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QUANTITATIVE GAS–LIQUID CHROMATOGRAPHY OF NON-PROTEIN AMINO ACIDS IN THE PRESENCE OF THE TWENTY PROTEIN AMINO ACIDS

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SUMMARY

The quantitative gas–liquid chromatographic analysis of eighteen non-protein amino acids, in the presence of the twenty protein amino acids, as their N-TFA-*n*-butyl esters, has been successfully achieved on a glass column packed with EGA. The relative molar responses of these amino acids, relative to ornithine, are presented.

INTRODUCTION

In the course of a long series of experiments on prebiological organic chemistry[1,2], the need for a fast and convenient method for separating non-protein amino acids became clear. The detection and separation of several non-protein amino acids, in the presence of protein amino acids, with an automated amino acid analyzer has been recently investigated in our laboratory[3,4]. Our samples have been analyzed by the gas–liquid chromatographic (GLC) method developed by GEHRKE *et al.*[5–8] for the complete and quantitative separation of the twenty protein amino acids, as their N-TFA-*n*-butyl esters. We here pursue the GLC separation of protein from non-protein amino acids.

The quantitative analysis of at least forty non-protein amino acids, as their N-TFA-*n*-butyl esters, by using the same GLC method used for the twenty protein amino acids, has been mentioned, in passing, by GEHRKE *et al.*[8], without additional data. Recently, the elution temperature of several non-protein amino acids, as their N-TFA-*n*-butyl esters, was determined by GEHRKE[9]. But, the literature does not as yet contain any description of *quantitative* studies on the GLC separation of non-protein amino acids, in the presence of the protein amino acids, as their N-TFA-*n*-butyl esters.

This paper describes the separation on an EGA column, from all the protein amino acids (including arginine, histidine and cystine, which are not eluted from this column), of the following eighteen non-protein amino acids, as their N-TFA-*n*-butyl

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esters: isovaline, sarcosine, α-amino-*n*-butyric acid, norvaline, alloisoleucine, β-amino isobutyric acid, β-alanine, pipecolic acid, norleucine, allothreonine, 4-aminobutyric acid, 2-aminooctanoic acid, iminodiacetic acid, δ-aminolevulinic acid, α-aminoadipic acid, ornithine, S-carboxymethyl cysteine, and methionine sulfone. The relative molar responses of these amino acids relative to ornithine are presented.

EXPERIMENTAL

Apparatus

Pyrex glass reaction vials, with Teflon-lined screw caps, were obtained from Analytical Biochemistry Laboratories, Columbia, Mo., U.S.A. The sand-bath was constructed in our laboratory. The ultrasonic cleaner was a Heat Systems Ultrasonics apparatus. The gas chromatograph was a Hewlett-Packard Model 5750, with two hydrogen flame detectors, one differential electrometer, and a linear temperature programmer.

Reagents

Trifluoroacetic anhydride (TFAA), was "Eastman Grade", from Distillation Product Industries. The solution of 3 *N* HCl in *n*-butanol was made in a glass–Teflon system, with HCl from a Matheson Company compressed gas cylinder. HCl was passed through a glass wool–silica gel filter and a sulfuric acid trap, before bubbling through *n*-butanol. The normality of HCl was determined by weighing a known volume of solution. CH_2Cl_2 was "certified A.C.S. spectroanalyzed" from Fisher and used without other distillation. All solvents used for the derivatization and for making the columns were GC analysed in our laboratory, on a Porapak Q column. They showed less than 0.01 % water.

Alloisoleucine, allothreonine, α-aminoadipic acid, isovaline, lanthionine, methionine, methionine sulfone, norleucine, ornithine·$(HCl)_2$, pipecolic acid, proline, and serine were purchased from Mann Research Laboratories; norvaline, from K&K Laboratories. β-Alanine, α-amino-*n*-butyric acid, arginine, aspartic acid, betaine·HCl, cysteine, cystine, glutamic acid, glycine, histidine·HCl, homocystine, hydroxyproline, isoleucine, leucine, lysine·HCl, phenylalanine, sarcosine, taurine, threonine, tryptophan, tyrosine, urea, and valine were purchased from Nutritional Biochemical Corporation, L-cysteic acid·H_2O from Sigma Chemical Company, and S-carboxymethyl cysteine from BDH Chemicals, Ltd.

α-Alanine and N-amidinoalanine were Eastman Organic Chemical products.

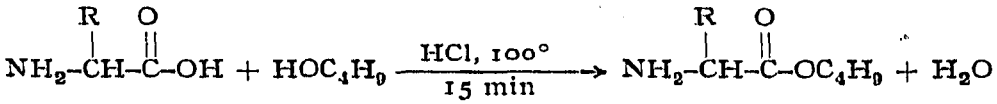
The sample of iminodiacetic acid was a gift of Dr. F. WOELLER. This compound was recrystallized from dilute HCl with the use of acetone, prior to use.

Solutions containing 2.5 mmoles/l of each non-protein amino acid individually in 3 *N* HCl were used for the preliminary calibrations. Then a standard solution containing the twenty protein amino acids and twenty non-protein amino acids, each 2.5 mmoles/l in 3 *N* HCl, was employed.

Derivatization

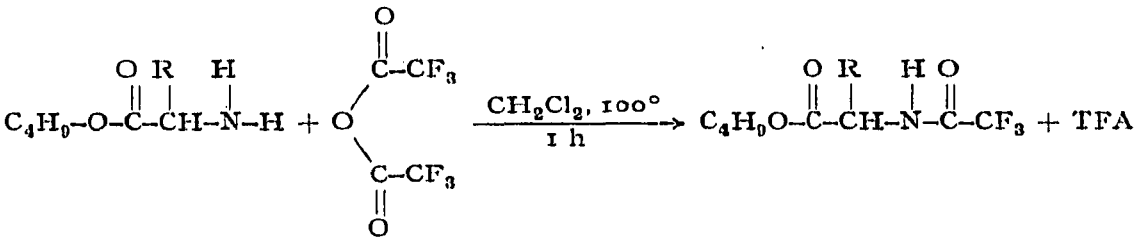
The derivatization was done at the microgram level, as described by ROACH AND GEHRKE[6]. 1 ml of sample is evaporated in a microreaction vial, and placed in a sand-bath. Then, 0.2 ml of CH_2Cl_2 is added to remove azeotropically the last trace of water.

Esterification:



For the direct butylation of the amino acids, 0.2 ml of *n*-butanol, 3 *N* in HCl, is added. The reaction vial is firmly closed, and, after 30-sec ultrasonic mixing and manual shaking, is heated for 15 min at 100°, in the sand-bath. Then the *n*-butanol is evaporated, and 0.2 ml of CH_2Cl_2 is again added to remove azeotropically the last trace of water.

Acylation:



0.2 ml of CH_2Cl_2 and 0.05 ml of TFAA are added, and the closed vial, after 30-sec ultrasonic mixing and manual shaking, is heated in the sand-bath for 1 h at 100°. When the vial is cool, 5 μl of the sample, after manual shaking, is injected into the gas chromatograph.

Gas–liquid chromatographic conditions

The packing material of the GC columns was prepared, as described by GEHRKE *et al.*[5], with 0.325 wt. % of EGA, stabilized grade, coated on 80–100 mesh a.w. HT Chromosorb G. The support material was heat-treated for 15 h at 550°. Two different glass columns, filled with this packing material, were employed: one dual column of 6 ft. long × 4 mm I.D. and one dual column of 11 ft. long × 2 mm I.D.

The temperature program was: isothermal at 80° for 10 min, then programmed temperature at 2°/min, and a final temperature of 212°, held for 20 min. The temperature of the injector was 210°; the temperatures of the detector (FID) was 260°. Flow-rates employed were: carrier nitrogen, 50 ml/min; hydrogen, 40 ml/min; and air, 500 ml/min.

RESULTS AND DISCUSSION

Each non-protein amino acid was first individually derivatized, and then injected into the gas chromatograph. Cysteic acid, lanthionine, homocystine, taurine and betaine gave no detectable peaks, because of non-elution from the EGA column (similar to the behavior of arginine, histidine, and cystine), or because of non-derivatization. 6-Aminohexanoic acid gave a very unsymmetrical peak; in addition it co-elutes with aspartic acid, as corroborated during the calibration.

The derivatives of all the remaining twenty non-protein amino acids which were tested — β-alanine, alloisoleucine, allothreonine, N-amidinoalanine, α-aminoadipic acid, 4-aminobutyric acid, α-amino-*n*-butyric acid, β-aminoisobutyric acid,

δ-aminolevulinic acid, 2-aminooctanoic acid, iminodiacetic acid, isovaline, methionine sulfone, norleucine, norvaline, ornithine, phenylalanine, pipecolic acid, sarcosine and S-carboxymethyl cysteine — were used for the calibration of the columns, by adding to the mixture of the derivatives of the twenty protein amino acids successively each non-protein amino acid as its N-TFA-*n*-butyl ester.

After this calibration, 1 ml of the solution of the twenty protein amino acids and these twenty non-protein amino acids, each 2.5 mmoles/l, was derivatized. Fig. 1 shows the gas chromatogram of 5 μl of this solution, after derivatization. Each peak corresponds to about 50 nmoles of amino acid, using a 6 ft. long × 4 mm I.D. glass column packed with 0.325 wt. % EGA coated on 80-100 mesh a.w. HT Chromosorb G. There is no separation between norvaline and alloisoleucine. Separation between the isoleucine and alloisoleucine/norvaline peaks, the leucine and pipecolic acid peaks, and the α-aminoadipic and tyrosine peaks are poor. But, of the twenty non-protein amino acids for which data are presented here, eighteen are distinguishable from the twenty protein amino acids.

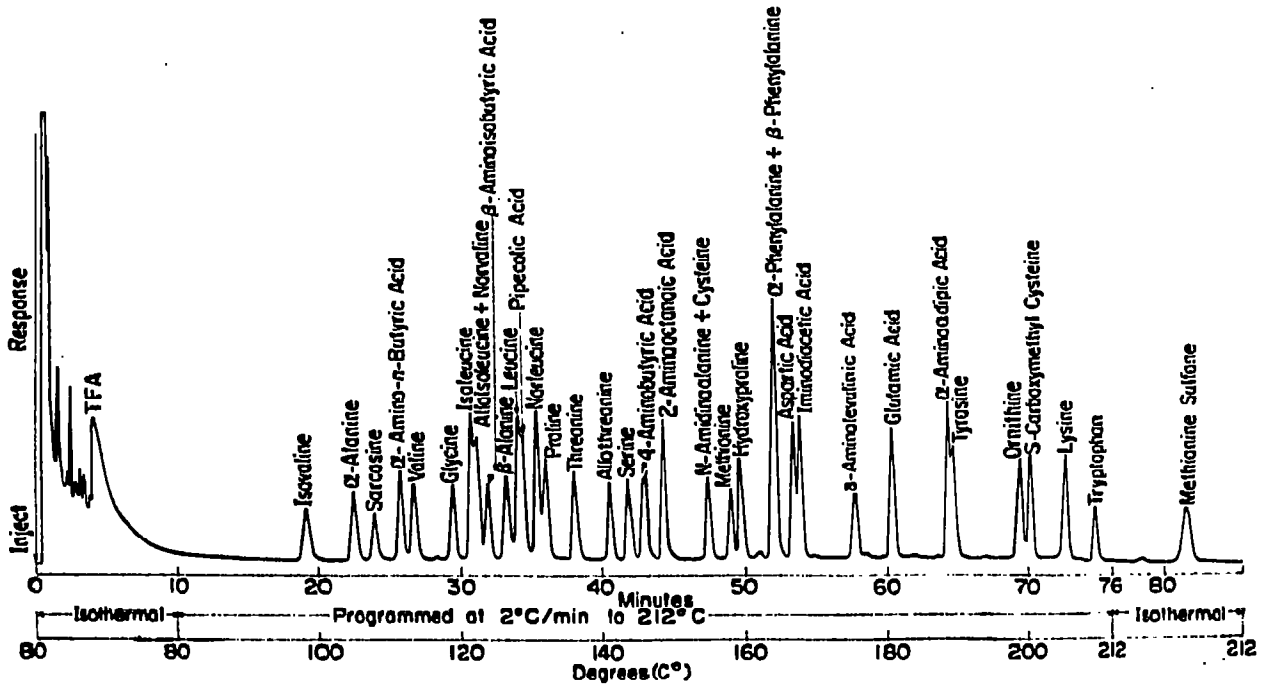


Fig. 1. GLC of an equimolar solution of seventeen protein amino acids and twenty non-protein amino acids. A 6 ft. × 4 mm I.D. glass column, packed with 0.325 wt. % EGA coated on 80–100 mesh a.w. HT Chromosorb G was employed. Programmed temperature: isothermal at 80° for 10 min; then 2°/min to a final temperature of 212° held for 20 min. About 50 nmoles of each amino acid were injected. Sensitivity: 4×10^{-9} a.f.s.

Urea was also tested. It gave a peak which is located between the α-amino-*n*-butyric acid peak and the valine peak. But the GC response of the urea derivative is very poor, and the position of the corresponding peak is very unstable.

In order to obtain a better separation, a longer column, with a smaller inside diameter, was used for the GLC of the same sample. Fig. 2 shows the gas chromatogram of 5 μl of this sample, using an 11 ft. long × 2 mm I.D. glass column, packed with 0.325 wt. % EGA coated on 80–100 mesh a.w. HT Chromosorb G. The resolution is not better than with the 6 ft. × 4 mm I.D. column. Particularly, the norvaline/al-

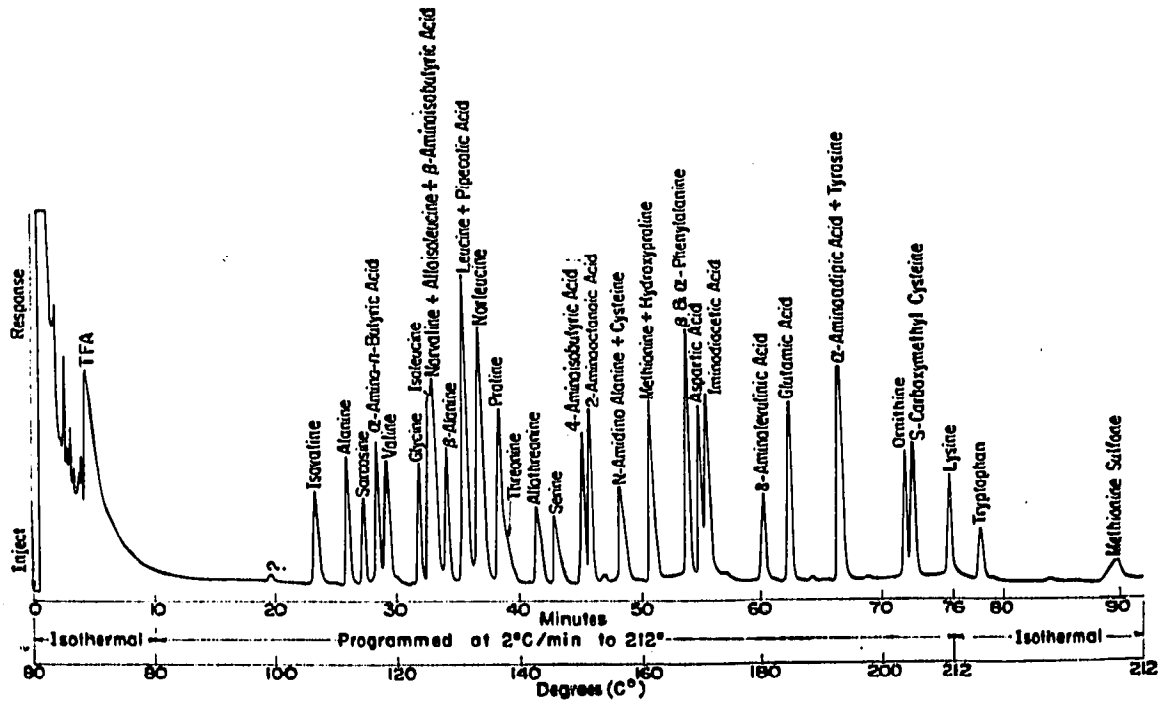


Fig. 2. GLC of an equimolar solution of seventeen protein amino acids and twenty non-protein amino acids. An 11 ft. × 2 mm I.D. glass column, packed with 0.325 wt. % EGA coated on a.w. HT Chromosorb G was employed. All other conditions are as for Fig. 1, except a sensitivity of 2×10^{-9} a.f.s.

loisoleucine and β-aminoisobutyric acid derivatives, leucine and pipecolic acid derivatives, and the tyrosine and α-aminoadipic acid derivatives are not separated. The methionine and hydroxyproline derivatives and the proline and threonine derivatives are also co-eluted.

A quantitative study of the GLC of these eighteen distinguishable non-protein amino acids was performed by determining the relative molar response (*RMR*) of each amino acid with respect to ornithine.

By definition, $RMR_{aa/orn} \equiv (A_{aa}/M_{aa})/(A_{orn}/M_{orn})$, where A_{aa} is the area of the amino acid peak, A_{orn} the area of the ornithine peak, M_{aa} the number of moles of amino acid injected, and M_{orn} the number of moles of injected ornithine. Since an equimolar solution was used, $RMR_{aa/orn} = (A_{aa}/A_{orn})$.

Table I displays the values of *RMR* for three different derivatization and GLC separation runs, and their average. The values for the protein amino acids are in good agreement (±10 %) with values calculated from the results of GEHRKE *et al.*[5], except for tryptophan which is not well-eluted on the EGA column (GEHRKE's value for tryptophan corresponds to an OV-17 column).

CONCLUSION

We have utilized the derivatization process and the EGA column of the GLC method described by GEHRKE *et al.*[3,4]. Of twenty-six non-protein amino acids studied, only two are co-eluted with a protein amino acid: N-amidinoalanine with cysteine, and 6-aminohexanoic acid with aspartic acid. The following eighteen non-protein amino acids are detectable and simultaneously distinguishable from all twenty

TABLE I

RELATIVE MOLAR RESPONSE OF N-TFA-*n*-BUTYL ESTERS OF SEVENTEEN PROTEIN AND EIGHTEEN NON-PROTEIN AMINO ACIDS

Amino acids	$RMR_{aa/orn}$				
	Three independent analyses			*Average*	*Values in ref. 5*
Isovaline	0.66	0.74	0.70	0.70	
Alanine	0.73	0.68	0.70	0.70	0.70
Sarcosine	0.50	0.45	0.53	0.49	
α-Amino-*n*-butyric acid	0.85	0.91	0.83	0.86	
Valine	0.85	0.85	0.83	0.84	0.84
Glycine	0.62	0.65	0.62	0.63	0.56
Isoleucine	0.99	0.97	0.96	0.97	0.97
Alloisoleucine	0.98	0.97	0.95	0.97	
Norvaline	0.77	0.79	0.75	0.77	
β-Aminoisobutyric acid	0.80	0.77	0.75	0.77	
β-Alanine	0.68	0.63	0.66	0.66	
Leucine	1.11	1.09	1.09	1.10	1.09
Pipecolic acid	1.10	1.25	1.14	1.16	
Norleucine	1.10	1.07	1.18	1.12	
Proline	0.94	1.02	1.00	0.99	1.01
Threonine	0.92	0.89	0.91	0.91	0.94
Allothreonine	0.67	0.72	0.74	0.71	
Serine	0.82	0.88	0.82	0.84	0.80
4-Aminobutyric acid	0.90	0.87	0.81	0.86	
2-Aminooctanoic acid	1.05	1.12	1.07	1.08	
Cysteine	0.58	0.60	0.56	0.58	0.58
Methionine	0.77	0.68	0.70	0.72	0.78
Hydroxyproline	1.06	1.13	0.96	1.05	1.05
Phenylalanine	1.67	1.60	1.49	1.59	1.58
Aspartic acid	1.21	1.17	1.19	1.19	1.23
Iminodiacetic acid	1.23	1.29	1.14	1.22	
δ-Aminolevulinic acid	0.77	0.76	0.70	0.74	
Glutamic acid	1.37	1.33	1.26	1.32	1.45
α-Aminoadipic acid	1.24	1.21	1.15	1.20	
Tyrosine	1.44	1.40	1.37	1.40	1.42
Ornithine	1.00	1.00	1.00	1.00	1.00
S-Carboxymethyl cysteine	1.15	1.17	1.14	1.15	
Lysine	1.15	1.17	1.08	1.13	1.04
Tryptophan	0.78	0.56	0.55	0.63	1.56
Methionine sulfone	1.17	1.10	0.91	1.06	

protein amino acids: β-alanine, alloisoleucine, allothreonine, α-aminoadipic acid, 4-aminobutyric acid, α-amino-*n*-butyric acid, β-aminoisobutyric acid, δ-aminolevulinic acid, 2-aminooctanoic acid, iminodiacetic acid, isovaline, methionine sulfone, norleucine, norvaline, ornithine, pipecolic acid, sarcosine and S-carboxymethyl cysteine. Of these eighteen amino acids, only alloisoleucine and norvaline are co-eluted.

We conclude that the method of GEHRKE and coworkers can be extended to the quantitative GLC analysis of non-protein amino acids, in the presence of the twenty protein amino acids, as their N-TFA-*n*-butyl esters. This method has been used successfully in our laboratory, for the quantitative analysis of amino acids formed during the long-wavelength UV photolysis of simulated primitive earth

atmospheres[1,2]. It can be used even for the analysis of very small (picomole) quantities of non-protein amino acids in the presence of protein amino acids.

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