %. N-TFA *n*-butyl esters. Sample: 8 mg in 4 ml, 4 μg total amino acids injected (2 μl). Closed tube acylation, 150°, 5 min. TFAA/CH_2Cl_2, 1:1 v/v. Column: 0.65 w/w % EGA on 80/100 mesh A.W. Chromosorb W, 1.5 m × 4 mm I.D. glass, conditioned 48 h at 220°; initial temperature 80°, 6°/min.

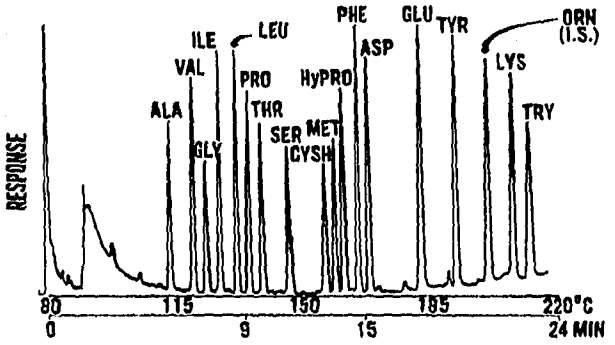


Fig. 4. GLC of equimolar standard amino acid solution with Chromosorb W, dried, EGA 0.65 w/w %. N-TFA *n*-butyl esters. Sample: 8 mg in 4 ml, 4 μg total amino acids injected (2 μl). Closed tube acylation, 150°, 5 min. TFAA/CH_2Cl_2, 1:1 v/v. Column: 0.65 w/w % EGA on 80/100 mesh dried (140°, 12 h) A.W. Chromosorb W, 1.5 × 4 mm I.D. glass; initial temperature 80°, 6°/min.

To remove primarily the water adsorbed on the surface, a temperature of 140° was selected. A low drying temperature was considered desirable, since drastic surface changes could occur at elevated temperatures, as previously noted with heat treated Chromosorb G. Heating A.W. Chromosorb W, 80/100 mesh, for 6 h resulted in a weight loss of 0.19 w/w %. This decrease in weight was assumed to be a loss of water adsorbed on the surface of the Chromosorb W particles. No appreciable further weight loss was observed when the Chromosorb W was heated at 140° for up to 72 h. Excellent resolution of seventeen of the protein amino acids was achieved with a 0.65 w/w % EGA column prepared by coating the stabilized EGA on dried A.W. Chromosorb W, 80/100 mesh, (140° for 12 h). Note that cysteine and methionine were well resolved in the chromatogram shown in Fig. 4.

Other A.W. Chromosorb W supports of different mesh size were then evaluated, including 60/80, 80/100, and 100/120 mesh. It was observed that mesh size contributed little to the separation. In another experiment, the effect of substrate loading on resolution was studied. Seventeen of the amino acids were well resolved on a 1.5 m × 4 mm I.D. U-shaped glass column consisting of 0.325 w/w % EGA on 60/80 mesh, dried (140° for 12 h), A.W. Chromosorb W (Fig. 5). Substrate loadings

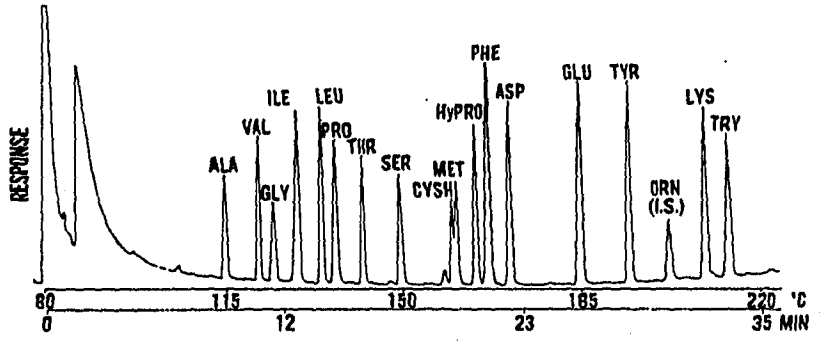
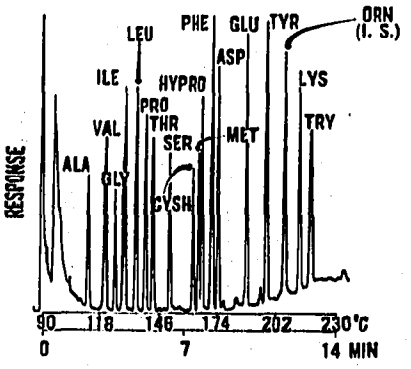


Fig. 5. GLC of equimolar standard amino acid solution with Chromosorb W, dried, EGA 0.325 w/w %. N-TFA *n*-butyl esters. Sample: 5 mg in 5 ml, 2 μg total amino acids injected (2 μl). Closed tube acylation, 150°, 5 min. TFAA/CH_2Cl_2, 1:1 v/v. Column: 0.325 w/w %EGA on 60/80 mesh dried (140°, 12 h) A.W. Chromosorb W, 1.5 m × 4 mm I.D. glass; initial temperature 80°, 4°/min.



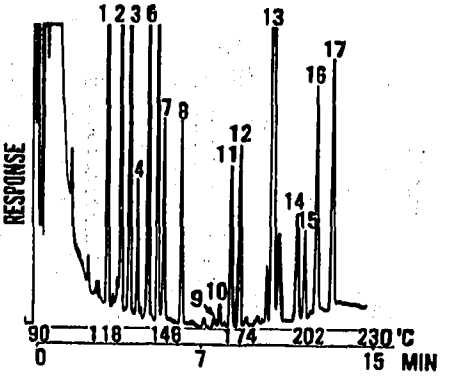


Fig. 6. GLC of equimolar standard amino acid solution with Chromosorb W, dried, EGA 0.65 w/w %. N-TFA *n*-butyl esters. Sample: 8 mg in 4 ml, 4 μg total amino acids injected (2 μl). Closed tube acylation, 150°, 5 min. TFAA/CH_2Cl_2, 1:1 v/v. Column: 0.65 w/w % EGA on 80/100 mesh dried (140°, 12 h) A.W. Chromosorb W, 1.5 m × 4 mm I.D. glass; initial temperature 90°, 10°/min.

Fig. 7. GLC of human blood plasma. N-TFA *n*-butyl esters. Sample: 3 mg in 6 ml, 2 μg total amino acids injected (4 μl). Closed tube acylation, 150°, 5 min. TFAA/CH_2Cl_2, 1:1 v/v. Column: 0.65 w/w % EGA on 80/100 mesh dried (140°, 12 h) A.W. Chromosorb W, 1.5 m × 4 mm I.D. glass; initial temperature 90°, 10°/min. Sample cleaned by cation-exchange. *n*-butyl stearate as I.S. 1, ALA; 2, VAL; 3, GLY; 4, ILE; 5, LEU; 6, PRO; 7, THR; 8, SER; 9, MET; 10, HyPRO; 11, PHE; 12, ASP; 13, GLU; 14, TYR; 15, I.S.; 16, ORN; 17, LYS.

TABLE I

RELATIVE MOLAR RESPONSE OF N-TFA *n*-BUTYL ESTERS OF AMINO ACIDS[a]

Amino acid	*$RMR_{a.a./glu.}$*							
	Chromosorb W, dried[b]						*Chrom W*[c] *non-dried*	*Chrom G*[d] *heat treated*
	1	*2*	*3*	*4*	*Av.*	*R.S.D. (%)*	*Av.*	*Av.*
Alanine	0.533	0.529	0.534	0.542	0.534	1.03	0.51	0.50
Valine	0.746	0.747	0.736	0.735	0.741	0.86	0.68	0.66
Glycine	0.428	0.438	0.437	0.430	0.433	1.15	0.40	0.39
Isoleucine	0.848	0.830	0.859	0.853	0.848	1.48	0.79	0.75
Leucine	0.821	0.830	0.828	0.820	0.825	0.57	0.80	0.77
Proline	0.684	0.698	0.691	0.697	0.692	0.67	0.70	0.68
Threonine	0.639	0.647	0.643	0.650	0.645	0.72	0.62	0.62
Serine	0.554	0.544	0.533	0.536	0.542	1.73	0.55	0.58
Methionine	0.556	0.554	0.579	0.587	0.569	2.11	0.55	0.63
Cysteine	0.407	0.402	0.426	0.418	0.413	2.62	0.38	0.40
Hydroxyproline	0.731	0.727	0.736	0.739	0.733	0.59	0.75	0.79
Phenylalanine	1.15	1.11	1.12	1.14	1.13	0.15	1.14	1.09
Aspartic acid	0.877	0.879	0.885	0.884	0.881	0.42	0.89	0.87
Glutamic acid	1.00	1.00	1.00	1.00	1.00	—	1.00	1.00
Tyrosine	0.930	0.917	0.894	0.901	0.910	1.72	0.93	0.95
Lysine	0.875	0.882	0.896	0.893	0.886	1.10	0.85	0.85
Tryptophan	0.772	0.757	0.714	0.726	0.742	3.21	0.76	—

[a] Relative molar response of glutamic acid assigned a value of unity.

[b] Each value represents an independent determination on 0.65 w/w % EGA coated on dried (140° for 12 h) Chromosorb W.

[c] Each value represents an average of two independent determinations on 0.65 w/w % EGA coated on non-dried Chromosorb W.

[d] Each value represents an average of three independent determinations on heat treated (550° ± 50° for 15 h) Chromosorb G.

of from 0.325 to 0.65 w/w % of stabilized EGA on both dried and non-dried A.W. Chromosorb W were then tested. The use of lighter loadings on non-dried support materials resulted in skewing of the threonine and serine peaks. As expected, the separations achieved with the lighter loadings were slightly poorer than those with a substrate loading of 0.65 w/w % EGA.

Excellent chromatographic separations and improved peak shapes were obtained when A.W. Chromosorb W which had been dried at 140° for 12 h was used as the support phase (Figs. 4–7). These improvements have resulted in very rapid analyses of the amino acids by GLC. The chromatograms shown in Figs. 6 and 7 required less than 14 min for completion.

A comparison of the relative molar response values obtained using dried and non-dried A.W. Chromosorb W was made with the corresponding data obtained using A.W. heat treated Chromosorb G. These experimental results are given in Table I.

The relative molar response (RMR) of glutamic acid was arbitrarily assigned a value of unity. The RMR of each amino acid (a.a.) relative to glutamic acid (glu.) $RMR_{a.a./glu.}$, was calculated as follows:

$$RMR_{a.a./glu.} = \frac{\dfrac{A_{a.a.}}{\dfrac{g_{a.a.}}{M.W._{a.a.}}}}{\dfrac{A_{glu.}}{\dfrac{g_{glu.}}{M.W._{glu.}}}}$$

where $A_{a.a.}$ = area in counts of amino acid peak, $g_{a.a.}$ = grams of amino acid in sample, $M.W._{a.a.}$ = molecular weight of amino acid.

The precision of the RMR data using dried A.W. Chromosorb W as the support material was well within the range of experimental error. The percent relative standard deviations which are included in Table I ranged from 0.15 to 2.62 with an overall average of 1.18. The RMR data for both dried and non-dried Chromosorb W were generally in agreement or slightly higher than the RMR values for A.W. heat treated Chromosorb G.

CONCLUSIONS

These experiments conclusively demonstrate that the N-TFA *n*-butyl esters of seventeen of the protein amino acids can be separated, and rapidly and quantitatively eluted from columns containing 0.65 w/w % stabilized EGA coated on 80/100 mesh A.W. Chromosorb W (dried at 140° for 12 h).

As reported earlier[2,3] columns containing 1.5 w/w % OV-17 coated on high performance Chromosorb G, 80/100 mesh, can be used for the quantitative analysis of arginine, histidine, tryptophan, and cystine.

A complete GLC chromatogram of seventeen of the protein amino acids can be completed in less than 14 min. Analyses are now routinely made on all kinds of biological substances (blood, urine, extracts) in the Experiment Station Chemical Laboratories.

ACKNOWLEDGEMENT

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3 C. W. GEHRKE, D. ROACH, R. W. ZUMWALT, D. L. STALLING AND L. L. WALL, *Quantitative Gas–Liquid Chromatography of Amino Acids in Proteins and Biological Substances*, Analytical Biochemistry Laboratories, Inc., Columbia, Mo., 1968.

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Page 308. The peaks in Fig. 7 were wrongly numbered. The correct figure is as follows: