QUANTITATIVE GAS CHROMATOGRAPHY OF AMINO ACIDS[1]

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Because of its speed, accuracy, and sensitivity, gas chromatography offers advantages over other chromatographic methods for the determination of amino acids. Among derivatives which have been investigated are the trimethylsilyl N-trimethylsilyl esters (Rühlmann and Giesecke, 1961), the n-amyl N-acetyl esters (Johnson, et al., 1961), the n-butyl N-trifluoroacetyl esters (Zomzely, et al., 1962), the phenylthiohydantoins and methyl N-dinitrophenyl esters (Pisano, et al., 1962), and the methyl N-trifluoroacetyl esters (Saroff and Karmen, 1960; Hagen and Black, 1964; Cruickshank and Sheehan, 1964). Each of these derivatives has proved to be useful for gas-chromatographic separations, but none has been studied in sufficient detail to permit general analytical use.

It was recognized that quantitative, reproducible derivative formation was an essential prerequisite for an accurate gas-chromatographic method, thus studies were made to investigate the organic reaction conditions necessary for quantitatively converting amino acids to volatile derivatives. This paper describes a general method for quantitative and

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reproducible preparation of the n-butyl N-trifluoroacetyl ester derivatives of 19 protein amino acids. Information is presented on the method, retention temperatures, relative molar response, quantitation, required chromatographic separation, and instrumental conditions.

Procedure. ANALYTICAL PREPARATION OF n-BUTYL N-TRIFLUOROACETYL ESTERS. The amino acid or mixture (<60 mg.) was placed in a 125-ml. flat-bottom flask, and 10 ml. of anhydrous methanol containing 1.20 ± 0.10 meq. per ml. of anhydrous HCl were added. The flask was then stoppered with a ground-glass stopper; the solution was stirred on a magnetic stirrer for 30 minutes at room temperature; and the methanol was removed by vacuum distillation at 60° ± 1° C. Ten (10) ml. of n-butanol containing 1.20 ± 0.10 meq. per ml. of anhydrous HCl were added; the solution was heated for 180 minutes with magnetic stirring in an oil bath at 90° ± 3° C.; and the butanol was removed by vacuum distillation at 60° ± 1° C. The n-butyl ester hydrochloride(s) was (were) then trifluoroacetylated by adding 5.00 ml. of methylene chloride and 0.50 ml. of trifluoroacetic anhydride and stirring for 120 minutes at room temperature on a magnetic stirrer. The trifluoroacetic anhydride and solvent were removed by vacuum distillation at room temperature, and the n-butyl N-trifluoroacetyl ester(s) was (were) dissolved in anhydrous chloroform prior to gas chromatography. Preparation of derivatives by this procedure required over 5 hours, but since only ca. 10 minutes of the analyst's time was needed per sample, a number of samples (6 with the apparatus employed) could be carried through the procedure simultaneously. Esterification in methanol was followed by inter-

esterification in n-butanol due to the insolubility of cystine and the basic amino acids in n-butanol.

Retention Temperature and Relative Molar Response.

Relative molar response in the flame detector, with glutamic acid arbitrarily assigned a value of 1.00, was determined for each of the 19 amino acids, and as shown by the last three columns of Table I, reproducibility of re-

TABLE I

RETENTION TEMPERATURES AND RELATIVE MOLAR RESPONSES OF THE n-BUTYL N-TRIFLUOROACETYL ESTERS

Amino Acid	Retention Temperature[a], °C.	Relative Molar Response[b] by Flame Ionization A	B	Ave.
Alanine	68	0.536	0.514	0.53
Valine	70	0.692	0.664	0.68
Isoleucine	78	0.786	0.801	0.79
Glycine	83	0.428	0.433	0.43
Threonine	86	0.615	0.633	0.62
Leucine	87	0.764	0.790	0.78
Proline	97	0.701	0.740	0.72
Serine	98	0.531	0.548	0.54
Cysteine	115	0.523	0.501	0.51
Hydroxyproline	120	0.755	0.766	0.76
Methionine	126	0.685	0.707	0.70
Aspartic Acid	130	0.940	0.898	0.92
Phenylalanine	130	1.127	1.114	1.12
Glutamic Acid	145			1.00[c]
Tyrosine	152	1.014	1.029	1.02
Lysine	173	0.886	0.830	0.86
Histidine	197	0.572	0.553	0.56
Tryptophan	176, 203			1.12[d]
Cystine	211	0.415	0.410	0.41

[a]Instrument: F and M Model 400. Column: 1.00-m. x 3-mm. i.-d. glass, packed with 1.00% (w./w.) neopentylglycol succinate on 60-80 mesh Gas-Chrom A. Column temp.: 67° C. for 6.0 min., then programmed at 3.3° C./min. to 218° C. Carrier gas: 38 ml./min. N_2 at 20.0 psig. Each sample was injected directly on the chromatographic column without the use of a flash heater.

[b]Amino acid molar response - area per mole of amino acid.

$$\text{Relative Molar Response} = \frac{\text{Amino Acid Molar Response}}{\text{Glutamic Acid Molar Response}}$$

[c]Arbitrarily set at unity.

[d]Two peaks were obtained, and the relative molar response was computed from the sum of the two areas.

sponse for amino acids carried through the entire chemical and chromatographic procedure was found to be excellent. Also, mixtures of representative amino acids with different functional groups gave the same relative molar responses as the individual amino acids taken through the chemical and chromatographic method singly. Thus no problems were encountered with interactions. Anhydrous conditions must be maintained during derivative preparation to prevent hydrolysis of the O-trifluoroacetyl esters of the hydroxy amino acids. Addition of trifluoroacetic anhydride to the solvent after acylation prevents hydrolysis.

Precision and Accuracy. The most important single factor affecting the accuracy of a gas-chromatographic method for the determination of amino acids is the yield of derivative. A low yield would probably result in poor precision and would severely limit the usefulness of the method for quantitative analysis. To assess the accuracy expected in an actual analysis, the pure n-butyl N-trifluoroacetyl esters were synthesized and were used as reference standards to determine the yield of derivative of 18 amino acids in the analytical method. Purity for each derivative was checked by boiling-point or melting-point, and gas chromatography. Conversion of amino acid to derivative was found to be quantitative (Table II). Interesterification of threonine and cystine at 100° C. increased the yield to 100%.

Glutamic acid was found to be converted into the dibutyl ester, and tyrosine and lysine gave the ditrifluoroacetyl derivatives. It had been anticipated that methionine would be converted into its sulfoxide, but elemental analyses showed that the sulfur was not oxidized during derivative

TABLE II

YIELD AND STRUCTURE OF n-BUTYL N-TRIFLUOROACETYL ESTERS

Amino Acid Class	Empirical Formula[a] of Derivative	footnote	Yield, %[b] and Range
1. Aliphatic			
Glycine	$C_8H_{12}NO_3F_3$	c	101 ± 0.1
Alanine	$C_9H_{14}NO_3F_3$	c	98 ± 0.4
Leucine	$C_{12}H_{20}NO_3F_3$	c	100 ± 0.2
Isoleucine	$C_{12}H_{20}NO_3F_3$	c	96 ± 0.2
Valine	$C_{11}H_{18}NO_3F_3$	c	99 ± 1.7
Phenylalanine	$C_{15}H_{18}NO_3F_3$	c	97 ± 0.4
2. Dicarboxylic			
Aspartic Acid	$C_{14}H_{22}NO_5F_3$	f	99 ± 1.6
Glutamic Acid	$C_{15}H_{24}NO_5F_3$	f	97 ± 1.6
3. Hydroxy			
Serine	$C_{11}H_{13}NO_5F_6$	d	102 ± 0.9
Threonine	$C_{12}H_{15}NO_5F_6$	d	91 ± 0.1
Tyrosine	$C_{17}H_{17}NO_5F_6$	d	100 ± 2.8
4. Sulfur			
Cysteine	$C_{11}H_{13}NO_4F_6S_1$	d	93 ± 0.3
Cystine	$C_{18}H_{26}N_2O_8F_6S_2$	e	88 ± 0.0
Methionine	$C_{11}H_{18}NO_3F_3S$	c	98 ± 1.8
5. Basic			
Lysine	$C_{14}H_{20}N_2O_4F_6$	d	100 ± 0.1
Arginine	$C_{14}H_{22}N_4O_5F_6$	g	
6. Heterocyclic			
Proline	$C_{11}H_{16}NO_3F_3$	c	97 ± 0.4
Hydroxyproline	$C_{13}H_{15}NO_5F_6$	d	98 ± 0.4
Histidine	$C_{14}H_{15}N_3O_4F_6$	d	97 ± 0.3

[a]Confirmed by elemental analysis for C, H, N, F, and S.

[b]Average and range for two independent determinations.

[c]Calculated for the trifluoroacetyl n-butyl ester.

[d]Calculated for the ditrifluoroacetyl n-butyl ester.

[e]Calculated for the ditrifluoroacetyl n-dibutyl ester.

[f]Calculated for the trifluoroacetyl n-dibutyl ester.

[g]Calculated for the α-trifluoroacetyl n-butyl ester ω-trifluoroacetate salt.

preparation. Elemental analyses of the derivative indicated that in the analytical method all carboxyl groups were es-

terified and all amino, imino, phenolic, hydroxy, sulfhydryl, and imidazole groups were trifluoroacetylated. Tryptophan gave two derivatives, the mono- and diacyl n-butyl esters which can be analyzed by establishing a response factor for the two peak areas.

Under these conditions arginine was converted to the α-trifluoroacetyl n-butyl ester ω-trifluoroacetate salt. Acylation of arginine in the presence of anhydrous Na_2CO_3 gave a derivative suitable for gas chromatographic analysis. Further research is necessary on the chemistry of arginine and tryptophan.

Chromatographic and Instrumental Conditions. Complete resolution of a mixture of 19 protein amino acids taken simultaneously through the analytical and chromatographic procedure was achieved using a mixed stationary phase column

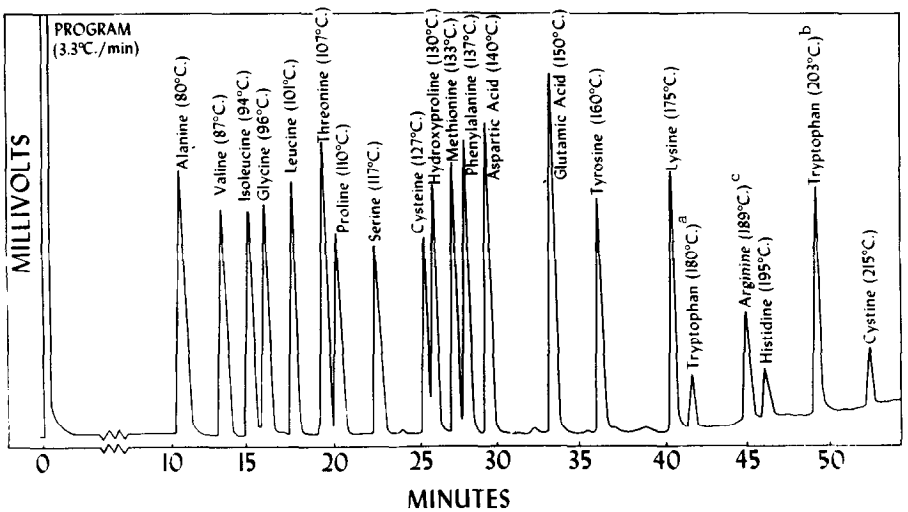

Figure 1. Chromatogram of 20 n-Butyl N-Trifluoroacetyl Amino Acid Esters. Sample size: 5 μl. Each peak represents 2.5 μg. of amino acid.

[a]Diacyl derivative; [b]Monoacyl derivative

[c]Acylated for 18 hours in presence of anhydrous Na_2CO_3

of 0.75/0.25 w./w.% of DEGS/EGSS-X and temperature programming. An acylated sample of arginine was added to this mixture prior to chromatography and it was also separated. The instrumental operating and chromatographic conditions are given on Figure 1 and as follows:

Instrument used: F and M Model 300 linear programmed temperature gas chromatograph, and F and M Model 1609 Flame Ionization attachment, with a F and M Model 400 column oven and detector module.

Column temperature: Initial 67° C., final 218° C.

Program Rate	3.3° C./min.
Detector Cell Temperature (at start)	123° C.
Sensitivity	1/32
Carrier Flow, N_2	38 ml./min.
Air (to detector)	450 ml./min.
Hydrogen (to detector)	36 ml./min.
Chart Speed	1/3 inch/min.

Column: 1.00 meter x 4 mm. i.d. borosilicate-glass column packed with 60/80 mesh acid-washed Chromosorb W and mixed substrate phases of 0.75/0.25 w./w.% of DEGS/EGSS-X.

It is concluded that the protein amino acids, as their n-butyl N-trifluoroacetyl esters, can be analyzed quantitatively by gas-liquid chromatography.

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